Appetite-regulating peptides and natural rewards: emphasis on ghrelin and glucagon-like peptide-1

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Cover illustration: A typical chromatogram in rainbow colors
by Jesper Vestlund

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Dedicated to all research animals that contributed to this work

“When you study natural sciences and the miracles of creation, if you do not turn into a mystic you are not a natural scientist”

Albert Hofmann, the discoverer of LSD

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Abstract

Evolutionary conserved natural behaviors, such as foraging and sexual behaviors, are strongly associated with reward processes. Brain areas important for reward processes include, but are not limited to, the nucleus accumbens (NAc) shell, the ventral tegmental area (VTA), the laterodorsal tegmental area (LDTg) and the nucleus of the solitary tract (NTS). The mechanisms that control natural rewards are complex, and appetite-regulating peptides, such as ghrelin and glucagon-like peptide-1 (GLP-1), have recently been identified as substrates involved in reward processes. The aim of the present thesis is therefore to elucidate the involvement of ghrelin and GLP-1 in natural rewards, by assessing how they mediate two different natural rewards, i.e. skilled reach foraging from the feeding-related domain and sexual behaviors from the social behavior domain, in preclinical behavioral models.

We showed in **paper I** that repeated treatment with a ghrelin receptor antagonist decreases the motivation of skilled reach foraging in rats with an acquired skilled reach performance tentatively through suppression of ghrelin receptors within the NAc shell. Repeated ghrelin increases, whereas a ghrelin receptor antagonist reduces, the motivation and learning of skilled reach foraging in rats during acquisition of this behavior. In **paper II**, we further established that GLP-1, as ghrelin, modulates the motivation and learning of skilled reach foraging. Indeed, the GLP-1 receptor (GLP-1R) agonists,

exendin-4 and liraglutide, decrease the motivation of skilled reach foraging in rats with an acquired skilled reach performance whereas another GLP-1R agonist, dulaglutide, increases the learning of this complex behavior. When it comes to GLP-1 and sexual behaviors we demonstrated in **paper III** that a systemic exendin-4 injection decreases social behaviors, mounting behaviors and self-grooming behaviors but does not influence preference for females or female odors in sexually naïve male mice. We also identified that activation of GLP-1R within the NTS suppresses social behaviors, mounting behaviors and self-grooming behaviors in sexually naïve male mice. In addition, in **paper IV** we further identified that activation of GLP-1R within the LDTg or the posterior VTA suppresses social behaviors and mounting behaviors whereas activation of GLP-1R within the NAc shell only reduces social behaviors, but not mounting behaviors, in sexually naïve male mice.

Collectively, these data support the emerging literature suggesting that ghrelin increases whereas GLP-1 decreases natural rewards, by showing that these peptides via reward-related areas modulate natural rewards from both the feeding-related and the social behavior domains of natural rewards.

Keywords: Reward, Gut-brain axis, Sexual behaviors, Skilled reach foraging

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Populärvetenskaplig sammanfattning

Aptitreglerande hormoner och naturliga belöningar: fokus på ghrelin och glukagonlik peptid-1

Drogberoende innebär ett stort lidande och hög risk för förtidig död för den drabbade. På senare år har beroende begreppet vidgats, och det inkluderar idag också beteenden som har beroendeliknande uttryck såsom hetsätning och sexberoende. Det neurobiologiska forskningsfältet har föreslagit att beroende till droger och beteenden till stor del drivs av maladaptiva belöningsmekanismer i hjärnan. Naturliga belöningar, såsom mat och sex, samt beroendeframkallande droger aktiverar belöningskretsar. Förmågan hos belöningarna att aktivera dessa kretsar påverkas av många olika mekanismer, och studier har visat att aptitreglerande hormoner som produceras i mag-tarmkanalen påverkar upplevelsen av belöningarna. Genom att kommunicera med hjärnan, har tidigare studier visat att dessa aptitreglerande hormoner kontrollerar energi- och matintag. Exempel på sådana aptitreglerande hormoner är ghrelin och glukagonlik peptid-1 (GLP-1). GLP-1 reglerar också blodglukosnivåerna, och substanser som liknar GLP-1 används därför vid behandling av diabetes typ II. Även om initiala studier pekar på att ghrelin och GLP-1 är involverade i belöningsreglering är det fortfarande inte helt klarlagt om och hur ghrelin och GLP-1 påverkar naturliga belöningar såsom motivationen att konsumera socker, samt sexuella beteenden. Denna avhandling syftar till att ytterligare klarlägga om och hur ghrelin och GLP-1 påverkar dessa naturliga belöningar med hjälp av etablerade djurmodeller.

I vår första studie i råttor visade vi att upprepad ghrelin behandling ökar motivationen och inlärningen att konsumera socker. Vidare visade vi att farmakologisk blockad av ghrelin signalering minskar detta belönings-relaterade beteende. Vi visade också att ghrelin genom att påverka ett område mycket centralt för belöning, dvs accumbenskärnan, minskar motivationen till att konsumera socker. I den andra motivationsstudien jämförde vi tre stycken olika GLP-1 verkande diabetesläkemedel, nämligen exendin-4, liraglutid och dulaglutid. Vi visade att upprepad behandling med exendin-4 eller liraglutid minskar motivationen att konsumera socker, medan dulaglutid ökar inlärningen av beteendet. I den tredje och fjärde studien visade vi att behandling med exendin-4 minskar hanens sexuella interaktion med en hona via områden som är associerade med belöning.

Sammanfattningsvis visar dessa studier att aptitreglerande hormoner reglerar naturliga belöningar från både den mat-relaterade och den sociala domänen. Vi anser därför att farmakologiska substanser som antingen blockerar ghrelin signalering eller aktiverar GLP-1 signalering har möjlig potential att testas kliniskt vid behandling av beroende-liknande beteenden såsom hetsätning och sexberoende.

List of papers

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

I. **Vestlund J**, Bergquist F, Eckernäs D, Licheri V, Adermark L, Jerlhag E. Ghrelin signalling within the rat nucleus accumbens and skilled reach foraging. *Psychoneuroendocrinology*. 2019; 106: 183-194.

II. **Vestlund J**, Bergquist F, Licheri V, Adermark L, Jerlhag E. Activation of glucagon-like peptide-1 receptors and skilled reach foraging. *Addiction Biology*. 2020: e12953. DOI: 10.1111/adb.12953.

III. **Vestlund J** and Jerlhag E. Glucagon-like peptide-1 receptors and sexual behaviors in male mice. *Psychoneuroendocrinology*. 2020; 117: 104687.

IV. **Vestlund J** and Jerlhag E. The glucagon-like peptide-1 receptor agonist, exendin-4, reduces sexual interaction behaviors in a brain site-specific manner in sexually naïve male mice. *Hormones and Behavior*. 2020; 124: 104778.

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Abbreviations

GLP-1	glucagon-like peptide-1
GABA	gamma-aminobutyric acid
LDTg	laterodorsal tegmental area
VTA	ventral tegmental area
NAc	nucleus accumbens
NTS	nucleus of the solitary tract
mPOA	medial preoptic area
aVTA	anterior ventral tegmental area
pVTA	posterior ventral tegmental area
MSN	medium spiny neurons
DMS	dorsomedial striatum
DLS	dorsolateral striatum
PPG neurons	preproglucagon neurons
GHSR-1A	growth hormone secretagogue receptor 1A
GLP-1R	glucagon-like peptide-1 receptor
IP	intraperitoneal
SC	subcutaneous
Ex4	exendin-4
Ex9	exendin-3 (9-39) amide
HPLC-ECD	high-pressure liquid chromatography with electrochemical detection
ELISA	enzyme-linked immunosorbent assay
ANOVA	analysis of variance

1 INTRODUCTION

1.1 Natural rewards and compulsive behaviors

The initial and continued reward from a behavior is necessary for animal survival (for review see [1,2]). These evolutionary conserved behaviors activate the reward systems of the brain [2-13] and are thus referred to as natural rewards. There are different domains of natural rewards including food-related behaviors, social behaviors, exercise behaviors and novelty-seeking behaviors (for review see [14]). Social behaviors are further sub-divided into behaviors such as pro-social behaviors, aggression behaviors, sexual behaviors, maternal behaviors, paternal behaviors and social play (for review see [15,16]).

These reward systems are also mediating reward from addictive drugs (for review [17]). Excessive use of addictive drugs causes neuroplasticity changes in these reward systems thereby causing drug addiction, a brain state characterized by compulsive drug-seeking and loss of control over intake (for review [17]). Interestingly, excessive use of natural rewards is also causing similar neuroplasticity changes as addictive drugs thereby causing them to become compulsive [18-21]. Example of these compulsive behaviors are binge eating disorder, internet addiction, gambling disorder, compulsive buying and compulsive sexual behaviors (for review see [22,23]). However, the development of these addictive disorders is complex and both inherited genetic predisposition and environmental factors contribute (for reviews see [24-26]). To date, cognitive behavioral therapy, serotonin-reuptake inhibitors and naltrexone are used for treatment of binge eating disorder, internet addiction, gambling disorder, compulsive buying and compulsive sexual behaviors with modest effects (for review see [23,27-29]). In addition, lisdexamfetamine is also used to treat binge eating disorder with modest effect (for review see [27]). These compulsive behaviors are largely understudied and more insight into the underlying neurobiological mechanisms driving these natural rewards to become compulsive could lead to improved pharmacotherapy. To understand the mechanisms driving natural rewards to become compulsive, we need to pinpoint neurocircuits and neuromodulators which guide these mechanisms. We have therefore focused on two different natural rewards, i.e. skilled reach foraging from the feeding-related domain and sexual behaviors from the social behavior domain, in preclinical behavioral models.

1.1.1 Foraging behaviors

Maintaining energy homeostasis is necessary for survival and animals invest a major part of their day to seek and consume nutrients (for reviews see [30,31]). Feeding is guided by homeostatic and hedonic signals (for reviews see [30,31]). Homeostatic signals affect brain areas such as the hypothalamus, and are responsible to maintain energy balance by influencing regular food intake (for reviews see [30,31]). Appetite-regulating peptides, such as ghrelin, glucagon-like peptide-1 (GLP-1), neuromedin U, amylin, leptin, insulin and peptide YY, are well known for their ability to guide homeostatic feeding (for review see [32]). Hedonic signals drive the animal to overeat by giving food incentive salience (for reviews see [30,31]). Albeit the neurocircuits regulating homeostatic and hedonic feeding often are considered as dissociable, recent findings suggest that these overlap to some extent (for review see [33]).

Hedonic feeding, driven by the reward systems, is typically divided into two components: "liking" and "wanting" [34]. Liking is associated with the palatability of the food, and the immediate response to their consumption, while wanting is associated with the drive to obtain certain types of foods [34]. Different nutrients, internal states and the context where they are consumed affect the processes of reward and consequently modulate the hedonic feeding [35-40]. Hedonic feeding is divided into motivational hedonic feeding and consummatory hedonic feeding which are assessed with different animal models (for review see [41]). The consummatory aspects are assessed by measuring palatable food intake while the motivational aspects are evaluated by using operant self-administration models for palatable foods (for review see [41]). There are different types of palatable foods that are rewarding in rodents, such as sucrose, chocolate, peanut-butter, high-fat diet, high sucrose/high fat diet and western-style cafeteria diet, which are causing overeating [42-45]. Interestingly after a period of palatable food extinction rodents' relapse to operant self-administration for palatable food seeking in response to palatable-food priming, food-associated cues or stress, sharing similarities with addictive drugs (for review see [46]). The Montoya staircase paradigm, classical used for evaluating motor learning and performance [47,48], utilize sucrose pellets, as a palatable food source, to motivate the rodent to learn this complex progressively more difficult motor task [47,48]. This test is therefore used to assess motivation and learning of skilled reach foraging by measuring the consumption of sucrose pellets and the success rate.

Hedonic feeding behaviors are complex but are modulated by various appetite-regulating peptides that originates in neurons, in the periphery (i.e. gut-brain peptides) or both (for review see [32]). Appetite-regulating peptides that increase hedonic feeding are ghrelin (for review see [49]) orexin [50] and neuropeptide Y

[51,52]. On the other side appetite-regulating peptides that decrease hedonic feeding are GLP-1 (for review see [53]), neuromedin U [54-56], insulin [57], leptin [57,58], amylin [59,60] and peptide YY [61]. In addition to appetite-regulating peptides various neurotransmitters, such as dopamine, enkephalin, serotonin, acetylcholine, gamma-aminobutyric acid (GABA) and glutamate, modulate hedonic feeding [62-71]. These appetite-regulating peptides and neurotransmitters act in various brain regions of the reward systems to modulate hedonic feeding such as classical reward areas including the laterodorsal tegmental area (LDTg), ventral tegmental area (VTA) and nucleus accumbens (NAc) [63-71] and areas which have previously not been associated with reward such as the nucleus of the solitary tract (NTS) [50,72,73] lateral parabrachial nucleus [74], paraventricular thalamic nucleus [75], supramammillary nucleus [76], ventral hippocampus [37], lateral septum [77] and lateral hypothalamus [78,79].

1.1.2 Sexual behaviors

Procreation is a necessary process for the survival of the species (for reviews see [80]). Sexual behaviors are to a large extent innate, and these behaviors are evoked in response to environmental cues (for reviews see [80,81]). Sexual behaviors are considered sexually dimorphic, as the behavior motor pattern differs to a high extent between the sexes (for review see [82,83]). First, during the pre-sexual interaction phase, males and females express sex-specific social behaviors where females attract males by emitting pheromones and males respond with sniffing, following and attending the females causing the females to engage in proceptive behaviors (for review see [81,82,84]). Secondly, during the sexual interaction phase, males engage in mounting behaviors which ends in ejaculation, while the females engage in lordosis behaviors to facilitate semination (for review see [81,82,84]). Finally, during the post-sexual interaction phase, both males and females rest and engage in self-grooming behaviors (for review see [81,82,84]). However, in contrast to the difference in behavioral motor pattern, the underlying reward processing of these behaviors, most likely, do not differ as both male and female behaviors during the pre-sexual interaction phase and the sexual interaction phase activate the reward systems (for review see [85]). However, the data presented in this thesis focuses on male sexual behaviors and all the references are about male sexual behaviors unless stated otherwise.

Sexual behaviors are divided into two components: motivational sexual behaviors and consummatory sexual behaviors (for review see [80,81,84]). Motivational sexual behaviors describe the urge to seek after a potential mate (for review see [80,81,84]) and can be further subdivided into sexual incentive

motivation and sexual conditioned motivation. The sexual incentive motivation describes the innate urge to seek a partner without prior experience and the preference for female test [86] and the straight-arm runway test [87] are used to asses sexual incentive motivation (for review see [84]). The sexual conditioned motivation describes the learned motivation that emerge from prior experience and the level searching paradigm [88,89] and the lever-pressing paradigms [90] are used to assess sexual conditioned motivation (for review see [84]). Consummatory sexual behaviors describe the behavior pattern of copulation (for reviews see [80,81,84]). These behaviors are assessed by measuring the interaction between a male rodent and a female rodent in estrus in an arena.

Sexual behaviors are complex and are influenced by hormones such as corticosterone and testosterone [81,91-96] and neurotransmitters including dopamine, serotonin, noradrenaline, acetylcholine, glutamate, GABA and oxytocin [4-6,97-106]. In addition, appetite-regulating peptides such as the orexigenic peptide orexin and neuropeptide Y inhibit sexual interaction behaviors [107-110] and anorexigenic peptides such as leptin or α-melanocyte stimulating hormone promote sexual interaction behaviors [110-113]. To modulate sexual behaviors, these hormones, peptides and neurotransmitters act at various brain areas including the medial preoptic area (mPOA) [89,114,115], ventromedial hypothalamus [116], lateral hypothalamus [107,117,118], paraventricular nucleus [119] amygdala [120,121], bed nucleus of stria terminalis [115], periaqueductal gray [122], central tegmental field [123] and dorsal raphe [124,125]. In addition, these signals also act in the LDTg, VTA and NAc to modulate sexual behaviors [3-8,118,126,127].

1.2 Brain regions associated with reward

The reward systems consist of brain regions and neurocircuits that processes incentive salience (motivation and desire for a reward) and associative learning (positive reinforcing and condition) of rewarding stimuli [1,128,129]. The VTA, NAc and LDTg are some of the brain regions which are part of the reward systems (for review see [130,131]).

1.2.1 Ventral tegmental area

The mesocorticolimibic dopamine system is an important part of the reward systems and processes both natural rewards and addictive drugs (for review see [130,131]). The origin of this dopamine system is the VTA, where high density of dopamine cell bodies are located [132]. The activity of dopamine neurons in the VTA are influenced by afferents including serotonin [71], noradrenalin [133], GABA [134,135], glutamate [136,137], acetylcholine [138,139], orexin [140] and GABA interneurons [141]. The VTA is a heterogenous brain structure and its subparts receive different inputs and have different outputs [142-144]. The VTA is commonly divided into the anterior (aVTA) and posterior VTA (pVTA) [142-144]. Both the aVTA and pVTA are involved in reward processing, but they also process negative valence from aversive stimuli [136,142,145]. A simplified schematic representation of afferents to and efferents from the VTA is summarized in Figure 1.

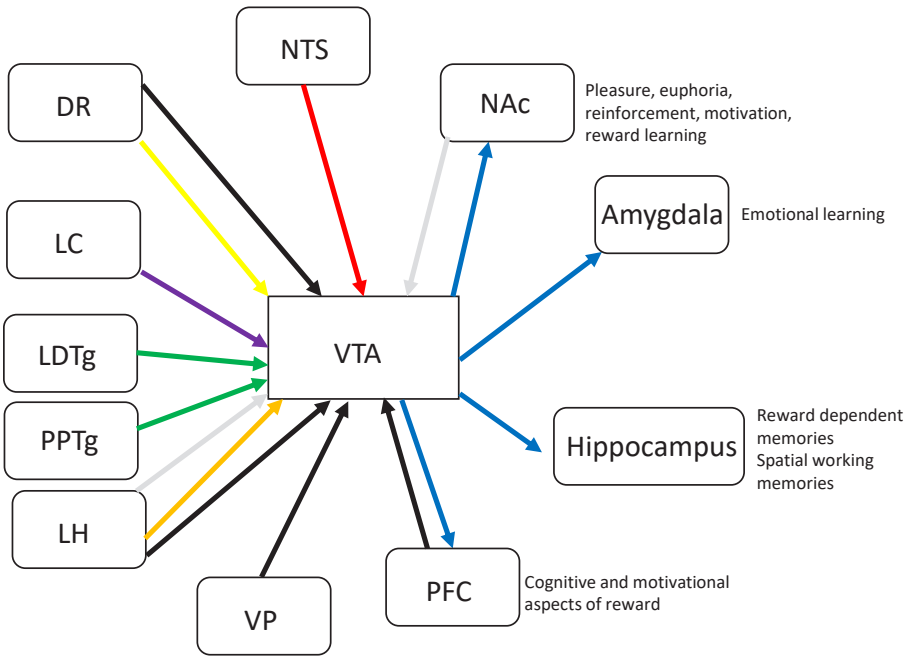

Figure 1. Schematic illustration of some of the afferents/efferents to the ventral tegmental area (VTA)
NAc=nucleus accumbens; PFC=prefrontal cortex; LH=lateral hypothalamus; DR=dorsal raphe; LDTg=laterodorsal tegmental area; PPTg=pedunculopontine tegmental nucleus; NTS=nucleus of the solitary tract; LC=locus coeruleus; VP=ventral pallidum. Blue line = dopamine; Yellow line = serotonin; Green line = acetylcholine; Red line = GLP-1; Orange line = Orexin; Purple line = noradrenaline; Black line = glutamate; Grey line = GABA

The VTA dopamine neurons project to the prefrontal cortex which are referred to as the mesocortical dopamine system and are associated with the motivational and cognitive aspects of reward (for review see [146]). The VTA dopamine neurons also project to limbic areas including the NAc, amygdala and hippocampus which is referred to as the mesolimbic dopamine system. This mesolimbic dopamine system is associated with pleasure, euphoria, positive emotional memories, stimulation, motivation and positive reinforcement (for review see [131,147,148]). The mesolimbic system can be further subdivided into the mesoamygdaloid dopamine projection (VTA-amygdala), a neurocircuit associated with emotional learning [149,150] and the mesohippocampal dopamine projection (VTA-Hippocampus) a neurocircuit associated with spatial working memories and reward-dependent memories [151-154] and the mesoaccumbal dopamine projection (VTA-NAc), a neurocircuit intimately associated with euphoria, stimulation, positive reinforcement, reward learning and motivational properties of rewards (for reviews see [130,147,155])

1.2.2 Nucleus accumbens

NAc (also called ventral striatum) is subdivided into two distinct structures; the NAc core and the NAc shell. These different subparts of the NAc have different inputs and outputs, and thus modulate different processes [156]. The NAc core modulates reward learning, while NAc shell is associated with reward processing [157]. The output neurons of the NAc are GABAergic medium spiny neurons (MSN). These MSN project to the VTA and ventral pallidum controlling reinforcement, motivation and movement initiation [158-161]. The MSN are divided into dopamine D1 receptor expressing MSN which when activated stimulate, whereas dopamine D2 receptor expressing MSN which when activated suppresses, reward from addictive drugs and natural rewards [162-164]. Besides dopamine, the activity of output neurons in the NAc are modulated by afferents including serotonin [97,165], glutamate [67,68,166-168], and GABA [169]. Moreover, the activity of these MSN is also modulated by cholinergic interneurons [170,171] and GABAergic interneurons [172,173]. A simplified schematic representation of afferents to and efferents from the NAc is summarized in Figure 2.

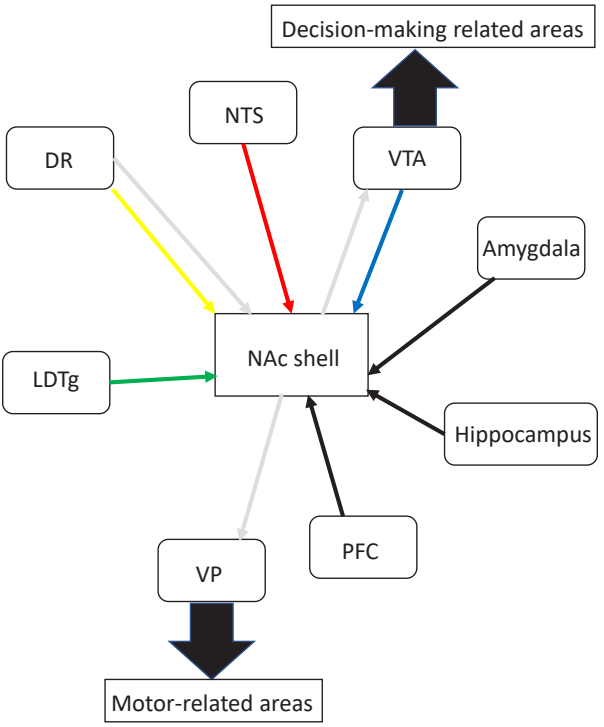

Figure 2. Schematic illustration of some of the afferents/efferents to the nucleus accumbens (NAc).
VTA=ventral tegmental area; PFC=prefrontal cortex; DR=dorsal raphe; LDTg=laterodorsal tegmental area; NTS=nucleus of the solitary tract; VP=ventral pallidum. Blue line = dopamine; Yellow line = serotonin; Green line = acetylcholine; Red line = GLP-1; Black line = glutamate; Grey line = GABA

1.2.3 Dorsal striatum

The nigrostriatal dopamine projection from substantia nigra pars compacta to the dorsal striatum mediates motor function and learning of motor skills [174,175]. Dorsal striatum consists of two subregions, *i.e.* the dorsolateral striatum (DLS) and the dorsomedial striatum (DMS) (for review see [176-178]). The DMS modulates goal-directed behaviors and the neuronal activity in this area is regulated by glutamatergic projections originated from the prefrontal cortex [179-181]. The DLS is associated with habitual behaviors, and excitability in this area is driven by glutamatergic projections from the sensory motor cortex [182,183]. The shift from goal-directed behaviors to habitual behaviors are, at least in part, guided by decreased activity in projections from the orbitofrontal cortex to DMS [184]. The dorsal part of striatum is therefore of interest when studying acquisition and consolidation of behaviors. Albeit glutamate is a

major regulator of the activity of these areas, also serotonin from dorsal raphe, and GABAergic and cholinergic interneurons are important [185,186].

1.2.4 Laterodorsal tegmental area

As mention above the activity of the mesoaccumbal dopamine system is regulated by various inputs to the VTA. One crucial afferent is the cholinergic projection from the laterodorsal tegmental area (LDTg) (for review see [187-189]). Activation of the cholinergic projection from the LDTg causes an acetylcholine release, followed by an activation of nicotinic acetylcholine receptors on dopamine neurons in the VTA thus leading to a subsequent dopamine release in the NAc shell [138,139]. Optogenetic activation of these LDTg cholinergic neurons induces expression of conditioned place preference in mice and induces operant responding in rats [190,191]. Recent advances also detected that the cholinergic projections of the LDTg target the NAc and that this link is associated with reward [192]. These projections that links the LDTg to the NAc are visualized in Figure 3.

Albeit various studies have established that this cholinergic projection to the VTA is central for intake of food and addictive drugs [193-197], glutamatergic and GABAergic projections from the LDTg to the VTA also exist [190,198]. In addition, GABAergic interneurons exist and they mediate food intake [199]. These projections and interneurons may also have a role in reward processing [190,198,199], however this has been studied to a lesser extent.

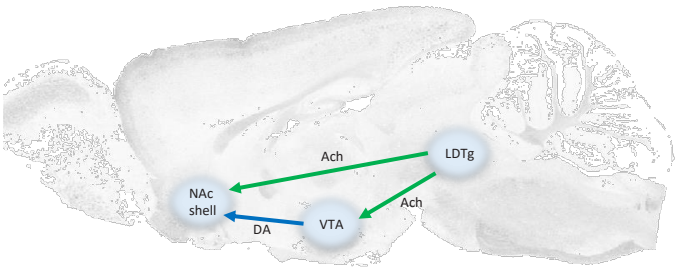

Figure 3. Projections linking the laterodorsal tegmental area (LDTg) with the nucleus accumbens (NAc) shell.
VTA=ventral tegmental area; Green line=Acetylcholine (Ach); Blue line=Dopamine (DA)

1.2.5 Nucleus of the solitary tract

The NTS is located in the brainstem and it receives innervation from vagal and splanchnic afferents from the gut (for review see [200]). The NTS is ideally located to integrate endocrine and mechanical signals from the periphery and transmit signals throughout the brain (for reviews see [53,200]). It is therefore considered as a central area for mediating homeostatic feeding (for reviews see [53,200]). Albeit historically not seen as a brain region involved in reward, recent advances have shown that peptides such as leptin, orexin and GLP-1 alter reward-related behaviors by acting in this brain region. Leptin infused into the NTS reduces hedonic feeding and infusion of a GLP-1 receptor (GLP-1R) agonist into NTS decreases reward from alcohol and palatable food [72,73,201]. Moreover, orexin infusion into the NTS increases hedonic feeding [50]. In addition, sexual interaction behaviors induce c-Fos expression in the NTS of male rodents [202,203] and noradrenergic signaling in the NTS is required for morphine reward [204]. The preproglucagon (PPG) neurons of the NTS project throughout the brain including to multiple brain areas processing reward such as the LDTg, VTA and NAc [205-207], and these projections are visualized in Figure 4. In further relevance for reward processing are the findings showing that noradrenergic neurons of the NTS project to the NAc shell [208,209]. These primary findings suggest that the NTS may be closely associated with reward processing, however this remains to be studied in detail.

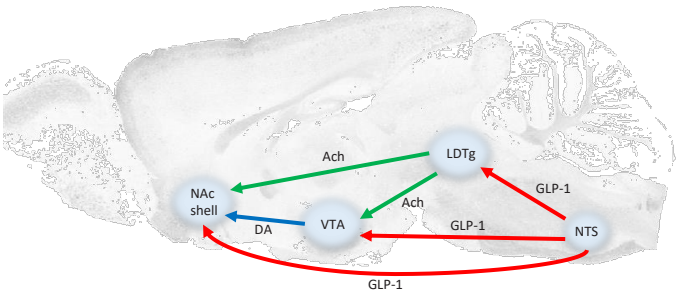

Figure 4. Projections linking the nucleus of the solitary tract with reward related areas such as the nucleus accumbens (NAc) shell, the ventral tegmental area (VTA) and the laterodorsal tegmental area (LDTg).
Green line=Acetylcholine (Ach); Blue line=Dopamine (DA); Red line=GLP-1

1.3 Appetite-regulating peptides and reward

Reward from natural rewards and addictive drugs share common neurobiological mechanisms, which mainly involve the mesolimbic dopamine system [1,128,129]. The mechanisms regulating the activity of the mesolimbic dopamine system are complex, but over the last decade extensive research has identified that appetite-regulating peptides, with origin in the gut, are important modulators of this system (for reviews see [210,211]). Indeed, appetite-regulating peptides, like ghrelin, GLP-1, neuromedin U, leptin and amylin, have all been shown to modulate reward for addictive drugs and natural rewards (for review see [210,211]). Additional research on the role of these appetite-regulating peptides on natural rewards will thus contribute to a further neurobiological understanding of these complex behaviors.

1.3.1 Ghrelin

The orexigenic peptide, ghrelin, is a 28-amino acid peptide with a post-translational octanoyl group at the third amino acid [212,213]. This acylated version of ghrelin, is often referred to active ghrelin or as herein; ghrelin (for review see [214]). Preproghrelin is encoded by the preproghrelin gene. Preproghrelin is cleaved into des-acyl ghrelin, and subsequently acylated by ghrelin-o-acyl transferase (GOAT) into ghrelin [212,213]. Ghrelin is hydrolyzed by esterases into des-acyl ghrelin [215-217]. Interestingly, one enzyme that hydrolyze ghrelin into des-acyl ghrelin is butyrylcholine esterase, and increased activity of this enzyme decreases ghrelin levels in plasma and subsequently suppresses aggression in male mice [215] and prevents re-bound obesity after caloric restriction in obese mice [217]. The synthesis and degradation of ghrelin are visualized in Figure 5.

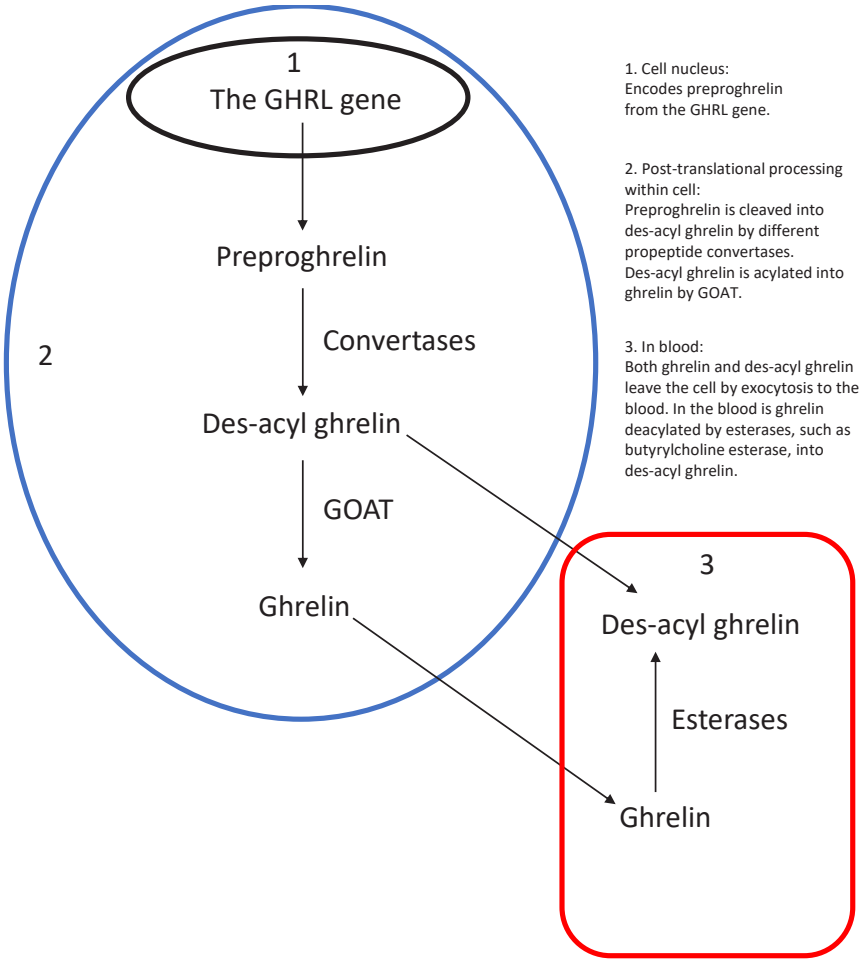

Figure 5. Schematic illustration of the synthesis and degradation of ghrelin.
GOAT=ghrelin-o-acyl transferase

Ghrelin is mainly produced and secreted from the stomach [218] and possibly in some parts of the brain [219-221]. Studies have shown that ghrelin in the periphery is released pre-prandially [222], however the plausible release in the brain has not been studied. Ghrelin circulating in the blood-stream may pass through the blood-brain barrier [223] and reach some, but not all, areas of the brain [224,225]. Ghrelin has multiple physiological effects in the body (for review see [214]) and these are to some extent summarized in Figure 6.

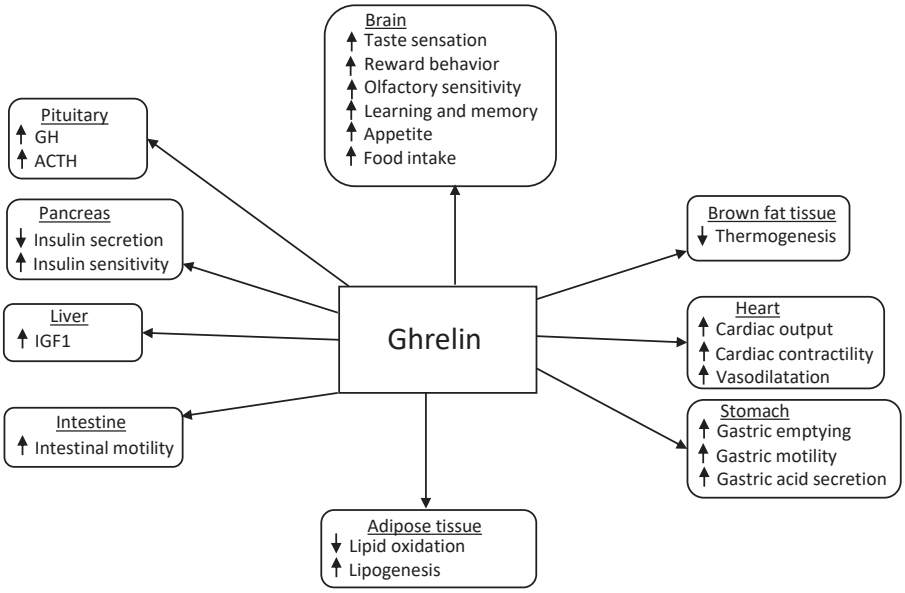

Figure 6. Some of the physiological effects of ghrelin.

The most well-known effect of ghrelin is its orexigenic properties. Indeed, ghrelin regulates homeostatic feeding behavior and appetite via ghrelin receptor (growth hormone secretagogue receptor 1A, GHSR-1A) in brain areas within the hypothalamus [222,226-229] and the brainstem [230-232]. Moreover, ghrelin infused into reward-related areas including the VTA and NAc increases homeostatic feeding [126,233-236].

1.3.2 Ghrelin, reward processing and drugs of abuse

Ghrelin has been elucidated as a potential mediator of reward processing. Indeed, both peripheral or central infusion of ghrelin activate the cholinergic-dopaminergic reward link as measured by increased accumbal dopamine release or increased locomotor stimulation [195,237-240]. The findings that GHSR-1A are expressed on dopaminergic neurons in the VTA [233], on cholinergic neurons in the LDTg [194] and densely expressed in the NAc [241,242], provide possible ghrelin sites of action. Indeed, local infusion of ghrelin into the VTA or LDTg causes robust locomotor stimulation and increases dopamine release in the NAc shell [233,239,243,244] but not core [239]. Although still loosely studied, the

underlying mechanisms through which ghrelin activates the mesoaccumbal dopamine system involve nicotinic acetylcholine receptors and N-methyl-D-aspartate receptors within the VTA, as pharmacological blockade of these receptors within the VTA suppresses ghrelin-induced NAc dopamine release [195,245]. Research over the last decade have shown that ghrelin signaling mediates various alcohol-mediated behaviors (for review see [210]). For instance, GHSR-1A antagonists reduce the intake of, preference for and motivation to consume alcohol [246-252]. In addition, genetical suppression of the GHSR-1A decreases alcohol consumption, alcohol preference and prevents alcohol-induced reward [253-255]. In addition to alcohol, suppression of the GHSR-1A attenuates reward induced by cocaine, amphetamine, morphine and nicotine [256-265] suggesting a general role of ghrelin in the regulation of reward induced by drugs of abuse.

1.3.3 Ghrelin and hedonic feeding

Substantial reports show that ghrelin signaling mediates hedonic feeding for palatable foods such as sucrose, chocolate, peanut-butter, high-fat diet, high sucrose/high fat diet and western-style cafeteria diet and thereby contributes to obesity (for review see [49]). When it comes to sucrose, an acute systemic injection of ghrelin increases, while a GHSR-1A antagonist decreases, sucrose intake in the two-bottle choice paradigm [266]. Furthermore, acute systemic injection of ghrelin increases, whereas a GHSR-1A antagonist decreases, operant progressive ratio self-administration of sucrose [266,267]. Acute intracerebroventricular injection of ghrelin increases, whereas a GHSR-1A antagonist decreases, operant progressive ratio self-administration of sucrose [267,268]. Interestingly dopamine receptors within the NAc and opioid receptors, GLP-1R and serotonin receptors within the VTA have been suggested as mediators of this ghrelin-enhanced operant progressive ratio self-administration of sucrose [268-270]. One area central for this ghrelin-sucrose link is the VTA. Indeed, intra-VTA ghrelin increases, whereas a GHSR-1A antagonist decreases, operant progressive ratio self-administration of sucrose [268-270]. Recent advances also suggest that GHSR-1A within lateral hypothalamus, ventral hippocampus and lateral septum are involved in sucrose motivation as infusion of ghrelin into these areas increase operant progressive ratio self-administration of sucrose [37,77,79]. Albeit these studies show that acute ghrelin signaling modulates the motivation to consume sucrose assessed in a simpler motor task, the role of repeated ghrelin on motivation and learning of skilled reach foraging for sucrose in a complex motor model has not been investigated. Initial studies shows that direct infusion of a GHSR-1A

antagonist into the NAc shell decreases intake of chow and peanut-butter [126,236], but the role of NAc shell-GHSR-1A for sucrose consumption is unknown.

1.3.4 Ghrelin and social behaviors

Initial studies have investigated the role of ghrelin in social behaviors with some conflicting data. The first study showed that systemic injection of ghrelin increases latency to attack another male mouse [271]. On the contrary, a second study showed that increasing the activity of the butyrylcholine esterase in plasma (which hydrolyze ghrelin) decreases, while knock-out of the enzyme enhances, spontaneous fighting in male mice [215]. In line are the data showing that systemic injection of a GHSR-1A antagonist decreases, whereas intracerebroventricular ghrelin enhances, aggressive behaviors in the resident-intruder paradigm in male mice [272]. Ghrelin increases, whereas a GHSR-1A antagonist decreases, social interaction induced place preference, but only in the heavier male rat in a social pair [273]. In line, sub-chronic GHSR-1A antagonist treatment with osmotic pumps increases latency to approach a stranger in an open field in male mice [274]. In addition, GHSR-1A knock-out mice, in comparison to wild-type, display enhanced social avoidance in response to repeated social defeat stress [275].

When it comes to sexual behaviors, one study suggested that intracerebroventricular ghrelin inhibits sexual interaction behaviors in sexually naïve male rats [276]. On the contrary, others showed that systemic ghrelin increases, whereas a GHSR-1A antagonist decreases, preference for female mice and sexual interaction behaviors in sexually naïve male mice [277]. In addition, infusion of a GHSR-1A antagonist into either the LDTg or the VTA decreases preference for female mice and suppresses sexual interaction behaviors in sexually naïve male mice [126]. Another group showed that genetic suppression of GHSR-1A decreases level changes in the level searching paradigm in sexually experienced male rats [89]. These rats also display decrease in sexual interaction behaviors when they are sexually inexperienced compared to wild-type, and these differences disappears when they acquire sexual experience [89]. In addition, infusion of a GHSR-1A antagonist decreases level changes in the level searching paradigm in sexually experienced male rats, whereas infusion of ghrelin into the mPOA decreases level changes in the level searching paradigm in sexually experienced male rats [89].

1.3.5 GLP-1

The anorexigenic peptide, GLP-1, is a 30-amino acid peptide encoded by the PPG gene [278-280]. GLP-1 is mainly produced and secreted in the intestinal L-cells after enzymatic cleavage of PPG in response to food intake [278-280]. GLP-1 is also produced in the PPG containing neurons originating in the NTS [207]. GLP-1 is foremost known for its ability to regulate blood-glucose by stimulating insulin release and inhibiting glucagon secretion [281,282]. The glucoregulatory ability of GLP-1 has led to the development of synthetic GLP-1R agonists for the treatment of diabetes type II (for review see [283]). GLP-1 also decreases both homeostatic and hedonic feeding (for review see [53]). GLP-1R agonists decrease homeostatic feeding via activation of GLP-1R in the hypothalamus, NTS, LDTg, VTA and NAc [205,206,284-291]. In addition, GLP-1R agonists also reduce body weight in rodents and in humans (for review see [53,292]). GLP-1 has in addition to glucoregulation and energy homeostasis pluripotent physiological roles in the body (for review see [293]) and these are to some extent summarized in Figure 7.

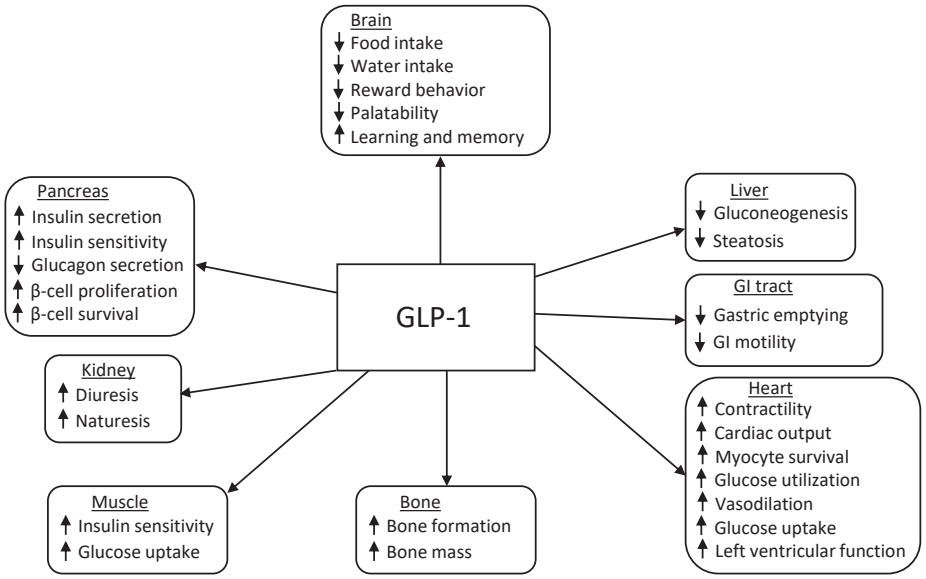

Figure 7. Some of the physiological effects of glucagon-like peptide-1 (GLP-1).

1.3.6 Clinically available GLP-1R agonists

GLP-1R agonists are approved for treatment of diabetes type II in humans (for review see [283]). These are divided into short-acting GLP-1R agonists such as exendin-4 (Ex4), and long acting such as liraglutide and dulaglutide (for review see [283]). Ex4 has longer half-life in plasma than endogenous GLP-1 [294] and is subcutaneously administered twice daily one hour before eating a meal (for review see [283]). This agonist has been extensively used preclinically to assess peripheral and central physiological functions following activation of the GLP-1R (for review see [53]). Liraglutide is subcutaneously administered once daily and is in addition to its approval to treat diabetes type II also approved for treatment of obesity (for review see [283,295]). Dulaglutide is subcutaneously administered once weekly (for review see [283]) and has therefore higher patient adherence than liraglutide [296,297]. Interestingly head-to head trial shows that liraglutide is superior to dulaglutide in weight loss in humans [298]. Some notable side-effects of GLP-1R agonists are nausea, diarrhea, hypoglycemia, pancreatitis and injection-site reactions (for review see [283]). On that note it is interesting that liraglutide-treated patients report lesser nausea than Ex4-treated patients [299].

1.3.7 GLP-1 and addictive drugs

Activation of GLP-1R have been found to attenuate reward induced by either alcohol or other drugs of abuse (for review see [210]). For instance, systemic injection of Ex4 decreases alcohol-induced reward, reduces alcohol intake and prevents relapse drinking [300,301]. Liraglutide, decreases alcohol drinking [302] and attenuates alcohol-induced withdrawal symptom (i.e. anxiety) in rats [303]. In addition, Ex4 or liraglutide decreases alcohol drinking in monkeys[304]. A general role of GLP-1R in reward processing is supported as peripheral Ex4 blocks reward induced by amphetamine, cocaine and nicotine in rodents [305-308].

1.3.8 GLP-1 and hedonic feeding

GLP-1 signaling modulates hedonic feeding as acute peripheral Ex4 decreases operant progressive ratio and operant fixed ratio self-administration of sweetened high-fat diet [309]. GLP-1R within various brain regions have been implicated in mediating motivation to palatable foods including NAc, VTA, NTS, lateral parabrachial nucleus, paraventricular thalamic nucleus,

supramammillary nucleus and lateral hypothalamus [72-76,78,205,310]. Acute and systemic injection of Ex4 reduces operant progressive ratio self-administration of sucrose [310]. When it comes to GLP-1 and sucrose have to date the NAc, VTA, NTS, paraventricular thalamic nucleus, supramammillary nucleus and lateral hypothalamus been implicated as infusion of Ex4 into these areas decreases operant progressive ratio self-administration of sucrose [72,75,76,78,310]. However, the role of repeated GLP-1R signaling on motivation and learning of skilled reach foraging for sucrose in a complex motor model is to date unknown, furthermore the role of NAc shell for such behaviors remains to be evaluated.

1.3.9 GLP-1 and social behaviors

In contrast to ghrelin [89,126,215,271-273,275-277] has the tentative role of GLP-1 in social behaviors not been investigated. Intracerebroventricular injection of amylin, another anorexigenic peptide, inhibits sexual interaction behaviors in sexually experienced male rats and decreases apomorphine-induced increase in sexual interaction behaviors [311], which suggest that GLP-1 may also inhibit sexual interaction behaviors. Sexual interaction behaviors are mediated by signals acting in the LDTg, VTA, NAc shell and NTS [126,202,203,312-317], and these brain areas could potentially be involved in GLP-1R mediated sexual behaviors.

2 AIMS OF THE THESIS

The overall aim of this thesis was to further investigate the role of ghrelin and GLP-1 in natural rewards in male rodents.

Specific aims

Paper I. To evaluate the effects of repeated ghrelin signaling on motivation and learning of skilled reach foraging in male rats.

Paper II. To investigate the effects of repeated administration of Ex4, liraglutide and dulaglutide on the motivation and learning of skilled reach foraging in male rats.

Paper III. To evaluate the effects of peripheral administration or NTS infusion of a GLP-1R agonist, Ex4, on sexual behaviors in sexually naïve male mice.

Paper IV. To pinpoint the effects of infusion of a GLP-1R agonist, Ex4, into brain areas associated with reward including the LDTg, VTA and NAc shell on sexual interaction behaviors in sexually naïve male mice.

3 MATERIALS AND METHODS

3.1 Animals

Adult outbred post-pubertal age-matched male rats from the Wistar Rcc Han strain (Envigo, Horst, Netherlands) were used in paper I and II, as this strain robustly pick sucrose pellets in the Montoya staircase test [48]. Outbred post-pubertal age-matched male mice from the NMRI strain (Charles River, Sulzfeld Germany) were used in paper III and IV. Sexually naïve male mice from this strain were selected since they display a robust and stable sexual behavior [126,277]. The neurocircuits that control sexual behaviors in sexually naïve and experienced male mice diverge but overlap to some extent (for review see [84]). The effects of Ex4 on sexual behavior in sexually experienced male mice falls outside the scope of the present thesis and are warranted for the future. In addition, we used estrus-induced post-pubertal ovariectomized sexually experienced female C57Bl/6N mice as stimuli mice in the sexual interaction paradigm (paper III-IV) and the preference for female paradigm (paper III). To ensure that stimuli females were receptive during sexual behavior experiments they were ovariectomized, and treated with estrogen and progesterone at defined time points before the interaction as extensively described in paper III-IV and in previously published articles [126,277].

The animal experiments were approved by the Swedish Ethical Committee on Animal Research in Gothenburg. All experiments were conducted in a way to minimize animal suffering and the principles of replace, reduce and refine animal experiments were considered when designing the experiments. The present behavioral studies cannot be conducted *in vitro* and performing this type of experiments in humans are neither ethical or practical. An option would be to study foraging and sexual behaviors in non-mammalian species such as the zebrafish and drosophila melanogaster (for review see [15,318]). Albeit intriguing to study behavior in these animal models, information about mammalian specific-behaviors which are shared between rodents and humans are lost when studying non-mammalian species (for review see [319]). In order to reduce the number of animals' a preliminary power calculation was performed based on data from previous studies [126,201,277]. In addition, *ex-vivo* analyses on tissues and blood collected from animals in the behavioral experiments were collected to increase the data output from every animal. In the sexual behavior experiments the stimuli ovariectomized female mice in artificial estrus were reused in multiple experiments to reduce the number of mice. The experiments were refined to mimic natural foraging behaviors and sexual behaviors as much as possible. The housing, handling, operations and injections were performed in a way to lower distress to the animals in accordance with the ethical permits.

3.2 Drugs and surgeries

The full description of drugs and chemicals used are found in paper I-IV, and are shortly summarized in Table 1. The pharmacological agents were either administered intraperitoneal (IP), subcutaneous (SC) or locally into the brain.

Table 1. Dose selection in Paper I-IV

Paper	Drug	Mechanism of action	Dose	Animal	Reason
Paper I	Rat ghrelin	GHSR-1A agonist	0.33 mg/kg (IP)	Rat	This dose activates the reward pathway and increases sucrose intake in rats [237,266,267]
Paper I	JMV2959	GHSR-1A antagonist	3 mg/kg (IP)	Rat	This dose decreases alcohol intake in rats without affecting gross behavior in rats [244,250,266]
Paper I	JMV2959	GHSR-1A antagonist	10 µg in 0.5 µl per side (NAc shell)	Rat	This dose reduces alcohol intake in rats [320] and food intake in mice without altering locomotor activity *per se* in mice [126]
Paper II	Ex4	GLP-1R agonist	1.2 µg/kg (IP)	Rat	This dose decreases alcohol intake and motivation to high-fat food without altering pica response or gross behavior in rats [300,309]
Paper II	Liraglutide	GLP-1R agonist	0.1 mg/kg (SC)	Rat	This dose decreases alcohol intake without altering gross behavior in rats [302]
Paper II	Dulaglutide	GLP-1R agonist	0.1 mg/kg (SC)	Rat	This dose decreases alcohol intake without altering gross behavior in rats[321]

Paper II	Ex4	GLP-1R agonist	0.05 µg in 0.5 µl per side (NAc shell)	Rat	This dose reduces the consumption of high-fat diet, alcohol intake and cocaine-seeking behaviour, without affecting malaise or gross behaviour in rats [205,320,322]
Paper III	Ex4	GLP-1R agonist	2.4 µg/kg (IP)	Mice	This dose decreases alcohol-related behaviors without altering locomotor activity *per se* in mice [300]
Paper III	Ex4	GLP-1R agonist	0.05 µg in 0.5 µl per side (NTS)	Mice	This dose decreases alcohol-related behaviors without altering locomotor activity *per se* in mice [323]
Paper III	Ex9	GLP-1R antagonist	5 µg in 0.5 µl per side (NTS)	Mice	This dose does not alter locomotor activity *per se* in mice [323]
Paper IV	Ex4	GLP-1R agonist	0.0025 µg in 0.5 µl per side (NAc shell)	Mice	This dose decreases alcohol-related behaviors without altering locomotor activity *per se* in mice [323]
Paper IV	Ex4	GLP-1R agonist	0.0025 µg in 0.5 µl per side (aVTA)	Mice	This dose does not alter locomotor activity *per se* in mice [323]
Paper IV	Ex4	GLP-1R agonist	0.0025 µg in 0.5 µl per side (pVTA)	Mice	This dose decreases alcohol-related behaviors without altering locomotor activity *per se* in mice [323]
Paper IV	Ex4	GLP-1R agonist	0.0025 µg in 0.5 µl per side (LDTg)	Mice	This dose reduces alcohol-related behaviors without altering locomotor activity *per se* in mice [323]

Brain surgeries were performed to allow local infusions of a pharmacological agent into a central brain nucleus (paper I-IV). Two guides were placed 1 mm below the skull surface and a cannula was at the experimental day inserted ventrally beyond the tip of the guide allowing local infusion. The stereotaxic surgery technique is old and has been extensively used in neuroscience since the 1970 [324]. A detailed description of the surgery technique is found in paper I-IV. The coordinates for surgery (Table 2) were based on a mouse and a rat brain atlas [325,326] and the drug infusions were always verified post mortem by gross observation [324]. We always stated the number of animals excluded, as a meta-analysis showed that only 15% of publications reported that they excluded rats with off-target implants [324].

Table 2. Coordinates for brain infusions in Paper I-IV

Paper	Paper I-II	Paper III	Paper IV	Paper IV	Paper IV	Paper IV
Brain region	**NAc shell** (rat)	**NTS** (mouse)	**LDTg** (mouse)	**pVTA** (mouse)	**aVTA** (mouse)	**NAc shell** (mouse)
Anterior-Posterior	+1.85 mm	-7.4 mm	- 5.0 mm	- 3.6 mm	- 3.4 mm	+ 1.4 mm
Lateral from Midline	± 1.0 mm	± 0.5 mm	± 0.5 mm	± 0.5 mm	± 0.5 mm	± 0.6 mm
Dorsal-Ventral	-7.8 mm	-4.3 mm	-3.2 mm	- 4.2 mm	- 4.3 mm	- 4.7 mm
Extension from guide	6.8 mm	3.3 mm	2.2 mm	3.2 mm	3.3 mm	3.7 mm

3.3 Behavioral, electrophysiological and biochemical experiments

3.3.1 The Montoya staircase test

A battery of behavioral assessments was set up to evaluate the effects of ghrelin signaling (paper I) and GLP-1R signaling (paper II) on skilled reach foraging in rats.

The Montoya staircase paradigm investigates the ability of ipsilateral forelimbs to reach sucrose pellets (45 mg; BioServ, Frenchtown, NJ, USA) at a descending staircase with progressively more difficult reach. This rodent model was originally used to evaluate motor function, but has also been established as a model that measures the motivation and learning of skilled reach foraging as underlying processes to motor performance in the test [47,48]. In rats without prior exposure to the Montoya staircase test, drug effects on alteration of pellets consumed and success rate provide insight into

motivational and learning processes during acquisition of the task. Whereas, in rats with an acquired skilled reach performance drug effects on alteration of pellets consumed and success rate provide insight into motivational processes. However, other underlying processes to motor performance such as motor coordination, gross motor performance, aversion processes and exploratory processes, which the drugs may alter, should also be taken into consideration when interpret the data. This test was performed in accordance with previous studies [47,48] and is described in detail in Paper I-II and Figure 8.

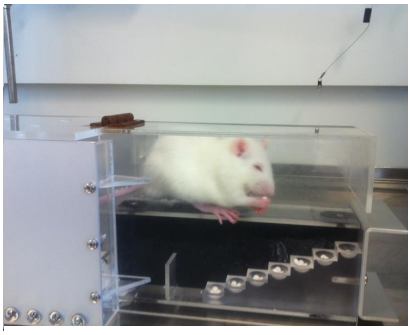

Figure 8. Montoya staircase
Before test: *The rat is exposed three times to sucrose to establish a liking for sucrose.*
During the test: *The Montoya box (9x6x30 cm) is placed inside a sound attenuating and ventilated cupboard. Three pellets are allocated to each well on both sides. The rat is placed in the box to forage for 15 minutes per session.*
Primary outcomes: *Consumed pellets; Success rate=(Consumed pellets/(Consumed pellets+Dropped pellets)*100)*

3.3.2 The Rotarod test

The rotarod test was used to stratify rats based on their learning and performance of gross motor behavior and to evaluate the effects of repeated drug treatment on gross motor performance. This test provides insight into the rat's gross motor learning and performance by measuring the latency to fall of a rotating rod. The protocol was conducted in accordance with previous studies [48,327] and is described in detail in Paper I-II and Figure 9.

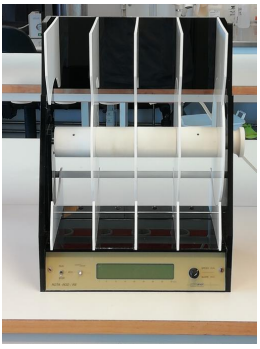

Figure 9. Rotarod test
During the test: *The rat is placed on the rod and the rod is accelerated (4-40 rpm during 5 minutes). The latency to fall of the rod is recorded.*
Primary outcome: *The mean latency to fall of the rod of four different trials per day.*
Purpose: *The mean latency to fall of the rod is used to stratify and divide rats into treatment groups for Montoya training. Gross motor behavior is recorded once per week to ensure that the drug regime does not alter gross motor behavior during the Montoya training.*

In addition, the 1-hour food-intake (paper I) and the body weight gain (paper II) were assessed to confirm previous known pharmacological effects of the drug.

3.3.3 Field potential recordings and whole cell recordings

These *ex vivo* electrophysiological recordings in brain slices from rats were conducted to study neurophysiological correlates underlying motivation and learning of skilled reach foraging (paper I and II). *Ex-vivo* electrophysiological methods are extensively used within neuroscience to evaluate the pharmacological effects of drugs on neuronal activity. In these methods some of the complexity of the intact brain is removed, thus allowing in-depth mechanistic studies within specific brain regions. Importantly, these mechanistical insight derived from brain slice electrophysiological studies should therefore not be directly translated into neuronal activity within the intact brain. For instance, population spikes are evoked by manually applied currents which may not or only partially reflect the true glutamatergic excitation pattern within the brain and substances which do not access a particular brain region *in.vivo* may still have receptors within that area and may thereby indicate findings which cannot be replicated *in-vivo.*

Brain slices were prepared by standard procedure of brain removal into ice-cold modified aCSF containing sucrose, followed by slice cutting and incubation in aCSF in 30°C for 30 minutes and thereafter stored in room temperature for the remainder of the day. The integrity of the neurons within the slice are dependent on these critical steps and variation in skills between researcher could biased the data. For detailed description see paper I and II and previous published studies describing the preparation in detail [328,329].

Field potential recordings measure evoked population spike amplitudes in the brain area using electrodes in a recording chamber perfused with aCSF. This protocol has been described before [330], in detail in Paper I-II and is summarized in Figure 10.

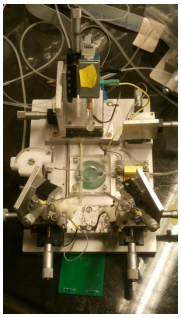

Figure 10. Ex-vivo physiology; field potential recordings *Paired pulse stimulation (50 ms interpulse interval; frequency of 0.05 Hz; Stimulus intensity of 0.01-0.04 mA) is used to evoke population spike amplitudes to half of the maximal response.*
Outcomes: *Population spike amplitudes. Paired pulse ratio= Population spike amplitude 2/ Population spike amplitude 1. Net synaptic output by increasing the stimulation strength (18-72 μA).*

Whole cell recordings measure transmitter-specific neuroadaptations within specific brain regions following acute perfusion of drug onto brain slices from rats with or without an acquired skilled reach performance (paper II) or after comparing slices from rats from different treatment groups which have acquired skilled reach behavior (paper I). Recording pipettes with internal solution with defined characteristics (Paper I and II) were used to detect currents. This protocol has been described before [329], in detail in Paper I-II and briefly in Figure 11.

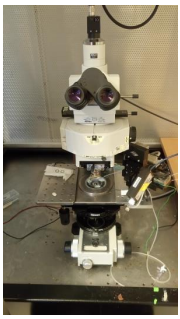

Figure 11. Ex-vivo physiology; whole cell recordings
Brain regions are identified using a 10x/0.30 objective attached to a Nikon FN-1 microscope. Medium spiny neurons (MSN) are localized using a 40x/0.80 water immersion objective. MSN are voltage clamped at -70 mV.
Outcomes: *Spontaneous inhibitory postsynaptic currents are recorded by blocking NMDA and AMPA receptor-mediated currents. Spontaneous excitatory postsynaptic currents are recorded by blocking GABA-mediated currents.*

3.3.4 Sexual interaction test

The sexual interaction test measures the full sexual encounter with an incentive ovariectomized female mouse in artificial estrus. This protocol has been described before [126,277,331,332], in detail in Paper III-IV and summarized in Figure 12. As mentioned in the introduction, the male sexual encounter with a female consists of three phases namely a pre-sexual interaction phase, a sexual interaction phase and a post-sexual interaction phase. All measured behaviors of this sexual interaction chain are described by duration (time invested in the behavior), frequency (the number of behavioral episodes) and latency (time to first behavioral episode). We scored social behaviors, such as sniffing, attending and following, as pre-sexual interaction behaviors, mounting and intromission i.e. mounting behaviors as sexual interaction behaviors and self-

grooming as a post-sexual interaction behavior in line with a previous study [333]. This sexual interaction test should be combined with other assays which measure processes such as sexual reward, sexual incentive motivation, sexual conditioned motivation, sexual aversion, odor processing and locomotion to pinpoint drug alterations in underlying processes which could contribute to the behavioral outcomes in the sexual interaction test.

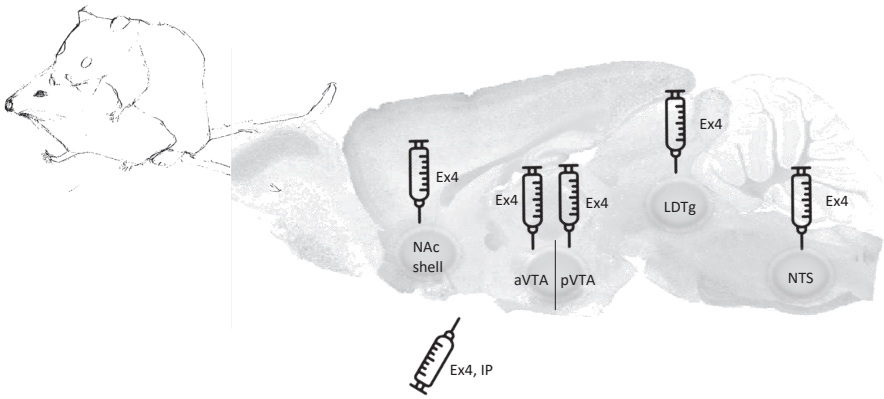

Figure 12. Sexual interaction test
The male mouse is single-housed for seven days to build up territory that enhances the probability that the mouse will engage in sexual activity. Food and nesting materials are removed from the home-cage 10 minutes before the test to remove stimuli that may trigger competitive behaviors. The male mouse is allowed to interact with the ovariectomized female mouse in artificial estrus for 20 minutes in his home-cage. The interaction is recorded and social behaviors, mounting behaviors and self-grooming behaviors are scored. Nucleus Accumbens (NAc) shell, anterior ventral tegmental area (aVTA) posterior ventral tegmental area (pVTA), laterodorsal tegmental area (LDTg), nucleus of the solitary tract (NTS), intraperitoneal injection (IP), exendin-4 (Ex4).

3.3.5 The preference for female test

The preference for female test was first described by Ågmo and is suggested to reflect sexual incentive motivation [86]. A male mouse is allowed to freely investigate an arena where a male mouse and a female mouse in artificial estrus are located on opposite side of the arena. It is important to note that the straight-arm runway test also reflects on sexual incentive motivation [87] and that the lever-pressing paradigm [90] and level searching paradigms [88,89] reflect on sexual conditioned motivation. In order to fully elucidate the drug effect on sexual motivation in rodents a battery of motivational test should therefore be used. The protocol for the preference for female test has been described before [86,126,277,334], in detail in Paper III and summarized in Figure 13.

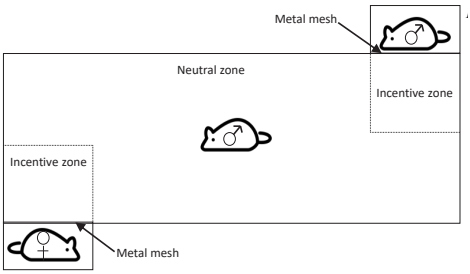

Figure 13. Preference for female test
The mouse is habituated to the arena three times before the preference test. An incentive female mouse and a male mouse are placed in cages on the opposite side of the arena behind metal meshes which creates two incentive zones. The mouse is placed on the midline and is allowed to investigate the cage for 15 minutes.
Outcomes: *Preference for female = (time in female zone /(time in female zone+time in male zone)); Total interaction time = (time in female zone+time in male zone)*

3.3.6 The olfactory preference test

The olfactory preference test measures preference to female odor by assessing how much time a mouse freely invest female soiled bedding or male soiled bedding as described previously [271,277,334], in Paper III and Figure 14. In our experiments this assay is used to evaluating one underlying process which might affect the behavioral outcomes in the sexual interaction test.

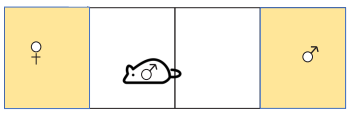

Figure 14. Olfactory preference test
After 5 minutes of habituation to an empty cage with a metal mesh floor, male and female bedding are placed on the opposite side under the metal mesh floor. The mouse is placed on the midline and is allowed to investigate the cage for 10 minutes.
Outcomes: *Time on top of female bedding; Time on top of male* bedding

3.3.7 High-pressure liquid chromatography with electrochemical detection

High-pressure liquid chromatography with electrochemical detection (HPLC-ECD) is a standard method to separate and detect molecules by their chemical and electrochemical properties [335]. We used this technique to evaluate the effects of Ex4 into the NTS on monoamines, metabolites and turnover in the

NAc, VTA and LDTg tissues *ex-vivo* from mice exposed to the sexual interaction test. The samples were prepared and analyzed using a standard procedure as described before [126,272,336] and in paper III. In order to convert the observed currents to the concentration of monoamines and metabolites, the measured values were compared to standard samples with known concentrations. HPLC-ECD has several advantages by being automated, quick and highly accurate compared to other techniques. On the downside is that HPLC-ECD has low sensitivity for certain compound, however the sensitivity is high for monoamines and their metabolites. Another limitation is that the amines are measured *ex-vivo* which means that the levels are only measured at one timepoint directly after the sexual interaction assay. An alternative approach is to measure the monoamines in brain-regions with *in.vivo* microdialysis in male mice before, during and after the encounter with the incentive female.

3.3.8 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a biochemical technique extensively used (for review see [337]). In paper III and IV we used commercially available ELISA kits which utilizing the colorimetric and competitive approach (AH Diagnostics, Stockholm) to detect corticosterone or testosterone in plasma. A spectrophotometer (Multiskan Go, Thermo Fisher) was used to detect the optical density of the colorimetric signal at a defined wavelength (405nm). In order to convert the observed optical density to the concentration of testosterone or corticosterone in the plasma samples, values were compared to a standard curve generated from standards with known concentrations. Colorimetric competitive ELISA has several advantages by being fast, standardized and commercially available. The test is highly specific and has high sensitivity, however on the downside is that other plasma constituents may affect the optical density.

3.3.9 Statistical methods

The statistical method was optimized to answer the research question. Thus, comparing behavior, electrophysiological and biochemical correlates from two treatment groups we used a two-tailed unpaired t-test. When assessing the effect of treatment on electrophysiological correlates before and after perfusion of substances we used a two-tailed paired t-test. To assess the treatment effect when using more than two treatments on behavior and biochemical correlates

we used a one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. When evaluating the effects of drug on behavior at multiple sessions or electrophysiological recordings over time we used a two-way repeated ANOVA. All analyses were conducted in and all graphs were generated with the GraphPad Prism Software (GraphPad Software Inc; CA, USA).

4. RESULTS

4.1 Paper I

In this paper, we investigated the effects of repeated activation or attenuation of GHSR-1A on skilled reach foraging in the Montoya staircase test, and the main findings are summarized in Figure 15. We showed that repeated peripheral administration of the GHSR-1A antagonist, JMV2959, decreased consumption of sucrose pellets in rats with an acquired skilled reach performance, and that the effect was higher in rats with a higher acquired skilled reach performance. In addition, JMV2959 into NAc shell decreased sucrose pellet consumption in rats with an acquired skilled reach performance. On the contrary, repeated ghrelin to rats with an acquired skilled reach performance did not influence on the number of pellets consumed. Repeated ghrelin increased, while JMV2959 decreased, the consumption of sucrose pellets when administered to rats throughout the entire Montoya training. In addition, ghrelin did not, whereas JMV2959 decreased, the success rate in these rats. *Ex-vivo* recordings in NAc shell from these rats showed that repeated ghrelin in combination with Montoya training decreased the NAc shell output via increased frequency of inhibitory post-synaptic currents. We also showed that neither ghrelin or JMV2959 did alter gross motor behaviors in the rotarod test. These data suggest that repeated suppression of the GHSR-1A decreased the motivation of skilled reach foraging and pinpointed suppression of NAc shell-GHSR-1A as a contributing mechanism to this behavior. In addition, these data indicated that ghrelin increased, whereas JMV2959 decreased, the motivation and learning of skilled reach foraging during the acquisition of the behavior and that alteration in inhibitory neurotransmission within NAc shell may tentatively be an underlying mechanism.

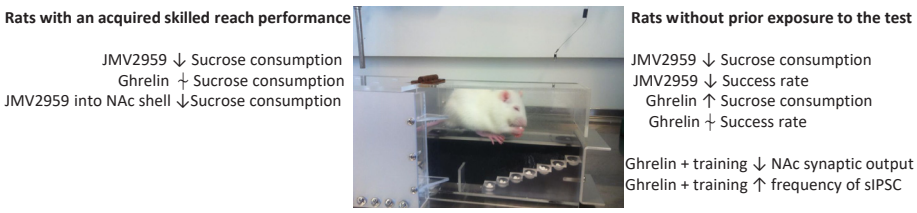

Figure 15. Schematic illustration of the main findings in Paper I.
Nucleus accumbens (NAc), sIPSC=spontaneous inhibitory post-synaptic currents, ┼=no difference, ↓=decrease, ↑=increase

4.2 Paper II

In the second paper, we investigated the effects of repeated activation of GLP-1R on skilled reach foraging in the Montoya staircase test, and the outcomes are summarized in Figure 16. We found that, in opposed to ghrelin, systemic administration of Ex4 or liraglutide, but not dulaglutide, decreased the consumption of sucrose pellets in rats with an acquired skilled reach performance. *Ex-vivo* field potential recordings in slices from rats with an acquired skilled reach performance showed that acute perfusion of Ex4 or liraglutide, but not dulaglutide, onto NAc shell brain slices suppressed evoked population spike amplitudes. Infusion of Ex4 into NAc shell, to rats with an acquired skilled reach performance, reduced the consumption of sucrose pellets. Repeated dulaglutide, but not liraglutide or Ex4, to rats without prior Montoya experience, increased the success rate in this paradigm. We also showed that neither Ex4, liraglutide or dulaglutide did alter gross motor behaviors in the rotarod test. These data highlighted that Ex4, liraglutide and dulaglutide provoked different responses on skilled reach foraging and neurotransmission. These data indicated that Ex4 or liraglutide decreased the motivation of skilled reach foraging in rats with an acquired skilled reach performance tentatively via NAc-shell-GLP-1R dependent mechanisms and that dulaglutide enhanced the learning of skilled reach performance during acquisition of the behavior.

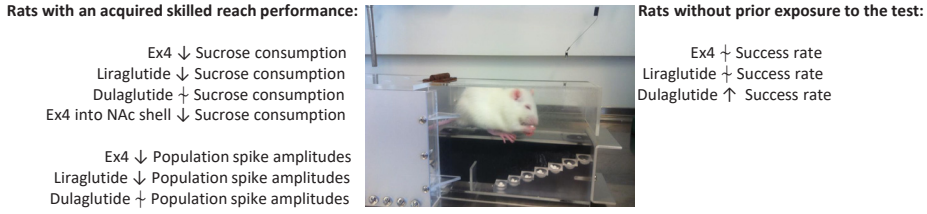

Figure 16. Schematic illustration of the main findings in Paper II.
Nucleus accumbens (NAc), exendin-4 (Ex4), ┼=no difference, ↓=decrease, ↑=increase

4.3 Paper III

In the third paper, we investigated the role of GLP-1R activation on another domain of natural rewards namely social behaviors, more precisely sexual behaviors in sexually naïve male mice and the main findings are shown in

Figure 17. An acute systemic injection of Ex4 reduced sexual interaction behaviors, but did not alter the preference for females or female bedding. More specifically, systemic Ex4 injection reduced social behaviors, mounting behaviors and self-grooming behaviors. On the contrary, six days of repeated treatment with Ex4 did not alter any of these sexual behaviors. In an attempt to define brain areas important for this GLP-1R mediated behavior, we found that local infusion of Ex4 into the NTS reduced social behaviors, mounting behaviors and self-grooming behaviors. *Ex-vivo* biochemical analysis from these mice showed that Ex4 into the NTS increased the serotonin levels and dopamine turnover in NAc. Furthermore, analysis of plasma from sexually exposed mice showed that NTS-Ex4 or systemic Ex4 increased the plasma levels of corticosterone, but not testosterone. To link the peripheral Ex4 effects with activation of GLP-1R in NTS, we showed that infusion of a GLP-1R antagonist, exendin-3 (9-39) amide (Ex9), into the NTS reversed some, but not all sexual behaviors, suppressed by peripheral Ex4. These data suggested for the first time that the GLP-1R system was involved in suppression of sexual interaction behaviors with a receptive female in sexually naïve male mice and pinpointed that NTS-GLP-1R were partly involved in this behavioral effect.

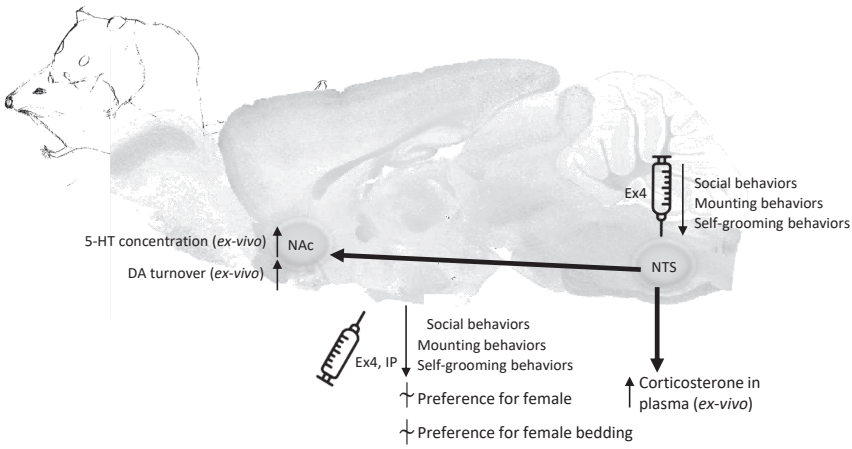

Figure 17. Schematic illustration of the main findings in Paper III.
Nucleus accumbens (NAc), nucleus of the solitary tract (NTS), intraperitoneal injection (IP) serotonin (5-HT), dopamine (DA), exendin-4 (Ex4), ┼=no difference, ↓=decrease, ↑=increase

4.4 Paper IV

In this forth paper, we explored more brain-region specific GLP-1R populations that contributed to the suppression of sexual interaction behaviors in sexually naïve male mice, and the main findings are demonstrated in Figure

18. We demonstrated that infusion of Ex4 into the LDTg decreased social behaviors and mounting behaviors in male mice. Infusion of Ex4 into the pVTA, but not the aVTA, reduced social behaviors and mounting behaviors in male mice. Ex4 infused into the NAc shell decreased social behaviors, but not mounting behaviors, in male mice. In addition, self-grooming behaviors were not altered following infusion of Ex4 into any of these brain areas. These data pinpointed that activation of GLP-1R within reward related areas decreased sexual interaction behaviors in a brain site specific manner in sexually naïve male mice.

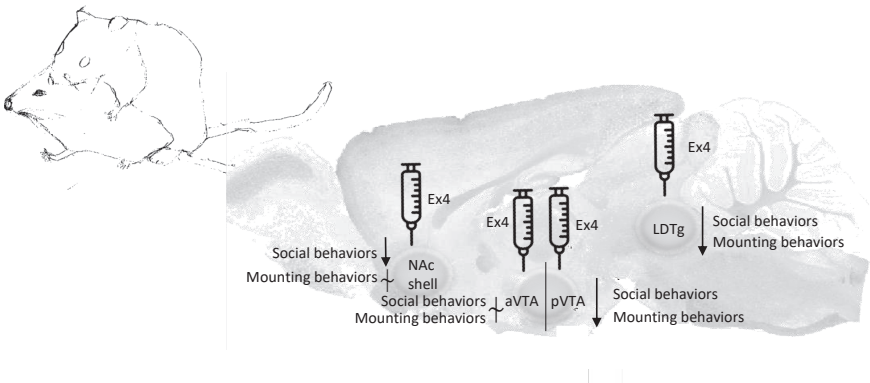

Figure 18. Schematic illustration of the main findings in Paper IV.
Nucleus accumbens (NAc) shell, anterior ventral tegmental area (aVTA), posterior ventral tegmental area (pVTA), laterodorsal tegmental area (LDTg), exendin-4 (Ex4), ┼*=no difference,* ↓*=decrease,* ↑*=increase*

5. DISCUSSION

The modulatory role of appetite-regulating peptides in reward processing have been described (for review see [210]). The studies included in the present thesis extend on these prior findings as they show that repeated activation of GHSR-1A or GLP-1R mediates the motivation and learning of skilled reach foraging in male rats and that activation of GLP-1R suppresses sexual interaction behaviors in sexually naïve male mice.

5.1 Gut-brain axis and motivation of skilled reach foraging

When the sucrose consumption and success rate of rats with an acquired skilled reach performance are monitored, the motivation to perform this behavior can be evaluated. We here identified that repeated systemic injections with JMV2959, Ex4 or liraglutide decrease the consumption of sucrose pellets in rats with an acquired skilled reach performance. These data indicate that both ghrelin and GLP-1 pathways modulate the motivation of skilled reach foraging. In support for this contention are the findings from operant motor models, showing that a single injection of either GHSR-1A antagonist [249,250,266,267] or a GLP-1R agonist [302,309,310] reduces the lever pressing for palatable foods and alcohol. We further reveled that local infusion of either JMV2959 or Ex4 into the NAc shell of rats with an acquired skilled reach performance reduces the number of pellets consumed. Collectively, this suggests that the motivation of skilled reach foraging is partly mediated through NAc shell dependent mechanism. This is in line with previous studies showing that ghrelin signaling within NAc shell-GHSR-1A mediates the intake of chow, peanut butter and alcohol [126,236,320]. When it comes to NAc shell-GLP-1R, previous data pinpointed that an infusion of a GLP-1R agonist into the NAc shell decreases the intake of either sucrose [310] or high fat diet [205]. In addition, activation of GLP-1R within NAc-shell attenuates other reward-related behaviors including alcohol-related behaviors, alcohol drinking, cocaine-seeking behavior and oxycodone-seeking behavior [320,322,323,338]. Our electrophysiological recordings further support that NAc shell is a target for Ex4 or liraglutide and provides a tentative insight into mechanisms that might modulate the consumption of sucrose pellets in rats with an acquired skilled reach performance. Indeed, Ex4 or liraglutide decrease the evoked population spike amplitudes in NAc shell slices from rats with an acquired skilled reach performance, tentatively through activation of putative GLP-1R located on

pre-synaptic glutamatergic inputs to the NAc shell [290] which are coupled to Gαi/O protein associated with decreased excitatory responses [339,340].

We further found that both JMV2959 and Ex4 reduces the consumption of sucrose pellets more profoundly in high versus low performing rats, indicating that the outcomes of these appetite-regulating peptides depend on prior learning of the task. On a similar note, repeated ghrelin increases consumption of sucrose pellets in rats with low, but not high, acquired skilled reach performance, indicating a ceiling effect in this paradigm. Supportively for a pharmacological ceiling effect for exogenous ghrelin is the data, evaluating the role of ghrelin in sexual conditioned motivation, showing that ghrelin into the VTA does not further increase level changes, whereas pharmacological suppression of GHSR-1A within the VTA decreases level changes, in the level searching paradigm in sexually experienced male rats [89]. Divergent effects of JMV2959 and Ex4 on low and high consumers are also evident when it comes to alcohol intake where JMV2959 or Ex4 reduces alcohol intake in high, but not low, alcohol consuming rats [250,302,341].

5.2 Gut-brain axis and learning of skilled reach foraging

With a different design of the Montoya staircase test, where rats are treated throughout the acquisition of the task, the learning of skilled reach foraging can be assessed by monitoring consumed pellets and success rate. Here, we found that repeated systemic injections of ghrelin increases, whereas JMV2959 decreases, the consumption of sucrose pellets in rats without prior Montoya experience. In addition, in these rats JMV2959 decreases, whereas ghrelin does not alter, success rate. As enhanced learning is hard to dissect from apparent motivation, we thus speculate that the ghrelin system both enhances motivation and learning of skilled reach foraging during acquisition of the task. Our *ex-vivo* recordings from these rats show that ghrelin in combination with training reduces the NAc shell output by selectively increasing sIPSC frequency, indicating that repeated ghrelin during acquisition of the task causes neuroadaptations in the GABAergic system in the NAc shell. These neuroadaptations in the GABAergic system may influence the motivational aspects to consume sucrose pellets. This contention is supported by the data showing that infusion of a $GABA_A$ receptor agonist into NAc shell increases chow intake [342-344]. As ghrelin signaling interacts with learning and memory processes by acting in other brain regions including the hippocampus and

amygdala [39,345] our electrophysiological data elucidate one of many possible mechanisms involved in ghrelin-induced learning of skilled reach foraging.

We also identified that repeated systemic injection with dulaglutide to rats without prior Montoya experience, increases the success rate during acquisition of the task. This improved performance driven by an increase in success rate may be linked to enhanced learning of skilled reach foraging. As GLP-1R agonists decrease operant self-administration of palatable foods [309,310] and improve reference memory and enhance associative and spatial learning in other learning paradigms [346,347], thus supporting that dulaglutide enhances learning of skilled reach foraging rather than augments motivational processes. As GLP-1R within the hippocampus control learning processes and motivated behaviors [38,40,346,347], this area may contribute to the effects of dulaglutide on learning of complex behaviors and are a research directive for upcoming studies.

5.3 Diverse pharmacological effects of GLP-1R agonists on skilled reach behavior

The present findings revealed that the behavioral and electrophysiological outcomes of the tested GLP-1R agonists varies. These divergent findings of various clinical available GLP-1R agonists have potential implications and this needs to be discussed in the context of our study. Ex4, liraglutide and dulaglutide have different pharmacokinetic properties, such as difference in half-life and distribution volume, in patients [348-352]. The difference in half-life was accounted for in the present study, and should always be accounted for, when selecting dose and dose interval of Ex4, liraglutide and dulaglutide. Preclinical studies support that Ex4 and liraglutide have discrete ability to penetrate and activate brain regions [353-355] whereas no study has assessed the distribution pattern of dulaglutide in the brain. The difference in distribution volume and distribution pattern in the brain may explain some variation in the behavioral effects of these drugs in the present study. These differences may also explain variation in efficacy and side-effects of these drugs in patients, where for example liraglutide-treated patients report lesser nausea than Ex4-treated patients [299]. Another mechanism that may explain the variation in effects on both behavior and neurotransmission are diverse ability of Ex4, liraglutide and dulaglutide to recruit different G-proteins coupled to intracellular pathways [340,356,357]. Indeed, Ex4 recruits the Gαi/O pathway to a higher degree than liraglutide [340] and differences in the effect by these agonists on hippocampal neurotransmission exist [358,359]. To this date, no study has

assessed the biased signaling of dulaglutide on the GLP-1R and in light of our data this is a tentative research directive.

5.4 GLP-1R signaling and various sexual behaviors

Different behavioral tests can be used to assess the effects of GLP-1R activation on various sexual behaviors. By means of these we showed that an acute systemic injection of Ex4 in a sexual interaction paradigm with an estrus female reduces social behaviors, mounting behaviors and self-grooming behavior in sexually naïve male mice, whereas it does not influence the preference for females or female odor in two different preference assays. Collectively, this indicate that peripheral Ex4 suppresses natural rewards. This contention is supported as Ex4 reduces other natural rewards such as sucrose and high-fat diet [309,310]. In addition, Ex4 attenuates reward induced by addictive drugs [300,305]. In addition to GLP-1, a role of appetite-regulating peptides in mediating sexual behaviors are supported as ghrelin, amylin, leptin, orexin and neuropeptide Y also regulate sexual behaviors [89,107,108,110,126,277,311,360]. The GLP-1 system affects sexual interaction behaviors and preference for female mice differently. Interestingly, endogenous orexin decreases sexual interaction behavior [107] but does not alter preference for female in sexually naïve male rats [360] and neuropeptide Y reduces sexual interaction behaviors without altering the proportion of rats showing erection in sexually experienced rats [108]. We thereby suggest that the GLP-1 system in sexually naïve male mice control neurocircuits that guides sexual interaction behaviors but not preference for female mice or female bedding. Interestingly, ghrelin increases, whereas JMV2959 decreases, both preference for females and sexual interaction behaviors in sexually naïve male mice [126,277] , indicating that the modulation of GLP-1 and ghrelin over sexual behaviors diverge to some extent.

5.5 Tolerance effects following repeated treatment with GLP-1R agonists

Interestingly, an acute injection of Ex4 reduces, whereas six days of repeated Ex4 injections do not alter sexual interaction behavior in sexually naïve male mice. This might indicate that there is a tolerance effect to some of the behavioral effects of Ex4. A tolerance pattern has also been observed when it

comes to Ex4's ability to reduce alcohol intake in rats and monkeys [300,304] and induce anxiety-like behaviors in rats [361]. However, this was not observed when it comes to the motivation and learning of skilled reach foraging (paper III) or to food-intake behavior (for review see [53]) and to glucose-homeostasis [362]. Experiments in cell culture have shown that repeated GLP-1R activation with pharmacological agents, such as Ex4, desensitize the GLP-1R in cell cultures [362-365], indicating one mechanism that may underly this tolerance pattern. A lower dose of Ex4 to mice decreases alcohol drinking without inducing a tolerance with repeated dosing [301], indicating that the tolerance effect *in-vivo* might be dose-dependent.

5.6 Brain region specific modulation of sexual interaction behaviors

By means of local injections of Ex4, we here found that activation of GLP-1R in a brain site specific manner suppresses various behaviors of the sexual interaction chain in sexually naïve male mice as visualized in Figure 19.

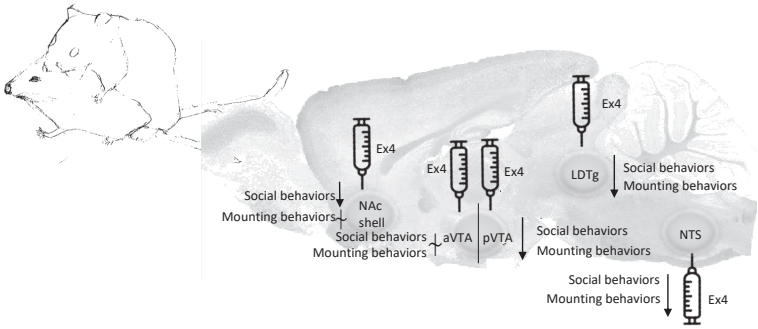

Figure 19. Exendin 4 (Ex4) decreases sexual interaction behaviors in a brain site specific manner in sexually naïve male mice.
Nucleus accumbens (NAc) shell, anterior ventral tegmental area (aVTA), posterior ventral tegmental area (pVTA), laterodorsal tegmental area (LDTg), nucleus of the solitary tract (NTS), ┼=no difference, ↓=decrease, ↑=increase

5.6.1 Nucleus of the solitary tract

Infusion of Ex4 into the NTS suppresses behaviors of the entire sexual interaction chain, namely social behaviors, mounting behaviors and self-grooming behaviors. A role of the NTS in sexual behaviors are supported as sexual interaction behaviors increases c-Fos expression in the NTS in male prairie voles and male Syrian hamsters [202,203]. A general role of GLP-1R within

the NTS in reward processing might be suggested as Ex4 into the NTS suppresses reward from alcohol and palatable food [72,73,201]. The location of GLP-1R within the NTS cannot be determined from the present study. However, as the GLP-1R are not directly expressed on post-synaptic PPG-neurons [366] other GLP-1R populations located pre-synaptic on astrocytes [284], on vagus nerve afferents (for review see [367] or on collaterals of the PPG neurons [368] might putatively mediate the effects of NTS-Ex4 on sexual interaction behaviors.

Here we found that NTS-Ex4 induced alterations in monoamine systems in NAc tissues, indicating that the behavioral outcomes following activation of GLP-1R within the NTS might involve neurotransmission in the NAc. Supportively these alterations in the NAc, increased serotonin levels and dopamine turnover, have been associated with a decrease in sexual interaction behaviors in rodents [4-6,97,98]. In addition, NTS-Ex4 increases corticosterone in plasma, but not testosterone, thus highlighting another underlying pathway that might contribute to the ability of Ex4 into the NTS to reduce sexual interaction behaviors. Indeed, enhanced plasma levels of corticosterone is associated with a decrease in sexual interaction behaviors [91,92,94,96]. As corticosterone levels in plasma are linked to an activated stress system (for review see [369]) we speculate that a GLP-1R mediated activation of the stress system [370-373] may contribute to the decrease in sexual interaction behaviors.

We further showed that Ex9 into the NTS blocks the decrease in social behaviors and self-grooming behaviors as well as the increase in corticosterone levels in plasma induced by systemic Ex4. On the contrary, Ex9 into the NTS does not reverse the decrease in mounting behaviors induced by systemic Ex4. Collectively, these findings suggest that activation of GLP-1R within the NTS only explain some of the ability of systemic Ex4 to reduce behaviors of the sexual interaction chain.

5.6.2 Laterodorsal tegmental area

GLP-1R activation within the LDTg decreases both social behaviors and mounting behaviors, but not self-grooming behaviors, in sexually naïve male mice. A role of the LDTg in sexual behaviors is supported as lesion of the LDTg suppresses sexual interaction behaviors [127] and JMV2959 into this area reduces, whereas ghrelin increases, mounting behaviors, in sexually naïve male mice [126]. We speculate that activation of GLP-1R within the LDTg might reduce such behaviors via a reduced activity of projections to the NAc shell [192,374] or indirectly via the VTA to NAc shell [190,191], thus activation of GLP-1R-

LDTg [206,207] decreases sexual interaction via inhibition of these neurocircuits. In line are the data showing that Ex4 into the LDTg reduces, whereas Ex9 into the LDTg increases, food intake [206], suggesting a general role of LDTg-GLP-1R in mediating natural rewards. As Ex4 into LDTg also decreases alcohol-related behaviors [323] a general role of LDTg-GLP-1R in reward processing might be suggested. In support are the data showing that cholinergic neurons of the LDTg target both the VTA and the NAc shell and that the activity of these neurons control reward-related behaviors [190-192,374,375]. Collectively, we showed for the first time that activation of GLP-1R within the LDTg suppresses social behaviors more precisely sexual interaction behaviors in sexually naïve male mice.

5.6.3 Ventral tegmental area

Ex4 infused into sub-regions of the heterogenous VTA [143,376] showed that activation of GLP-1R within pVTA, but not aVTA, suppresses both social behaviors and mounting behaviors in sexually naïve male mice. These data are supported by a previous study showing that Ex4 into pVTA, but not aVTA, suppresses alcohol-mediated behaviors in rodents [323]. On the other hand, infusion of a GHSR-1A antagonist into aVTA decreases mounting behaviors in sexually naïve male mice [126]. A general role of the VTA in sexual interaction behaviors are extensively described in the literature (for review see [81]). Activation of dopaminergic neurons of the VTA by sexual interaction behaviors [312-315] which are associated with dopamine release in the NAc shell and subsequently reward [3-6] are one pathway through which VTA dopamine controls sexual interaction behaviors. This study did not assess the exact location of the GLP-1R, however a previous study showed that both neurons and astrocytes within the VTA are Ex4-responsive [377] and an electrophysiological study has postulated that the GLP-1R is putatively located on presynaptic glutamatergic neurons within the pVTA that control dopamine neurons [289]. We therefore speculate that GLP-1R activation prevents sexual activity to activate dopamine neurons projecting to the NAc shell. A general role of pVTA-GLP-1R in mediating natural rewards are supported as Ex4 suppresses intake of sucrose, chow or high-fat diet [205,289,310,378]. As Ex4 into pVTA also decreases seeking or self-administration of cocaine [377,379] and suppresses alcohol-mediated behaviors in rodents [323] a general role of pVTA-GLP-1R in reward processing should be suggested. Collectively, we postulated for the first time that activation of GLP-1R within the pVTA decreases behaviors of the sexual interaction chain in sexually naïve male mice.

5.6.4 Nucleus accumbens shell

Ex4 infusion into the NAc shell decreases social behaviors, but not mounting behaviors or self-grooming behaviors in sexually naïve male mice. As activation of NAc-GLP-1R reduces another natural reward, namely operant progressive ratio self-administration of sucrose [310] and skilled reach foraging for sucrose (Paper II), this indicates that NAc shell-GLP-1R suppresses natural rewards from various domains differently. Interestingly, Ex4 into NAc shell also decreases alcohol and cocaine-related behaviors [56,320,322]. In support of discrepancy between control of sexual interaction behaviors from other reinforcers within the NAc shell are the data showing that infusion of a GHSR-1A antagonist into the NAc shell does not alter sexual interaction behavior in sexually naïve male mice [126], whereas a GHSR-1A antagonist into the NAc shell decreases intake of chow [236], peanut-butter [126], sucrose (Paper I) and alcohol [320] in rodents. It has been suggested that the dopamine system within the NAc controls anticipatory rather than consummatory aspects of sexual behavior [316,380]. As our data showed that Ex4 into NAc shell decreases social behaviors, but not mounting behaviors, one might therefore speculate that NAc shell-GLP-1R mediates anticipatory rather than consummatory aspects of sexual behavior via interaction with dopamine. Collectively, we postulated for the first time that activation of GLP-1R within the NAc shell decreases social behaviors with an estrous female in sexually naïve male mice.

5.7 Discussion about limitations with the current studies

There are some general limitations in our studies evaluating skilled reach foraging and sexual behaviors that needs to be discussed. We argue that reward processes are the main construct involved in driving the behavioral outcomes. However other constructs such as anxiety-like behaviors, depression-like behaviors, impulsivity and stress that GLP-1R signaling and ghrelin signaling are influencing might also contribute to the behavioral outcomes [40,361,372,381-384]. It is therefore interesting that systemic Ex4 or NTS-Ex4 increases corticosterone in plasma in male mice exposed to an estrous female whereas the same increase is not evident after infusion of Ex4 into the LDTg, VTA or NAc shell. This indicates that activation of stress pathways might have contributed to the behavioral suppression following systemic Ex4 and NTS-Ex4, but not in the suppression of behaviors of the sexual interaction chain following activation of GLP-1R within the LDTg, pVTA and NAc shell. Likewise, drug-altered locomotor activity and gross motor behavior would

definitely alter behavioral outcomes. However, this appears very unlikely given that we showed that gross motor behaviors in rats are unaltered on the rotarod following JMV2959, ghrelin, liraglutide, dulaglutide and Ex4 treatments and that the Ex4 doses, at any administration routes used, do not alter locomotor activity in mice [201,300,305,323,385]. Factors such as sex would probably affect the outcomes in the behavioral assays [76,78,98,386-390] and that female animals were not included are a major limitation in the present studies. In addition, nausea may affect the behavioral outcomes of GLP-1R agonists [391]. However, this appears less likely since we use doses and treatment regimens to limit this confounder [72,73,206,300,302,309,321,322,377].

5.8 Concluding remarks

The data presented in this thesis arrived from experiments evaluating the role of ghrelin and GLP-1 in controlling natural rewards. Previous studies have shown that acute activation of the GHSR-1A or the GLP-1R mediates operant self-administration of sucrose. We herein showed for the first time that repeated activation of the ghrelin system or the GLP-1 system mediates the motivation and learning of skilled reach foraging in rats via tentative accumbal mechanisms. Our novel findings, that activation of the GLP-1 system suppresses behaviors of the sexual interaction chain in sexually naïve male mice via brain site specific mechanisms involving the NTS, LDTg, pVTA and NAc shell, support a role of the GLP-1R in social behaviors.

6 FUTURE DIRECTIVES

The data in this thesis provide additional insight into how appetite-regulating peptides mediate natural rewards. GLP-1R agonists are used in the clinic for treatment of diabetes type II and obesity (for reveiew see [283,295]). Thus, highlighting the value for the clinic to understand how these pharmacological agents alter neurobiological mechanisms and thereby causes various pharmacological effects in patients.

The findings that repeated ghrelin signaling increases, while a repeated GLP-1R agonist decreases, the motivation of skilled reach foraging via accumbal mechanisms should motivate research into repeated administration of GHSR-1R antagonist, GLP-1R agonist or the combination on motivation in more established operant models of reinforcements for natural rewards (for review see [46]). Interestingly, combining Ex4 with JMV2959 into the NAc shell decreases alcohol intake in female rats [320]. The data from this thesis provide some mechanistical insight into NAc shell-GHSR-1A activation and NAc shell-GLP-1R activation, additional insight into the molecular mechanisms that drives the GLP-1R or GHSR-1A dependent modulation of reward from sucrose and other reinforcers are needed. Many regions including but not limited to the VTA and the LDTg have been implicated in feeding-related behaviors following activation of GLP-1R or activation of GHSR-1A [37,72-79,242,310], upcoming studies should therefore focus on additional brain targets and how these brain targets are connected. In addition, more research into how the endogenous ghrelin system and the GLP-1 system, in opposed to exogenous administration of these peptides, are influencing natural rewards are needed.

The data showing that ghrelin in combination with training putatively causes neuroadaptations in the GABAergic system within the NAc shell, provided one tentative mechanism underlying the ghrelin-induced increase in motivation and learning of skilled reach foraging. Future studies should also assess additional brain areas, such as the amygdala and the ventral hippocampus [39,345], that may mediate the learning of complex behaviors induced by ghrelin. The finding that dulaglutide enhances learning of skilled reach foraging needs to be replicated in other learning models. It is also warranted to study the mechanisms for this effect, a tentative site of action is the ventral hippocampal-GLP-1R which are involved in learning processes and motivated behaviors [38,40,346,347]. It is also noteworthy that GLP-1R agonists and ghrelin are evaluated as potential treatments of neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (for review see [392-394]). In addition to the vast literature showing neuroprotective effects of GLP-1R agonists and ghrelin in rodents

[346,347,395-399], a recent RCT study in humans showed that Ex4 indeed improves motor symptoms in Parkinson's disease [400].

The findings that various GLP-1R agonists affected behaviors differently depending on the context in the Montoya staircase, warrants further studies into the pharmacological effects of different GLP-1R agonists. As previous studies have shown that GLP-1R agonists exert biased agonism on the GLP-1R this may be an underlying cause to the variability in pharmacological effects seen with different agonists [340,356,357]. Interestingly, also agonists on the GHSR-1A exhibit biased agonism and recruit diverse intracellular pathways contributing to difference in cellular responses [401]. The findings that liraglutide and Ex4 have different ability to access and activate brain regions [353-355] emphasize that future studies need to assess the distribution pattern for dulaglutide. Interestingly, after systemic treatment with Ex4, the brain concentration of Ex4 are only 2-3% of the levels in plasma in both rats [402] and humans [400]. As GLP-1R agonists have emerging therapeutic potential in various brain disorders there is thus a need to develop GLP-1R agonists with higher brain penetrance which could result in higher efficacy and fewer side-effects. Future studies should also investigate where Ex4, liraglutide and dulaglutide act in the brain with use of immunofluorescence methods. Initial studies show that peripheral Ex4 reaches the NTS, LDTg, pVTA and NAc shell and localize and internalize in both neurons and astrocytes [206,284,322,377].

Feeding-regulating peptides have previously been implicated in sexual behaviors. Indeed, both ghrelin and amylin mediate sexual behaviors [89,126,277,311]. This thesis shows for the first time that the GLP-1 system is involved in sexual behavior and the effect is specific to sexual interaction. Future studies should further pinpoint which brain pathways are shared and divergent in the control of different aspects of sexual behavior including odor preference, female preference, pre-sexual interaction behaviors, sexual interaction behaviors and post-sexual interaction behaviors. Upcoming studies should share additional insight into the molecular and neural mechanisms following GLP-1R-dependent suppression of sexual interaction behavior. Given that Ex4 increases serotonin and dopamine turnover in NAc *ex*-vivo, a tentative research directive is to use *in-vivo* microdialysis to measure how Ex4 affects the levels of monoamines in NAc before, during and after the novel interaction with an incentive female. Previous studies have shown that Ex4 into the NAc core decreases natural rewards such as intake of palatable food and chow [205,291] indicating that NAc core-GLP-1R may be involved in sexual interaction behaviors, a future research directive. Additional brain areas such as the mPOA, ventromedial hypothalamus, lateral hypothalamus, paraventricular nucleus, amygdala, bed nucleus of stria terminalis, periaqueductal gray, central tegmental field and dorsal raphe (for review see

[80,81,85]), that might be associated with GLP-1R mediated suppression of sexual behaviors should also be investigated. Interestingly, ghrelin infused into the mPOA decreases level changes in the level searching paradigm whereas a GHSR-1A antagonist into the VTA decreases level changes in the same paradigm in sexually experienced male rats [89], indicating that appetite-regulating peptides, at least ghrelin, have opposite roles on sexual behaviors depending on brain circuit modulated. If this is true for other appetite-regulating peptides remains to be elucidated.

Importantly, future studies need to investigate the potential role of GLP-1R agonists for mediating sexual behavior in sexually experienced male rodents, as the neural control over sexual behaviors in naïve and experienced rodents diverge to some extent [84]. For instance, genetic suppression of the GHSR-1A suppresses sexual interaction behaviors in sexually naïve male rats, but this is not evident when they acquire sexual experience [89] and lesion of the LDTg decreases sexual interaction behaviors in sexually naïve male rats, but not in sexually experienced male rats [127]. It is even more complex, as lesion of the NAc, before mating experience, produces deficits in sexual interaction behaviors that sustain after acquisition of the behavior [118], whereas lesion of the NAc in rats, with mating experience at the time of operation, show no deficit in sexual interaction behaviors [380]. Future research directives are therefore to 1) evaluate the role of Ex4 on sexual conditioned motivation and sexual interaction behaviors in sexually experienced male rodents and 2) the effects of repeated Ex4 on sexual interaction behaviors on more than one sexual interaction session to study the effects of activation of the GLP-1R on the acquisition of sexual conditioned motivation and sexual interaction behaviors in male rodents.

The effects of GLP-1R activation on female sexual incentive motivation, sexual conditioned motivation and sexual interaction behaviors need to be assessed in upcoming studies. Interestingly, ghrelin decreases duration of lordosis in sexually experienced female Syrian hamsters [403] and suppress receptivity in sexually experienced female mice [404], contrasting the data from male rodents suggesting that ghrelin enhances aspects of sexual behaviors in both sexually naïve and sexually experienced rodents [89,126,277]. As female rodents invest more energy in maternal behaviors, whereas male rodents only invest energy in paternal behavior during the sexual act in promiscuous species such as the house mouse (Mus musculus) and the rat (Rattus norvegicus)(for review see [16]), it is not surprising that a orexigenic signal, such as ghrelin, appears to influence sexual behaviors differently between sexes in promiscuous species. Future studies should therefore assess the effects of appetite-regulating peptides on sexual behaviors, paternal behaviors and maternal behaviors in monogamous rodents, such as

the prairie vole (Microtus ochrogaster) and the California mouse (Peromyscus californicus)(for review see [16]).

Ghrelin enhances both spontaneous aggression and isolation-induced aggression in male mice [215,272] and in-light of our findings that Ex4 decreases sexual interaction behaviors, future studies should evaluate the role of the GLP-1 system in various models of aggression in male mice. New evidence suggest that ghrelin is involved in various aspects of social behaviors [273-275]. Taken together with our novel data showing that Ex4 decreases social behaviors with an estrous female in sexually naïve male mice, these data highlight the need to assess the potential role of appetite-regulating peptides in social reward, social exploration and social avoidance.

Regarding other domains of natural rewards namely novelty seeking and exercise (for review see [14]) the data are sparse when it comes to appetite-regulating peptides. However, initial studies have showed that ghrelin enhances novelty-seeking behavior in rats [382] and ghrelin knock-out mice display less voluntary running than wild-type mice, whereas an acute injection of ghrelin increases voluntary running in the ghrelin knock-out to wild-type levels [405]. Future studies should evaluate the effects of appetite-regulating peptides on these other domains of natural rewards.

Previous studies investigating the role of appetite-regulating peptides in sexual behaviors have focused on normal sexual behavior, however GLP-1R agonists and GHSR-1A antagonists might possibly be beneficial for individuals with compulsive sexual behaviors as these patients have heighten activation in various reward-related areas including the NAc following pornographic cues compared to healthy controls [406,407]. In addition, intravenous injection of ghrelin enhances the activity of both the VTA and the NAc in humans exposed to images of palatable foods [408]. Indeed, manipulating appetite-regulating peptides to alter natural rewards constitute a tentative treatment prospective for various compulsive disorders such as binge eating disorder and sexual compulsive behaviors.

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