Glucagon-like peptide-1 and alcohol-mediated behaviors in rodents

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For science

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ABSTRACT

Alcohol use disorder (AUD) is a serious cause of morbidity and mortality. However, due to the limited efficacy of existing pharmacotherapies, further investigations of potential neurochemical targets are required to define new pharmacological interventions. In recent years, a pivotal role of the appetite regulatory peptide glucagon-like peptide-1 (GLP-1) in drug reinforcement and addiction processes has been identified. However, the ability of GLP-1 receptors (GLP-1R) to influence various alcohol-related behaviors and the downstream mechanisms for this interaction remains to be further evaluated. The aim of the present thesis was to investigate the mechanisms of action of GLP-1R agonists on alcohol-mediated behaviors in rodents.

Our studies firstly investigated the GLP-1R agonist, liraglutide, which suppressed the well-documented effects of alcohol on the mesolimbic dopamine system, namely alcohol-induced accumbal dopamine release and conditioned place preference (CPP) in mice. Also, acute administration of liraglutide prevented the alcohol deprivation effect and reduced alcohol intake in outbred rats, while repeated treatment decreased alcohol intake in outbred rats and reduced operant alcohol self-administration in selectively bred Sardinian alcohol-preferring rats. Secondly, we found that injections of exendin-4 (Ex4) into brain regions of the cholinergic-dopaminergic reward link are important for regulating alcohol-induced behaviors. Ex4 into the nucleus accumbens shell blocked alcohol-induced locomotor stimulation and alcohol reward-dependent memory retrieval in the CPP model in mice as well as decreased alcohol intake in rats. Moreover, Ex4 did not alter alcohol-induced behaviors when infused into the anterior ventral tegmental area

(VTA). On the other hand, Ex4 into the posterior VTA blocked alcohol-induced locomotor simulation without altering alcohol-CPP in mice or alcohol intake in rats. Furthermore, Ex4 into the laterodorsal tegmental area attenuated alcohol-induced locomotor stimulation in mice and reduced alcohol intake in rats, but did not affect alcohol reward-dependent memory retrieval in the CPP model in mice. Thirdly, obtained results showed that Ex4 into the nucleus of the solitary tract (NTS), a food-intake regulating area that is linked to the cholinergic-dopaminergic reward link, attenuated alcohol-induced locomotor stimulation, accumbal dopamine release and alcohol reward-dependent memory retrieval in the CPP model in mice. In addition, NTS-Ex4 decreased alcohol intake in rats consuming alcohol for 12 weeks. Fourthly, we found that both nine as well as five weeks of treatment with the GLP-1R agonist dulaglutide reduced alcohol intake in male and female rats. The decrease in alcohol consumption was prolonged in male rats following discontinuation of the nine-week dulaglutide treatment.

Collectively, findings in the present thesis demonstrated that different GLP-1R agonists attenuate various alcohol-mediated behaviors in rodents and that this involves subpopulations of central GLP-1R. As GLP-1 and its receptor seem to play an important role in the pathophysiology of alcohol-mediated behaviors, clinically available GLP-1R agonists deserve to be examined as potential treatments in patients with AUD.

POPULÄRVETENSKAPLIG SAMMANFATTNING

"Glukagonlik peptid-1 och alkoholmedierade beteenden i gnagare"

Alkoholberoende är en kronisk och ofta progressiv sjukdom som innebär ett stort lidande för de drabbade såväl som deras närstående. Alkoholberoende orsakar även, utöver den individuella ohälsan, stora kostnader för samhället. Idag existerar både farmakologiska samt psykologiska terapier för behandlingen av alkoholberoende. I Europa finns idag fyra stycken godkända läkemedel för alkoholberoende, men studier visar att dagens läkemedel varierar i effekt mellan patienter och fler behandlingsalternativ är önskade. Forskning har visat att alkohol aktiverar hjärnans belöningssystem samt att en överkonsumtion av alkohol förändrar dessa. En viktig del av hjärnans belöningssystem är det mesolimbiska dopaminsystemet som består av dopaminerga neuron från ventrala tegmentala arean (VTA) till nucleus accumbens (NAc). Vidare verkar även kolinerga projektioner från laterala dorsala tegmentala arean (LDTg) till VTA vara viktiga för de belönande egenskaperna hos alkohol. De kolinerga projektionerna från LDTg till VTA kallas tillsammans med de länkade projektionerna från VTA till NAc för den kolinerga-dopaminerga belöningslänken. Genom att studera interaktionen mellan alkohol och den kolinerga-dopaminerga belöningslänken kan nya måltavlor för behandling av alkoholberoende identifieras.

På senare år har födointagsreglerande hormoner uppmärksammats som modulatorer för belöning och beroendeframkallande processer. Ett sådant hormon är glukagonlik peptid-1 (GLP-1), som ger effekter på kroppen såsom att sänka glukosnivåer och signalera för aptitdämpning. Dessa effekter har lett till att GLP-1 receptor (GLP-1R) agonister används för behandling av typ II diabetes samt fetma. Tidigare studier har visat att kortvarig GLP-1R aktivering förhindrar de belönande egenskaperna av alkohol och minskar alkoholintag hos gnagare. Men effekten av olika GLP-1R agonisters inverkan på olika alkoholrelaterade beteenden samt mekanismerna bakom effekterna är ännu inte helt utstuderade. För att studera alkoholens belönande effekt har vi använt oss utav djurexperimentella försök. Målet med denna avhandling var att undersöka GLP-1R agonists inverkan på alkoholens belönande effekter. Detta har vi undersökt genom att, i studie ett, studera effekten av GLP-1R agonisten liraglutid och dess inverkan på alkoholrelaterade beteenden. Studie två var ämnad för att studera lokala injektioner av GLP-1R

agonisten exendin-4 (Ex4) i områden av den kolinerga-dopaminerga belöningslänken. I studie tre var målet att studera effekten av lokal injektion av Ex4 i solitärkärnan (NTS), ett område involverat i födointagsreglering, och dess inverkan på alkoholrelaterade beteenden. I studie fyra ville vi studera långtidsbehandling av GLP-1R agonisten dulaglutid och effekten på alkoholintag i han- och honråttor.

I vår första studie visade vi att akut tillförsel av liraglutid minskade belöningen av alkohol genom att blockera alkoholinducerad frisättning av dopamin i NAc och blockera alkohol inducerad konditionerad plats preferens (CPP) i möss, samt minskade alkoholintag hos råttor. Dessutom reducerade upprepad liraglutid-behandling alkoholintag hos råttor och även motivationen för att dricka alkohol i alkoholprefererande råttor. I studie två fann vi att Ex4 i NAc minskade alkoholinducerad lokomotoraktivitet och CPP i möss samt minskade alkoholintaget hos råttor. Däremot, Ex4 tillförsel till den främre delen av VTA påverkade inte alkohol-relaterade beteenden. I en bakre del av VTA minskade Ex4 endast lokomotoraktivitet inducerad av alkohol. Slutligen visade vi även att Ex4 i LDTg minskade alkoholinducerad lokomotoraktivitet i möss och alkoholintag hos råttor men ingen effekt sågs på minnet av alkoholbelöning i CPP-modellen i möss. I studie tre visade vi att Ex4 tillförsel till hjärnområdet NTS minskade alkoholinducerad frisättning av dopamin i NAc, minskade lokomotoraktivitet samt blockerade CPP i möss. Utöver det så minskade Ex4 behandlingen i NTS alkoholintaget hos råttor. I den fjärde och sista studien visade vi att både nio och fem veckors behandling med dulaglutid minskade alkoholintaget i han- och honråttor. Ytterligare så sågs en minskning av alkoholintaget tre veckor efter avslutad behandling för hanråttorna.

Sammanfattningsvis pekar dessa fynd på att GLP-1R agonister är av stor betydelse för att blockera alkoholens belönande effekter i gnagare. Fynden kan med fördel leda till att GLP-1-baserad behandling skall ses som intressant och ska kunna användas som framtida potentiella läkemedel för alkoholberoende.

LIST OF PAPERS

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

I.   Vallöf D, Maccioni P, Colombo G, Mandrapa M, Jörnulf JW, Egecioglu E, Engel JA, Jerlhag E. The glucagon-like peptide-1 receptor agonist liraglutide attenuates the reinforcing properties of alcohol in rodents. Addict Biol. 2016, 21(2):422-37

II. Vallöf D, Kalafateli AL, Jerlhag E. Brain region specific glucagon-like peptide-1 receptors regulate alcohol-induced behaviors in rodents. *Submitted*

III. Vallöf D, Jerlhag E. Glucagon-like peptide-1 receptors within the nucleus of the solitary tract regulate alcohol-mediated behaviors in rodents. *Submitted*

IV. Vallöf D, Kalafateli AL, Jerlhag E. Alcohol intake in male and female rats following long-term treatment with a glucagon-like peptide-1 receptor agonist. *Manuscript*

CONTENT

ABBREVIATIONS

ACh	Acetylcholine
ANOVA	Analysis of variance
AUD	Alcohol use disorder
aVTA	Anterior ventral tegmental area
BBB	Blood brain barrier
CNS	Central nervous system
CPP	Conditioned place preference
DPP-IV	Dipeptidyl-peptidase IV
Ex4	Exendin-4
FR	Fixed ratio
GABA	Gamma-aminobutyric acid
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
HPLC	High performance liquid chromatography
LDTg	Laterodorsal tegmental area
mAChR	Muscarinic acetylcholine receptors
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptors
NMDA	N-Methyl-D-aspartic acid
NMU	Neuromedin U
NTS	Nucleus of the solitary tract
PPG	Preproglucagon
PR	Progressive ratio
pVTA	Posterior ventral tegmental area
sP	Sardinian alcohol-preferring
VTA	Ventral tegmental area

INTRODUCTION

Alcohol use disorder

According to DSM-5, the diagnose criteria for alcohol use disorder (AUD) is defined by the presence of two or more of the eleven following symptoms within 12-months:

1. Alcohol is often taken in larger amounts or over a longer period of time than intended.
2. There is a persistent desire or unsuccessful effort to cut down or control alcohol use.
3. A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects.
4. Craving, or a strong desire or urge to use alcohol.
5. Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home.
6. Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.
7. Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
8. Recurrent alcohol use in situations where it is physically dangerous.
9. Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
10. Tolerance as defined by either of the following: i) a need for markedly increased amounts of alcohol to achieve intoxication or desired effect, ii) a markedly diminished effect with continued use of the same amount of alcohol.
11. Withdrawal as manifested by either of the following: i) the characteristic withdrawal syndrome for alcohol, ii) alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms.

The severity of AUD is defined as the number of criteria met: mild if two to three criteria are presented, moderate if four to five symptoms are presented and severe if six or more criteria are met.

AUD is a heterogeneous, chronic and relapsing brain disorder, and is one of the leading causes of mortality and morbidity worldwide (Koob and Le Moal 2001; Lim et al. 2012). AUD represents one of the most disabling psychiatric disorders for the individual, has major negative consequences to the family and is a great economical and societal burden (Ferrari et al. 2014; Grant et al. 2015). Long-term drinking can negatively affect heart muscle, (Fogle et al. 2010) lead to arrhythmias and also lead to damage of other organs connected to the heart. Alcohol is furthermore a contributing factor in developing hypertension and stroke (Kawano 2010). Another organ that is highly affected by heavy drinking is the liver, which has a major role in detoxification process of alcohol. Conditions of the liver developed from a heavy alcohol consuming behavior are steatosis, alcoholic hepatitis, fibrosis and cirrhosis (Gao and Bataller 2011). There is a connection between alcohol consumption and an increased risk for cancer, such as colon-, rectum-, throat- and mouth cavity cancer (Bagnardi et al. 2001), as well as breast cancer for women is well known (Smith-Warner et al. 1998).

Studies have shown that both men and women develop brain atrophy in the same extent, even if women had been addicted for a slightly shorter period (Mann et al. 2005). Furthermore, women start to consume alcohol later in life and have a later onset of continuous alcohol consumption and AUD (Diehl et al. 2007). Males have a higher rate of lifetime prevalence of AUD compared to females (36.0% for men and 22.7% for women)(Grant et al. 2015). However, this difference has in recent years become smaller and younger females tend to consume more alcohol with higher incidence of AUD presented (Colell et al. 2013; Keyes et al. 2008).

Treatments

Both pharmacological and psychosocial interventions are used to treat AUD and a combination of both is common (Garbutt et al. 1999). To date, there are three available and approved treatments for AUD by both the European Medical Agency and the US Food and Drug Administration: disulfiram, acamprosate and naltrexone. Additionally, a fourth treatment, nalmefene, was in 2013 approved by the European Medical Agency. Disulfiram inhibits

aldehyde dehydrogenase in the metabolism of alcohol and thereby causes accumulation of the metabolite acetaldehyde, which in turn gives an unpleasant feeling (Barth and Malcolm 2010). Albeit the mechanisms of action are not fully understood when it comes to acamprosate. Studies have proposed acamprosate to be an N-Methyl-D-aspartic acid (NMDA) receptor modulator (Cano-Cebrian et al. 2003), and it is thought to promote abstinence by restoring the imbalance between excitatory and inhibitory neurotransmitters, glutamate and gamma-aminobutyric acid (GABA) (Chau et al. 2010; Plosker 2015). It has also been suggested that acamprosate modulates the extracellular dopamine levels in nucleus accumbens (NAc) primarily via glycin receptors in the NAc and, secondarily, via nicotinic acetylcholine receptors (nAChR) in the ventral tegmental area (VTA) (Chau et al. 2010). Naltrexone is an un-selective opioid receptor antagonist on mu-, kappa-, and delta-opioid receptors (Swift 2013). Nalmefene is a similar drug to naltrexone, however it slightly varies since it is an antagonist on mu- and delta-opioid receptors and partial agonist on the kappa-opioid receptor (Swift 2013). Naltrexone and nalmefene are suggested to reduce alcohols rewarding effects and reduce cravings (Pettinati et al. 2006; Rosner et al. 2010). In addition, other pharmacological treatments such as baclofen (GABA$_B$ receptor agonist), topiramate (possible via NMDA and GABA$_A$ interaction (Motaghinejad et al. 2017)) and varenicline (partial nAChR agonist) have been shown to reduce alcohol consumption in clinical trials (Addolorato et al. 2002; de Beaurepaire 2012; de Bejczy et al. 2015; Johnson et al. 2003).

Albeit the variety of choices in treatment strategies, patients with AUD remain greatly undertreated. Possibly due to the heterogeneity and complexity of the disease, patients respond differently to treatments (Heilig and Egli 2006). It is therefore a need for new and personalized pharmacological treatment options. Consequently, it is of great importance to further study neuro-biological mechanisms involved in AUD, and thereby identify novel targets in order to develop new improved pharmacological treatments.

Alcohol

Alcohol is an organic, chemical compound consisting of a hydroxyl functional group bound to a carbon. The alcohol used in this thesis is ethanol (ethyl alcohol), which is the main and only drinkable alcohol in alcoholic beverages. The alcohol mentioned through out this thesis will always be ethanol, unless

otherwise specified. Alcohol is a small molecule with both hydrophilic and lipophilic characteristics and when ingested, it is quickly absorbed (Mitchell et al. 2014), spreads rapidly (Holford 1987) and passes the blood brain barrier (BBB) (Lee 1962) into the central nervous system (CNS). Unlike many other addictive drugs, alcohol's mechanism of action does not seem to involve the binding of the drug to a specific and identified neurotransmitter receptor or transporter and is therefore described as a "dirty drug" with several effects on the CNS which will be mentioned shortly below.

Alcohol and ligand-gated ion channels

Alcohol affects a variety of different neurotransmitters in the brain, by which it exerts either excitatory or inhibitory effects (Koob and Volkow 2016; Vengeliene et al. 2008). Studies illustrate that alcohol as an allosteric modulator on diverse ligand-gated ion channels, such as serotonin type 3 receptor, GABA$_A$, NMDA and nAChR (Koob 1992b; Lovinger and White 1991; Lovinger and Zhou 1994; Volkow et al. 2012; Yoshida et al. 1982). Acute effects of alcohol cause both stimulating and sedative effects which work in a biphasic manner (Addicott et al. 2007; Engel et al. 1988). Acute stimulating and reinforcing effects of alcohol are partly suggested to involve glycine receptors (Mascia et al. 1996; Molander and Soderpalm 2005; Molander et al. 2007) and potentiation of nAChR (Blomqvist et al. 1992; Blomqvist et al. 1993; Narahashi et al. 1999). The sedative effects are mainly a result of an increased transmission of the inhibitory transmitter GABA and mediated increased influx of chloride ions via the GABA$_A$ receptor (Suzdak et al. 1986). In addition, these sedative effects of alcohol may also involve decreased transmission of the excitatory neurotransmitter glutamate by inhibiting the NMDA glutamate receptor (Lovinger et al. 1989).

The reward system

The reward system and addiction pathology are closely related. Areas of the brain that mediate central stimulation, reward, pleasure and euphoria are activated naturally by natural behaviors such as food intake and sex (Kelley and Berridge 2002). These are important aspects for the survival of our species, regarding the evolutionary role on the need to search for food and desire for sex and reproduction. Moreover, drugs of abuse and addictive

behaviors also stimulate these areas in the brain that mediate rewards (Chen et al. 2010).

The mesocorticolimbic dopamine system

The reward system consists of several brain areas in the midbrain, the medial forebrain and parts of the cortical structures and the limbic system (Wise and Rompre 1989). A part of the reward systems is the mesocorticolimbic dopamine system, which can be divided into the mesocortical and the mesolimbic dopamine system (Wise and Rompre 1989). These systems are separated by their ability to project into different brain areas, which makes their neurobiological function vary. The mesocortical dopamine system consists of dopaminergic neurons that projects from VTA to the prefrontal cortex and is of importance for cognitive control, motivational behaviors and emotional response (Cools 2008; Russo and Nestler 2013; Volkow et al. 2004). The mesolimbic dopamine system is considered to be an important part of the reward systems with dopamine neurons originating in the VTA and projecting to NAc (Dahlstrom and Fuxe 1964; Koob 1992a). These components are implicated in the shaping of behaviors driven by conscious or unconscious motivation (Schultz et al. 1997). The NAc can anatomically be split into two separate regions, the central core and the surrounding shell (Voorn et al. 1989; Zahm and Brog 1992). The dopaminergic innervation of the shell has been suggested to link more to mesolimbic system while the core link more to nigrostriatal system (Deutch and Cameron 1992). NAc shell is associated with drug-induced reward, whereas the NAc core is crucial for goal-directed behaviors (for review see (Shirayama and Chaki 2006)). Moreover, VTA has diverse effects, as it is a heterogeneous brain area (Holly et al. 2016; Lammel et al. 2012; Menegas et al. 2017). Two distinct parts of the VTA are the anterior VTA (aVTA) and the posterior VTA (pVTA).

Acute alcohol and the mesolimbic dopamine system

Dopamine changes in the mesolimbic dopamine system lead to reinforcement and reward, a first step in consuming drugs and developing addiction (Volkow and Fowler 2000; Volkow et al. 2012). Further understanding on how alcohol interacts with the mesolimbic dopamine system would give a greater insight in the mechanisms behind AUD. Findings that there is a relationship between alcohol and the mesolimbic dopamine system in mice and rats show that acute alcohol injection elevates accumbal dopamine

release (Blomqvist et al. 1993; Blomqvist et al. 1997; Di Chiara and Imperato 1986; Engel et al. 1988; Ericson et al. 1998; Imperato and Di Chiara 1986; Jerlhag et al. 2006; Larsson et al. 2004), an effect that that is observed in the NAc shell but not in the core (Bassareo et al. 2003; Cadoni et al. 2000). Furthermore, intravenous administration of low doses of alcohol produce a dose-dependent increase in the firing rate of dopamine neurons in the VTA (Gessa et al. 1985) and voluntary alcohol-intake increase accumbal dopamine levels (Doyon et al. 2003; Ericson et al. 1998; Larsson et al. 2005). Different areas of VTA shows diverse effects on alcohol reward as perfusion of a low dose of alcohol into the aVTA increases accumbal dopamine in rats (Jerlhag and Engel 2014) and nAChR in the anterior part of the VTA are important for alcohol reinforcement (Jerlhag et al. 2006; Larsson et al. 2002; Lof et al. 2007). However, studies shows that microinjections of alcohol into pVTA increase dopamine release in the NAc shell (Ding et al. 2009) and increase alcohol-seeking in the operant chamber (Hauser et al. 2011). Followed up with rats that self-administer alcohol into the pVTA (Rodd et al. 2004b; Rodd et al. 2004a; Rodd-Henricks et al. 2000) suggest the posterior part of VTA as an alcohol target.

Initial data from animal studies have later been verified in the clinical setting. Alcohol increases dopamine release in the striatum of human subjects (Boileau et al. 2003; Urban et al. 2010). Self-reported behavioral measures of rewarding stimulus, euphoria and craving for alcohol (Ramchandani et al. 2011; Urban et al. 2010; Yoder et al. 2007) correlate with the established increase of dopamine release in the NAc. Together, both animal- and human data support the role of alcohol in activation of the mesolimbic dopamine system and induction of euphoria and reward.

Chronic alcohol and the mesolimbic dopamine system

Chronic alcohol intake and constant challenge of the mesolimbic dopamine system might eventually lead to neuroadaptive changes. These changes may then lead to loss of control over alcohol and thereby development of AUD (Volkow et al. 2002, 2003a). Findings in animal studies, supports a role of chronic alcohol consumption on the mesolimbic dopamine system, as rats that consume low levels of alcohol have an down-regulated expression of dopamine D2-receptor within the NAc (Jonsson et al. 2014). Also, long-term voluntary alcohol consumption reduces mRNA levels of the long dopamine D2-receptor isoform in NAc (Feltmann et al. 2018). Additionally, a

microdialysis study in rats, that voluntary have been drinking alcohol for a longer period, shows a decrease in dopamine output within the NAc (Feltmann et al. 2016). In corroboration are the findings that the sensitivity of the pVTA to the reinforcing effects of alcohol is enhanced in chronic alcohol drinking alcohol-preferring rats (Rodd et al. 2005).

Accordingly, reduction of dopamine D2-receptors has been found in AUD patients (Balldin et al. 1993; Volkow et al. 1996). The reduction of dopamine D2-receptors within the striatum is suggested to play a significant role in the severity of cravings for alcohol in AUD patients (Heinz et al. 2004). Additionally, a functional magnetic resonance imaging study show that alcohol-associated cues activate the NAc and VTA of high-risk drinkers but not low-risk drinkers (Kareken et al. 2004). Furthermore, chronic alcohol intake leads to lowered baseline levels of dopamine, but dopamine elevation in response to further alcohol consumption remains high (Diana et al. 1993).

The cholinergic-dopaminergic reward link

The cholinergic-dopaminergic reward link (Figure 1) (Larsson and Engel 2004) appears to be important for alcohol reward. It involves afferent acetylcholine neurons projecting from the laterodorsal tegmental area (LDTg) into the VTA. The projecting neurons from LDTg activate nAChR and muscarinic acetylcholine receptors (mAChR) localized in the VTA, resulting in a concomitant release of acetylcholine (for review see (Larsson and Engel 2004)). This stimulates VTA-dopamine neurons in the mesoaccumbal dopamine system, which causes dopamine release in NAc (Forster and Blaha 2000). Additionally, it is suggested that nAChR's in the VTA, rather than mAChR's, are more important for mediating the stimulatory effects of alcohol (Blomqvist et al. 1997; Ericson et al. 1998; Soderpalm et al. 2000).

The importance of the cholinergic-dopaminergic reward link in reward modulation is highlighted by investigations demonstrating that optogenetic activation of the cholinergic-LDTg projection induces a conditioned place preference (CPP), operant self-administration to reward as well as induces accumbal dopamine release (Lammel et al. 2012; Steidl and Veverka 2015; Steidl et al. 2017a). Accordingly, alcohol intake in high alcohol-consuming rats concomitantly increases acetylcholine in VTA and dopamine in NAc (Larsson et al. 2005), suggesting that the cholinergic-dopaminergic reward link

(Larsson and Engel 2004) are crucially involved in the rewarding effects of alcohol. Moreover, a direct link between cholinergic LDTg afferents to NAc (Cornwall et al. 1990; Dautan et al. 2014) (Figure 1) adds an additional pathway for alcohol to exert its rewarding effects.
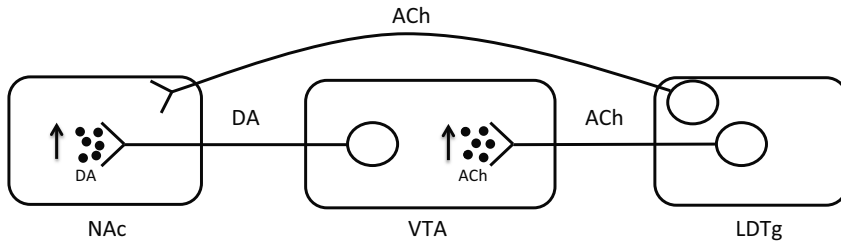

Figure 1 The cholinergic-dopaminergic reward link is composed of cholinergic neurons projecting from the LDTg to the VTA and the mesoaccumbal dopamine system, which consists of dopamine neurons projecting from the VTA to the NAc. Activation of cholinergic neurons originating in the LDTg causes a release of acetylcholine (ACh) into the VTA, which by interactions with nAChR (and/or mAChR) activates the mesolimbic dopamine system causing dopamine release in NAc. Moreover, a direct ACh afferent link from LDTg to NAc could be involved in this activation. Picture created with information from (Larsson and Engel 2004) and (Dautan et al. 2014).

Investigations of mechanisms, neurotransmitters and transmitter systems involved in the ability of alcohol to activate the cholinergic-dopaminergic reward link may thus contribute further to the knowledge on the development of AUD as well as to the detection of new pharmacological targets to treat AUD.

Alcohol and other neurotransmitter systems

Chronic use of a drug are suggested to alter synaptic connectivity between brain regions (Robinson and Kolb 2004) and chronic alcohol intake can lead to neuroadaptations of circuits within different regions of the CNS. Some changes include decreased GABA$_A$ receptor function and increased excitatory activity of the NMDA receptor (Korpi et al. 2015; Ravan et al. 2014). These are opposite effects as from acute alcohol intake. The decrease in GABA$_A$ function may be a result of a decreased number of receptors or decreased receptor sensitivity. Also, glutamate receptors appear to adapt to the inhibitory effects of alcohol and hence increase their excitatory activity (Hoffman et al. 1990; Mihic 1999). Moreover, alcohol alters levels of endodgenous opioids and the

mu-opioid receptor (Gianoulakis 1996; Oslin et al. 2003), which influence mood, pleasure and alcohol-seeking (Costardi et al. 2015).

AUD and the addiction cycle

The step from recreational alcohol use to excessive use differs among individuals, and the risk to develop AUD is influenced by individual genetic, as well as environmental factors and exposure to alcohol. The heritability and the risk to develop AUD has for instance been studied and confirmed in twin studies (Cloninger et al. 1981; Kendler and Baker 2007; Prescott and Kendler 1999) where genetic factors might explain the increased vulnerability. Further, total weighted mean heritability for environmental factors such as stressful life events, family environment, parental warmth, control and support, is estimated to a number of 27% (Kendler and Baker 2007). Moreover, in a meta-analysis of twin and adoption studies on heritability estimates, the heritability of AUD was confirmed to be 49% (Verhulst et al. 2015).

Individual personality traits such as impulsivity, novelty-seeking, conduct problems and high reward sensitivity, often seen in adolescence, are associated with excessive alcohol use and are thus risk factors and indicators for AUD later in life (Chartier et al. 2010; Cloninger et al. 1988). The availability of alcohol, alcohol attitudes among peers and family as well as general alcohol norms in society, act as factors to increase the risk of developing AUD (Chartier et al. 2010). Also age, gender and hormonal status are additional factors that could be influencing the risk of an individual to develop addiction (Engel et al. 1992).

In the early stages of alcohol use the consumption is motivated via impulsive drinking with positive reinforcing effects. The individual returns to a motivational state after the end of intoxication and external stimuli may again associate alcohol intake with its pleasurable reinforcing effects (Brown et al. 1980). However, as alcohol consumption continues and escalates over time, it may result in repeated episodes of heavy drinking followed by abstinence and, later on, dependence (for review see (Heilig and Koob 2007)). The behavioural change from recreational rewarding alcohol use to the compulsive and habitual use is suggested to involve a neuronal shift from NAc shell to the dorsal striatum (Ostlund and Balleine 2008). From the initial

reinforcing effects of alcohol an addictive process sets in and progresses over time. The impulsivity that first drove the individual to consume alcohol now turns to compulsivity and the reinforcement that first was positive shifts to negative (Koob 2003). The negative reinforcement is driven by the motivation to remove the negative effects from alcohol, such as withdrawal (for review see (Koob 2013)). Continuing further from impulsivity to compulsivity, the view on addiction has been conceptualized as three stages connected to each other and recur in a cycle (Figure 2): binge/intoxication, withdrawal/negative affect and preoccupation/anticipation (for reviews see (Koob and Volkow 2010, 2016)).
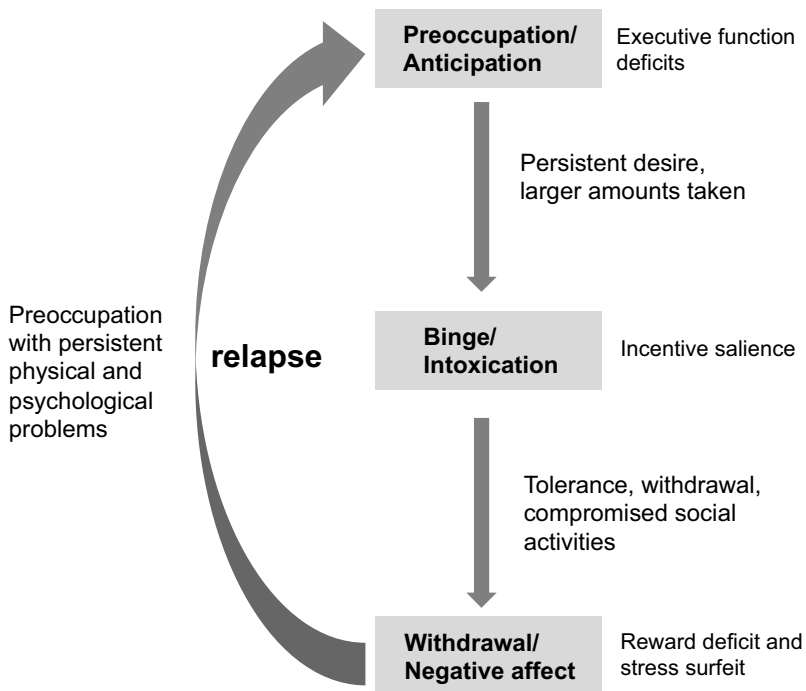

Figure 2. A schematic representation of the three stages in the addiction cycle: binge/intoxication, withdrawal/negative affect and preoccupation/anticipation. Modified from (Koob and Simon 2009; Koob and Volkow 2016).

During binge/intoxication state brain areas involved in rewarding effects of alcohol are activated. These areas are NAc, VTA and medial prefrontal cortex

and are known for dopamine and opioid release resulting in a euphoric feeling (Dichiara and Imperato 1988; Everitt et al. 2008; Volkow et al. 2007). Withdrawal/negative affect occurs after chronic alcohol exposure, which induce neurochemical changes. The mesocorticolimbic dopamine system is inhibited and stress response is activated to further enhance incentive salience (motivation and craving for a reward) via areas such as amygdala and the bed nucleus of stria terminalis (Delfs et al. 2000; Koob et al. 2014). The preoccupation/anticipation stage of addiction is seen as the drug- seeking state and is based on contextual cues proceeded in hippocampus, conditioned stimulus cues in basolateral amygdala and how these communicate with the frontal cortex (for review see (Koob and Volkow 2016). Also, habit formation of compulsive alcohol seeking followed by extended exposure to alcohol is suggested to involve the dorsal striatum (Barker and Taylor 2014; Everitt and Robbins 2013; Robbins and Everitt 2002).

Relation between food intake and alcohol consumption

Evidence from human and animal studies show that drugs of abuse and consumption of foods share similar pathways within the reward system (for review see (Kelley and Berridge 2002; Thiele et al. 2003)). Both natural and addictive drugs activate areas of the mesolimbic dopamine system, such as NAc and VTA (Nestler 2005). These routes regulates hedonic feeding, which is of importance for reward-based feeding (Volkow et al. 2011). The hedonic pathway can override homeostatic feeding, which controls energy balance by increasing the motivation to eat following depletion of energy stores (Lutter and Nestler 2009). However, appetite regulation is complex and involves various peptides (Fulton 2010; Zheng et al. 2009) where some peptides, such as ghrelin, stimulate feeding and others like neuromedin U (NMU), glucagon-like peptide-1 (GLP-1) and amylin inhibit food intake (for review see (Arora and Anubhuti 2006)). Over the last years, a pivotal role of these appetite regulatory peptides, in reinforcement and addiction processes has been identified (for reviews see (Engel and Jerlhag 2014; Jerlhag 2018a, 2018b)). In addition, more recent work identifies GLP-1 as an important regulator of food and drug reward (Egecioglu et al. 2013c; Egecioglu et al. 2013b, 2013a; Erreger et al. 2012; Graham et al. 2013; Shirazi et al. 2013).

Glucagon-like peptide-1

GLP-1 is a 30 amino acid long incretin hormone that regulates blood glucose through increased insulin production and secretion (Kreymann et al. 1987) as well as inhibition of glucagon secretion (Orskov et al. 1988). Also, GLP-1 signals for inhibition of gastric emptying (Flint et al. 1998) and food intake (Pannacciulli et al. 2007). GLP-1 is produced in the enteroendocrine L-cells (Novak et al. 1987) as well as in the olfactory bulb and in neurons of the hindbrain which originates in the nucleus of the solitary tract (NTS) (Jin et al. 1988; Merchenthaler et al. 1999). The precursor of GLP-1 is preproglucagon (PPG) and has post-translational processing in the pancreas and in the gut/brain (George et al. 1985; Mojsov et al. 1990).

The beneficial effects of GLP-1 on blood glucose and glucagon secretion have led to approval of GLP-1R agonists as treatment for type II diabetes (Holst 2004). However, GLP-1 is rapidly metabolized by dipeptidyl-peptidase IV (DPP-IV) in the body and data are showing that almost all of subcutaneously administered GLP-1 becomes degraded and inactive (Vilsboll et al. 2003). Hence, GLP-1 agonists such as exendin-4 (Ex4), liraglutide and dulaglutide, with longer half-life have been developed and are less prone to be degraded by the DPP-IV compared to GLP-1 (Holst 2004; Jackson et al. 2010; Smith et al. 2016; Thorens et al. 1993), thus providing pharmacological substances with longer half-life. GLP-1 has its own receptor (GLP-1R), which is a G protein-coupled receptor (Drucker et al. 1987) that is widely distributed in pancreas, brain, heart and the gastrointestinal tract. However, the function of the receptor in all these locations is not yet known (for review see (Holst 2007)).

Effects of glucagon-like peptide-1 on food intake and food reward

The anorexigenic properties of GLP-1 have been identified in rodents following central (Tang-Christensen et al. 1996; Turton et al. 1996) as well as systemic GLP-1 administration (Abbott et al. 2005; Chelikani et al. 2005). Systemic administration of GLP-1 also reduces food intake in humans (Flint et al. 1998; Gutzwiller et al. 1999a; Gutzwiller et al. 1999b; Verdich et al. 2001). Food intake reduction is accompanied by a decrease in body weight, which has led to the approval of GLP-1R agonists for the treatment of obesity in humans (for review see (Srivastava and Apovian 2018)). Areas of importance for GLP-1 to reduce food intake have been suggested to include the NTS, amygdala and the hypothalamus (Hayes et al. 2008; Hayes et al. 2009; McMahon and Wellman 1998; Tang-Christensen et al. 1996).

Regarding the rewarding aspects of food consumption, systemic administration of GLP-1R agonists prevents reward and reduces motivation to consume palatable food in rats (Dickson et al. 2012). Also, the GLP-1R agonist liraglutide, shifts food preference from rewarding food to regular chow in rats (Raun et al. 2007). Regarding areas regulating hedonic feeding, expression of GLP-1 is found in reward-related areas including the VTA and NAc (Alvarez et al. 1996; Merchenthaler et al. 1999). Peripheral and local administration of a GLP-1R agonist into the VTA or NAc decreases food reward and CPP as well as the motivation for sucrose consumption in rats (Alhadeff et al. 2012; Dickson et al. 2012). However, there seems to be different effects regarding which area of NAc that is activated. Hence, GLP-1R activation in the NAc core is suggested to be involved in free-feeding behavior (Dossat et al. 2011), which does not seem to be the case in NAc shell, an area crucial for controlling the rewarding behavior of feeding (Dickson et al. 2012). Regarding VTA, activation of GLP-1R in the posterior part has shown to reduce intake of sucrose, chow as well as high fat diet and cause a significant decrease in body weight (Alhadeff et al. 2012; Mietlicki-Baase et al. 2013; X. F. Wang et al. 2015). However, the effect of GLP-1 on food reward within the anterior part of the VTA is not fully evaluated. Further, GLP-1R´s within the LDTg (Merchenthaler et al. 1999), specifically located on axon terminals on NTS projection (Reiner et al. 2018), have shown to be of importance for food intake. LDTg is anatomically connected to mesolimbic areas (Dautan et al. 2014; Forster and Blaha 2000; Larsson et al. 2005), suggesting that GLP-1 might act via this route to regulate food reward.

Effects of glucagon-like peptide-1 on alcohol and addictive drugs

Findings that appetite regulatory peptides are expressed in reward-related areas (Thiele et al. 2004) collectively support that food and drug intake share overlapping neurobiological mechanisms. Co-morbidity between AUD and binge eating (Bulik et al. 1997) (for review see (Wolfe and Maisto 2000)) and characteristics of binge eating disorder such as loss of control and cravings for food (Ng and Davis 2013), are similar with substance use disorders (Schreiber et al. 2013). Comparatively to AUD patients, lower density of dopamine D2- receptors has been demonstrated in patients suffering from compulsive overeating (Volkow et al. 2003b; G. J. Wang et al. 2004). Regarding GLP-1 and alcohol, the first published study showed that acute peripheral injection of the GLP-1R agonist Ex4 attenuated alcohol

specific properties on the mesolimbic dopamine system as measured by accumbal dopamine release, CPP as well as locomotor activity in mice (Egecioglu et al. 2013c). Additionally, acute treatment with Ex4 decreased alcohol intake in the intermittent access two-bottle-choice model and prevented alcohol-seeking behavior, as measured by the progressive ratio test in the operant self-administration model in rats (Egecioglu et al. 2013c). These effects of GLP-1 and Ex4 were later collaborated by others. Indeed, it was shown that Ex4 reduced alcohol intake in rats and that a blockade of the GLP-1R resulted in increased alcohol intake (Shirazi et al. 2013). The ability of Ex4 to block alcohol-induced CPP was also demonstrated in this follow up study (Shirazi et al. 2013). Further pre-clinical findings have shown that repeated treatment with the GLP-1R agonist AC3174 reduced increased drinking in alcohol-drinking mice (Suchankova et al. 2015). Furthermore, the GLP-1R agonist liraglutide has shown to attenuate withdrawal-induced anxiety as well as potentiate anti-anxiety effects caused by alcohol (Sharma et al. 2015b). Same results were demonstrated by increasing endogenous levels of GLP-1 via pharmacological inhibition of DPP-IV (Sharma et al. 2015a).

Studies evaluating the effects of GLP-1, and GLP-1R agonists, in humans on alcohol consumption and alcohol reward are limited. However, a first study supporting a possible effect is data from a preliminary report that show a reduction of alcohol intake in type II diabetic patients treated with liraglutide (Kalra S 2011). Only one human genetic study reveals association between polymorphisms in the GLP-1R gene and alcohol dependence (Suchankova et al. 2015). In the same study, an additional experiment examining intravenous self-administration, displays that a polymorphism in the GLP-1R gene is associated with enhanced intravenous infusion of alcohol and increased measurement of breath alcohol in social drinkers (Suchankova et al. 2015).

Moreover, systemic administration of the GLP-1R agonist Ex4 blocks the rewarding properties of various drugs of abuse. Indeed, Ex4 has been shown to block the ability of amphetamine to increase locomotor activity in mice (Erreger et al. 2012) and to prevent the rewarding effects of cocaine in mice as measured by CPP (Graham et al. 2013). The findings in these two reports were replicated in another study where Ex4 reduced the ability of amphetamine as well as cocaine to increase locomotor stimulation, accumbal dopamine release as well as to induce a CPP in mice (Egecioglu et al. 2013a). GLP-1R activation of Ex4 also blocked the ability of nicotine to increase locomotor stimulation, accumbal dopamine release, induce a CPP as well as attenuate nicotine-induced expression of locomotor sensitization in mice

(Egecioglu et al. 2013b). Furthermore, GLP-1R stimulation with Ex4 reduces acute and chronic cocaine self-administration, attenuates cocaine-induced hyper locomotion as well as striatal dopamine elevation in mice (Sorensen et al. 2015). Collectively, these data imply that GLP-1Rs are important for reward induced by drugs of abuse.

Albeit initial studies show that short-lasting GLP-1R agonists attenuate alcohol-mediated behaviors, the effects of long-acting agonists are still unknown. Hence, a substantial need for additional studies evaluating possible circuits through which GLP-1 exerts its effect on alcohol-mediated behaviors are still warranted, and will be pursued in this thesis.

AIMS OF THE THESIS

The overall aim of the present thesis was to investigate the effects of long-acting GLP-1R agonists on alcohol intake and identify brain regions for GLP-1R activation on alcohol-mediated behaviors in rodents. Further, to identify if GLP-1R agonists could constitute the basis for development of new pharmacological treatment strategies for AUD.

Specific aims

Paper I. To evaluate the effects of the GLP-1R agonist, liraglutide, on alcohol-mediated behaviors in rodents.

Paper II. To investigate the role of GLP-1R in brain areas of the cholinergic-dopaminergic reward link on alcohol-mediated behaviors in rodents.

Paper III. To study the impact of NTS-GLP-1R stimulation on alcohol-mediated behaviors in rodents.

Paper IV. To determine the effects of long-term systemic treatment of the GLP-1R agonist, dulaglutide, on alcohol intake in male and female rats.

MATERIALS AND METHODS

Animals

All animal experiments conducted in Sweden were approved by the Swedish Ethical Committee on Animal Research in Gothenburg. Experiments at the CNR Neuroscience Institute, Monserrato, Italy were conducted in accordance to the Italian law on the "Protection of animals used for experimental and other scientific reasons" and approved by the Ethical Committee of the University of Cagliari. All efforts were made to minimize animal suffering and to reduce the number of animals used. Each experiment used an independent set of animals. All animals were allowed to acclimatize at least one week before the start of the experiments and none of the animals were ever food- or water-deprived in the experiments carried out at the University of Gothenburg. In the Italian lab, food and water were available *ad libitum*, except for short periods during initial training in the operant self-administration model.

Adult post-pubertal age-matched male NMRI mice (B&K Universal AB, Sollentuna, Sweden, paper I; Charles River, Susfeldt, Germany, paper II and III) (8–12 weeks old and 25–35 g body weight) were used for the locomotor activity, in vivo microdialysis, CPP, blood alcohol concentration and dose-response studies. Mice were used for these experiments as our lab has extensive experience with mice and we have previously obtained robust locomotor stimulation, CPP and accumbal dopamine release in response to alcohol and other addictive drugs (Jerlhag et al. 2009).

Adult post-pubertal age-matched male outbred Rcc Han Wistar rats (Harlan, Horst, Netherlands, paper I; Envigo, Horst, Netherlands, paper II, III and IV) were used in the intermittent access 20 percent alcohol two-bottle-choice drinking model and the alcohol deprivation test (paper I). These rats were selected because they display a voluntary high and stable alcohol intake causing pharmacologically relevant blood alcohol concentrations in this drinking model (Simms et al. 2008) and they have shown to have higher voluntary alcohol intake and alcohol preference than other Wistar rats (Palm et al. 2011). The reason for using an outbred rat strain was to obtain a better translational aspect of the results reflecting the general population with different genetic background.

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It is well known that females are largely underrepresented in pre-clinical trials in most fields as well as in the alcohol field. A possible explanation why that is could be regarding alterations in behavior during different stages in the estrous cycle. For instance, alcohol intake in female rats varies depending on the stage of their estrous cycle (Forger and Morin 1982). Adult post-pubertal age-matched female outbred Rcc Han Wistar rats were therefore included for an intermittent access 20 percent alcohol two-bottle-choice drinking model (paper IV).

Operant and oral self-administration of alcohol experiments were performed in selectively bred Sardinian alcohol-preferring (sP) rats (CNR Neuroscience Institute, Monserrato, Italy, paper I). The sP rat is selectively bred for high alcohol intake and preference, whose alcohol-seeking and -taking behaviors have been proposed to model several aspects of excessive alcohol consumption in humans (Colombo et al. 2006).

Dose-response studies in rats were carried out in male Wistar rats (Charles River, Germany) that were group housed in rooms under 20°C and 50% humidity and maintained on a 12/12-hour light/dark cycle (paper III).

Drugs

For studies investigating alcohol-induced activation of the mesolimbic dopamine system in mice, 96% ethanol (96%; VWR International AB, Stockholm, Sweden) was diluted in saline (0.9 percent NaCl) to 15 percent vol/vol for intraperitoneal (ip) injections and was administered at a dose of 1.75 g/kg, 5 minutes prior to initiation of the experiments. For the intermittent access alcohol two-bottle-choice drinking model, alcohol was diluted to a 20 percent vol/vol solution using tap water. 96% ethanol (Silvio Carta Srl, Oristano, Italy) was diluted to a 10 or 15 percent vol/vol solution using tap water for the operant self-administration experiments.

GLP-1 is rapidly degraded and has in plasma a short half-life of about two minutes (Vilsboll et al. 2003). To avoid short-lasting effects different GLP-1R agonists with longer half-life and more prolonged biological activity were used in the present studies. The GLP-1R agonist, Ex4 (Tocris Bioscience, Bristol, England) (Thorens et al. 1993) was in each experiment (paper II and III) diluted in Ringer solution (NaCl 140 mM, Ca Cl2 1.2 mM, KCl 3.0 mM and

MgCl2 1.0 mM; Merck KGaA, Darmstadt, Germany). Ex4 was in these experiments locally administrated into different brain areas at selected doses derived from our previous dose response studies. The selected doses of Ex4 in mice were 0.0025 μg per side into the NAc shell, aVTA, pVTA and LDTg as well as 0.05 μg per side into the NTS. In rats, a dose of 0.05 μg per side were selected for NAc shell and NTS and a dose of 0.025 μg per side were selected for aVTA, pVTA and for LDTg. Ex4 was always administered 10 minutes before initiation of experiment. In paper I, the long-acting GLP-1R agonist liraglutide (Victoza®, Novo Nordisk, Copenhagen, Denmark)(Jackson et al. 2010) was dissolved in vehicle (0.9% sodium chloride) and a dose of either 0.05 or 0.1 mg/kg was used. Liraglutide was always administered subcutaneous one hour before start of experiment. For paper IV, a third GLP-1R agonist was tested, namely dulaglutide (Trulicity®, Kronans Apotek, Gothenburg, Sweden). Dulaglutide is a long-acting GLP-1R agonist (for review see (Smith et al. 2016)) and was always subcutaneously injected once-weekly, one hour prior to introduction of experiment. Dulaglutide was dissolved in vehicle (0.9% sodium chloride) and a dose of 0.1 mg/kg, was after a pilot study, selected.

Importantly, in all experiments the selected doses did not affect the rodents' gross behavior to avoid such influence on obtained results.

Experimental procedures

Guide cannula and probe implantations

To be able to inject a drug of interest, directly into specific sites of the brain in rodents, is a really valuable technique that could be used within the field of pre-clinical neuroscience. Direct and site-specific drug administration, in freely moving and awake rodents, can be used to test outcomes in behavioral studies. It is a way to test the effects of a drug that may otherwise not be able to pass the BBB.

In the conducted experiments, probe implantations for microdialysis studies (Paper I and III) and guide cannula implantations for local injections of Ex4 into specific brain areas (Paper II and III) were used. The set up for implantation of either guide cannulas or probes was the same in both mice and rats. Briefly, rodents were anesthetized with isoflurane (Isoflurane

Baxter; Univentor 400 Anaesthesia Unit, Univentor Ldt., Zejtun, Malta), placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA, USA) and kept on a heating pad to prevent hypothermia. Xylocain adrenalin (5 µg/ml; Pfizer Inic; New York, USA) was used as local anesthetics and carprofen (Rimadyl®, 5 mg/kg ip, Astra Zeneca; Gothenburg, Sweden) was used to relieve pain. The skull bone was exposed and holes for either guide cannula or probe, plus one hole for anchoring screw, were drilled. In order to administer Ex4 or vehicle solution (Paper II and III) guide cannulas (stainless steel, length 10 mm, with an o.d./i.d. of 0.6/0.45 mm) were implanted 1 mm below the surface of the brain and first at time of experiment extended ventrally beyond the tip of the guide cannula for direct drug administration. The probe for dialysis was immediately placed in NAc shell. Full presentation of coordinates for guide cannula or probe placements is given in table 1. The dialysis probe and/or the guide cannulas were then anchored to a screw and the skull bone with dental cement (DENTALON® plus; Agntho's AB, Lidingö, Sweden). After surgery were the animals kept in individual cages for four days until the initiation of each experiment.

Table 1. Coordinates used for each specific brain region. (Franklin and Paxinos 1996; Paxinos and Watson 1998)

Brain region	Anterior/Posterior (Relative to bregma)	Lateral/Medial (Relative to midline)	Dorsal/Ventral (Relative to skull)	*Paper*
Mouse				
NAc	+1.4 mm	±0.6 mm	-4.7 mm	I, II, III
aVTA	-3.4 mm	±0.5 mm	-4.3 mm	II
pVTA	-3.6 mm	±0.5 mm	-4.2 mm	II
LDTg	-5.0 mm	±0.5 mm	-3.2 mm	II
NTS	-7.4 mm	±0.5 mm	-4.3 mm	III
Rat				
NAc	+1.85 mm	±1.0 mm	-7.8 mm	II
aVTA	-5.3 mm	±0.5 mm	-8.3 mm	II
pVTA	-6.8 mm	±0.5 mm	-8.6 mm	II
LDTg	-8.8 mm	±1.0 mm	-7.0 mm	II
NTS	-13.4 mm	±1.2 mm	-8.2 mm	III

On the experiment days, one hour before initiating the experiment, a dummy cannula was carefully inserted into the guide cannula to remove clotted blood

and to hamper spreading depression. At the proceeding drug challenge, the drug was administered over one minute at a volume of 0.5 μl and the cannula was left in place for another minute and was then retracted (5 μl Kloehn, microsyringe; Skandinaviska Genetec AB, V. Frölunda, Sweden). The injection sites were verified following the termination of the experiment (see *Verification of guide cannulas and probe placements*).

Verification of guide cannulas and probe placements

After locomotor activity (paper II and III), CPP (paper II and III) and micro-dialysis experiments in mice (paper I and III) as well as alcohol intake in rats (paper II and III) were completed, the locations of the probe and/or cannula/s were confirmed post mortem. The mice and rats were decapitated, probes were perfused with pontamine sky blue 6BX to facilitate probe localization, and the brains were mounted on a vibroslice device (752M Vibroslice: Campden Instruments Ltd., Loughborough, UK). The brains were cut in 50 μm sections and the location of the probe and/or cannula was determined by gross observation using light microscopy. Only mice and rats with correct placements were included in the statistical analysis.

Behavioral procedures

Development of addiction largely depends on the effects of drugs of abuse on the mesolimbic dopamine system (Blomqvist et al. 1993; Blomqvist et al. 1997; Egecioglu et al. 2013b, 2013a; Engel et al. 1988; Ericson et al. 1998; Jerlhag et al. 2006; Larsson et al. 2004; Larsson et al. 2005). The methods used in this thesis reflect reward and activation of the mesolimbic dopamine system. They are therefore of great value when investigating mechanisms involved in AUD. Indeed, these models have been the basis for the pharmacological agents approved today for AUD in humans (for review see (Spanagel 2000).

Conditioned place preference (CPP) in mice

CPP is a well-established model reflecting activation of the mesolimbic dopamine system. The CPP test involves a two-chambered CPP apparatus with distinct visual and tactile cues. Depending on design of the CPP-

experiment, this model can be used to evaluate the rewarding effects of alcohol (paper I) or alcohol reward-dependent memory retrieval (paper I, II and III).

CPP on alcohol reward-dependent memory retrieval

To evaluate the effect of alcohol on alcohol reward-dependent memory retrieval, the procedure consists of preconditioning (day 1), where the mouse is free to explore both cambers for 20 minutes and the least preferred compartment is identified. Before initiation of the pre-conditioning test, the mouse is injected with vehicle. Throughout conditioning (days 2-5), the least preferred compartment is paired, through a biased procedure, with an alcohol injection. During days 2-5, each mouse is subjugated to two sessions on each day. In the morning session, the mouse is either given an alcohol injection in its least preferred compartment or a vehicle injection in its preferred compartment. In the afternoon session, the treatments and compartments are switched. At post-conditioning (day 6), the mouse is acutely treated with the selected GLP-1R agonist or vehicle and placed on the midline between the two compartments where it is free to explore both compartments for 20 minutes.

CPP on acute rewarding effects of alcohol

To test the rewarding effects of alcohol, the experiment is slightly modified in three ways compared to the alcohol reward-dependent memory retrieval CPP mentioned above. i) On the pre-conditioning day, no vehicle treatment is given, ii) GLP-1R agonist or vehicle is administered prior to the alcohol injection on each of the four conditioning days and iii) at post-conditioning, the mouse is untreated and then placed on the midline between the two compartments with free access to both compartments for 20 minutes.

In both paradigms, the expression of CPP is calculated as the percentile difference of total time spent in the drug-paired (i.e. less preferred) compartment during the post-conditioning and the pre-conditioning session.

Locomotor activity in mice

Alcohol causes locomotor stimulation in rodents. This process is suggested to involve the ability of drugs to enhance extracellular concentrations of

accumbal dopamine (Engel et al. 1988). For this experiment, a plexiglas made arena is placed in a sound attenuated, ventilated and dim lit box. For the first set of experiments (paper II), the locomotor activity was measured in boxes (420 x 420 x 200 mm, Kungsbacka mät- och reglerteknik AB, Fjärås, Sweden) with five by five infrared beam detectors calculating for movement patterns and distance. In the second set of experiments (paper III), the same arena but a different activity system was used (420 x 420 x 200 mm; Open Field Activity System; Med Associates Inc, Gerogia, Vermont, USA) to record locomotion. In this system, 15 x 15 infrared beams at the bottom of the floor allow a computer-based system to register the distance travelled. The same experimental set up has been followed in all the papers, despite the use of different systems. Both systems measure and calculate locomotion in five minutes bins. Briefly, during these experiments, the animals were let freely to habituate to the arena for one hour. After habituation they were given an injection of GLP-1R agonist and shortly after they were administered alcohol. The infrared beams register the activity of the rodents, which reflects the stimulatory effect of the given substance, in these cases alcohol. By elucidating the ability of a pharmacological agent to attenuate the alcohol-induced locomotor stimulation, a point in the right direction is given on the effect off possible blocking effects of alcohol reinforcement.

In vivo microdialysis and dopamine release measurements in mice

In vivo microdialysis in awake and freely moving mice allows measurements of extracellular levels of neurotransmitters in the brain. The method allows us to study how dopamine responds in NAc shell after a systemic alcohol injection, which has shown to stimulate dopamine release in the NAc (Blomqvist et al. 1993; Blomqvist et al. 1997; Larsson et al. 2002; Larsson et al. 2004; Yoshimoto et al. 1992). With this knowledge it is possible to investigate if a drug has the ability to attenuate alcohol's ability to increase accumbal dopamine.

In paper I and III, the mice were implanted with a microdialysis probe (further explained under Guide cannula and probe implantation) positioned in NAc shell. We target this specific part of NAc since we have seen a more robust dopamine release in response to alcohol (Blomqvist et al. 1993; Blomqvist et al. 1997; Egecioglu et al. 2013c; Larsson et al. 2002; Larsson et al. 2004). On the day of the experiment the probe was, via freely rotating swivel, connected to a microperfusion pump (U-864 Syringe Pump; AgnThós

AB) and perfused with Ringer solution at a rate of 1.5 µl/minute. After one hour of habituation to the microdialysis set-up, perfusion samples were collected every 20 minutes. The baseline dopamine levels were defined as the average of three consecutive samples before the first drug of interest or alcohol challenge (Time 0). In paper I, an initial alcohol-challenge, after the last baseline sample, was given to establish that all mice included in the experiment would respond to alcohol-induced accumbal dopamine release. Then were seven consecutive samples collected before liraglutide or vehicle challenge, and then after another 60 minutes a second alcohol or vehicle challenge was administered. In paper III, Ex4 or vehicle challenge was induced 10 minutes after the last baseline sample, following another 10 minutes with alcohol or vehicle challenge (Time 20). Samples were in both studies, directly after the 20 minutes collection, placed in a high performance liquid chromatography (HPLC) apparatus for dopamine separation and quantification. Only subjects with correct probe placement within the NAc shell were used in the analysis (further explained in *Verification of guide cannulas placement*). The induced increase in accumbal dopamine was thereafter calculated as the percentage of increase from the baseline samples. The initial alcohol-challenge that was performed in study I was not undertaken in paper III study, since we throughout the years have established that all mice respond to alcohol.

Biochemical assay following in-vivo microdialysis

Dopamine was separated and quantified using two different HPLC apparatuses with electrochemical detection. In brief, a pump (UltiMate 3000 Pump; Thermo Scientific, Darmstadt, Germany), an ion exchange column (Nucleosil SA, 2.0 x 150 mm, 5 µm diameter, pore size 100 Å; Phenomenex Scandinavia, Västra Frölunda, Sweden) and a detector (Decade, Kovalent AB, Sweden) operated at 400 mV versus the cell were used. The mobile phase was delivered at 0.3 ml/min and consisted of 58 mM citric acid, 135 mM NaOH, 0.107 mM Na2–EDTA and 20% methanol. The second system consisted of a pump (UltiMate 3000 Pump; Thermo Scientific, Darmstadt, Germany), a reversed phase column (2.0 x 50 mm, 3 µm diameter; pore size 100 Å; Phenomenex Scandinavia, Västra Frölunda, Sweden) and a detector (Dionex, Västra Frölunda, Sweden) operated at 220 mV versus the cell. The mobile phase was delivered at 0.3 ml/min and consists of f 150 mM NaH2PO4, 4.76 mM citric acid, 3 mM sodium dodecyl sulphate, 50 µM EDTA, as well as 10% MeOH and 15% acetonitrile. To identify the peaks of dopamine and for

calculating dopamine concentrations, an external dopamine standard (500 µg/ml) was used.

Intermittent-access 20% alcohol two-bottle-choice drinking model in rats

This is a drinking paradigm that induces high alcohol consumption in rats and was already introduced in the early 70s (Wise 1973), showing different drinking patterns from various drinking schedules. The model was later refined to the specific intermittent-access 20% alcohol two-bottle-choice model, which demonstrates good validity and pharmacological relevant blood alcohol concentrations in rats and mice (Carnicella et al. 2009; Simms et al. 2008).

In general, rats are given access to one bottle of 20% alcohol and one bottle of water for three 24-hour-sessions per week (Mondays, Wednesdays, Fridays) for ten to twelve weeks (Figure 3)(Paper I-IV). The bottles are introduced on the onset of the dark cycle in the reversed light/dark cycle room, providing that rats are nocturnal animals and drink more during the dark phase (Wise 1975). With the dark phase starting in the mornings makes it easier for us researchers to test drugs for challenging the alcohol intake, right before the rats drinking bouts starts. Throughout the experiment, the rats have unlimited access of food and water. During the non-alcohol day, two bottles of water are available. Bottles and food are weighed at 24 hours after the bottles are presented. The body weight of each rat is measured weekly, to allow for calculating the grams of alcohol intake per kilogram of body weight (g/kg). The preference for alcohol over water (the ratio of alcohol to total fluid intake) is calculated at all time points.
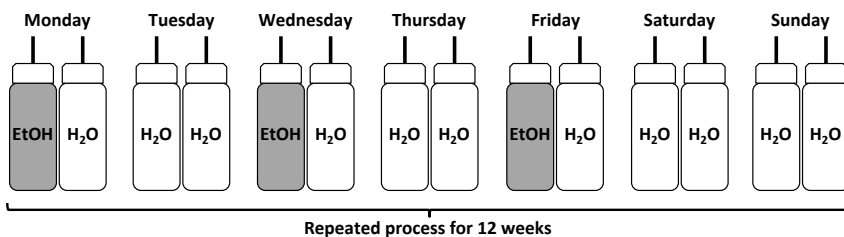

Figure 3. Schematic figure over the intermittent-access 20% alcohol two-bottle-choice drinking model. This is a repeated process for a maximal time of 12 weeks. Experiments with acute or repeated treatment of pharmacological agents are conducted after this drinking period (paper I-III). For the study in paper IV, the treatment started in the beginning of this period.

The effect of acute treatment of liraglutide (paper I), at a dose of either 0.1 mg/kg or 0.05 mg/kg, on alcohol intake was investigated. In the first drinking experiment, the daily effects of acute administration of liraglutide or vehicle on alcohol, water and food intake as well as body weight were investigated. The injection was administered 60 minutes before the rats were given access to alcohol and water. In addition, the effects of repeated liraglutide treatment on daily alcohol intake, the rats were repeatedly administered with liraglutide or vehicle for another seven days. In every experiment (paper I), a three day pre-treatment with liraglutide was subjected to lower the risk of possible aversion. In paper II, the effects of local treatment with Ex4 into specific brain regions on alcohol intake were investigated. The brain areas of interest were NAc, aVTA, pVTA and LDTg. The rats were subjected to 12 weeks of intermittent access to alcohol and then treated locally and bilaterally with Ex4 or vehicle into any of the aforementioned brain areas. In all experiments, measurements were registered 24 hours after bottle presentation. The effect of bilateral intra-NAc, intra-aVTA, intra-pVTA or intra-LDTg administration of Ex4 on alcohol, water, total fluid and preference for alcohol over water (the ratio of alcohol to total fluid intake) as well as food intake and body weight change was measured. In the experiments for aVTA and pVTA, a one-day break between administrations was conducted and each animal served as its own control. In paper III, the effects of local treatment of Ex4 in NTS on alcohol intake were investigated. The rats voluntarily consumed alcohol for 12 weeks and were subsequently subjected to local administration of Ex4 into the NTS, or an equal volume of vehicle, in a balanced design, on an alcohol-drinking day. There was one day between each injection, and each animal served as its own control. In this experiment, the effect of bilateral intra-NTS administration of Ex4 on alcohol, water, total fluid, food intake and preference for alcohol over water (the ratio of alcohol to total fluid intake) as well as body weight change was registered at 1, 4 and 24 hours after bottle presentation.

Effects of repeated treatment of dulaglutide on alcohol intake using the intermittent access model in male and female outbred rats were investigated in paper IV. A pilot experiment was first carried out to test two different doses of dulaglutide, with repeated three-week treatment on alcohol intake, in male rats. The effects of repeated administration of dulaglutide on the daily alcohol, water, and total food intake were investigated on three alcohol drinking days in the intermittent access model. The next series, of four separate experiments, were designed to study the effect of repeated treatment of

dulaglutide on alcohol intake in male and female rats. Male rats were after two alcohol sessions subjected to their first injection of dulaglutide or vehicle. The rats were then treated with dulaglutide for a i) longer period (nine weeks), or a ii) shorter period (five weeks). Similarly, female rats were after two alcohol sessions subjected to their first injection of dulaglutide or vehicle. The female rats were also then treated with dulaglutide for a iii) longer period, or a iv) shorter period. This design allows investigation of nine or five weeks of treatment to influence alcohol intake in both male and female rats. Also, the protracted effect of discontinued treatment on alcohol consumption gets evaluated in both sexes.

Alcohol deprivation model in rats

The alcohol deprivation model is based on the observation that voluntary alcohol intake temporarily increases following forced abstinence in alcohol-experienced rats, when compared to baseline drinking conditions. Thus, the alcohol deprivation model has been suggested to reflect relapse caused by craving in the clinical setting (Spanagel 2000). Rats are subjected to the intermittent access model for ten weeks and are thereafter deprived of alcohol for ten days. Prior to the reintroduction of alcohol, the rats are treated with either liraglutide (paper I), or vehicle. Liraglutide or vehicle sixty minutes before the reintroduction of alcohol. Thereafter, bottles and food were weighed at 24 hours after presentation. The ability of the liraglutide to block relapse drinking was investigated.

Operant self-administration in rats

Operant self-administration is a model that reflects the motivation to consume alcohol in rats. The higher the motivation to obtain the rewarding alcohol the harder the rat is willing to work for it. The meaning of work is in the case for the rat to press a lever, which delivers the reward when pressed. In the present study (paper I), the self-administration experiments were performed in the lab of professor G. Colombo (at Cittadella Universitaria di Monserrato, Cagliari, Sardinia, Italy) and conducted with the sP rats. Self-administration sessions were conducted in modular chambers (Med Associates, St. Albans, VT, USA) equipped with two retractable response levers, one dual-cup liquid receptacle positioned between the two levers, and two stimulus lights (one green and one white) mounted above each lever. Rats were initially exposed to the home cage 2-bottle choice regimen with

unlimited access for 24 hours/day over 10 consecutive days. The alcohol solution was presented at the concentration of 10% (v/v). This initial phase was a part of the conventional procedure of alcohol self-administration employed in the Italian laboratory with sP rats (Maccioni et al. 2012) and conducted to allow the rats to become accustomed to the taste of alcohol and start to experience its pharmacological effects. After the 2-bottle choice model, rats were introduced to the operant chambers and trained to lever-respond for alcohol. This type of experiment can include two paradigms, the progressive ratio (PR) and fixed ratio (FR). PR gives a value of the motivation to access alcohol, as it progressively increases the number of lever responses that are needed for successful alcohol delivery. Last completed ratio that gives the final alcohol presentation is set as break point. The higher break point, the more the rat is willing to work to receive the alcohol reward. For FR, there is a fixed number of lever responses that are needed for the rat to obtain alcohol. The FR gives a measure of reinforcement, indicating the amount of times that the rat is willing to press the lever in order to receive alcohol. Rats in the present study were initially exposed to an FR1 (single press on the lever results in delivery of alcohol) schedule of reinforcement for 10% alcohol (v/v) for four consecutive daily sessions. FR was then increased to FR2 and FR4 over four consecutive sessions. In sessions 9 and 10, the alcohol solution was presented at a final concentration of 15% (v/v). After 20 self-administration sessions of the maintenance phase the test sessions started. In test sessions, response requirement on the alcohol and water lever was kept at FR4 and FR1, respectively. Test sessions lasted 30 minutes and were conducted for five consecutive days. Vehicle or liraglutide was adminis-tered to independent groups of rats. After completion of the treatment phase, rats were exposed to four additional daily self-administration sessions (post-treatment phase). This was performed to evaluate if treatment had any persisting effect on the alcohol self-administration.

Biochemical procedures

Blood alcohol concentration in mice

A simple method to exclude the possibility that differences in alcohol metabolism influence the obtained results is the measurement of blood alcohol concentrations. In the present study (paper I), mice were injected with liraglutide or an equal volume of vehicle solution. Sixty minutes later the

animals were injected with alcohol, decapitated 20 minutes later and trunk blood was collected in micro tubes (Vacuette; Greiner Bio-one, Florence, Italy). The analysis of blood alcohol concentration from the experiment was outsourced to Sahlgrenska University Hospital (Gothenburg, Sweden; study agreement BML-NEURO).

Gene expression in alcohol-consuming rats

Analysis of gene expression is a commonly used method to get an insight in the regulation of a gene in any specific tissue of interest. With this as background, we tested if there were any alterations in gene regulation of the PPG gene (GCG) and Glp1R in rats that had been drinking alcohol for a longer period. Following 12 weeks of intermittent access to alcohol (paper II), the rats were decapitated, and brain areas implicated in AUD (Koob 1992a; Nestler 2001; Wise and Rompre 1989) were dissected: NAc, VTA, amygdala, hippocampus, prefrontal cortex and striatum. Tissue from each area was punched out from cold brain slices of 1 mm thickness and was snap frozen again for later RNA tissue preparation. For the analysis, the selected reference genes (RG) were HMBS and YWHAZ and the genes of interest were Glp1R (TaqMan™ assay ID Rn00562406_m1) and GCG (TaqMan™ assay ID Rn00562293_m1). The corrected CT values raw data were analysed using the comparative CT method as previously described (Livak and Schmittgen 2001) with low alcohol-consuming rats set as the internal calibrator.

Statistical analysis

When comparing parameters between two treatment groups such as for the experiments on CPP (paper I, II and III), blood alcohol concentration levels (paper I), gene expression data (paper II) and the effects of acute treatment on alcohol intake (paper I, II and III), an unpaired t-test was used. The unpaired t-test is used to compare the means of two unmatched groups. In two of the experiments, in the intermittent alcohol access model (paper II), we used a paired sample t-test. A paired t-test is used to compare two means that are from the same individual. Comparisons between two, or more treatment groups, such as for locomotor activity experiments (paper II and III), statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Bonferroni or Tukey's post-hoc test for comparisons between different treatments. When comparing parameters between two, or

more, treatment groups on one or more continuous dependent variables, a two-way ANOVA was used. Microdialysis experiments (paper I and III), repeated treatment on alcohol intake (paper I and IV), the effect of treatment on the alcohol deprivation effect (paper I) and operant alcohol self-administration (paper I) were analyzed with a repeated two-way ANOVA followed by Bonferroni or Tukey's post-hoc test for comparisons between treatments and at specific given time points. To analyze the correlation between gene expression and mean values of alcohol intake (paper II) the Pearson correlation test was performed.

RESULTS AND DISCUSSION

Paper I

In paper I, a series of experiments was undertaken to investigate the acute and repeated treatment effects of the GLP-1R agonist, liraglutide, on several alcohol-related behaviors in rodents

Liraglutide attenuates the rewarding properties of alcohol in mice

In the present study, we found that peripheral injection of the GLP-1R agonist liraglutide attenuates the ability of a second injection of alcohol to increase the release of accumbal dopamine (Figure 4A). The dopamine changes in the NAc shell are known to lead to reinforcement, central stimulation, euphoria and reward, a first step in consuming and developing addiction (Boileau et al. 2003; Volkow et al. 2004; Volkow et al. 2012). Hence, liraglutide blocking this effect, and given that GLP-1Rs are expressed in this area (Merchenthaler et al. 1999), supports a modulatory role for liraglutide in reinforcement. In agreement with the present data are previous studies reporting that the GLP-1R agonist Ex4 attenuates the ability of alcohol, amphetamine, cocaine and nicotine to induce accumbal dopamine release (Egecioglu et al. 2013c; Egecioglu et al. 2013b, 2013a).
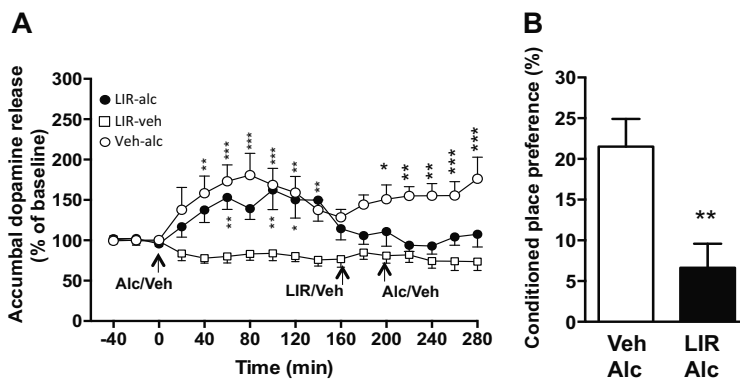
Figure 4. (A) Initial injections of alcohol cause significant increase in accumbal dopamine release compared to vehicle treatment. In the subsequent part of the experiment, administration of liraglutide attenuates alcohol-induced accumbal dopamine release. (B) Alcohol-induced CPP is significantly attenuated by concomitant injection of liraglutide on each conditioning day.

Additionally, liraglutide blocks alcohol-induced reward in the CPP model (Figure 4B), an effect that is seen when liraglutide is administrated prior to alcohol during the conditioning, and consequently attenuates expression in the following post-conditioning session (Sanchis-Segura and Spanagel 2006). This is in accordance with previous studies establishing that Ex4 attenuates alcohol-induced reward in the CPP model (Egecioglu et al. 2013c). The ability of GLP-1, it self, to block expression of alcohol-induced CPP was verified in a follow up study (Shirazi et al. 2013). In the present study, liraglutide failed to block alcohol reward-dependent memory retrieval in the CPP model. However, acute treatment of GLP-1 and Ex4 at post-conditioning attenuates alcohol reward-dependent memory retrieval (Egecioglu et al. 2013c; Shirazi et al. 2013). This divergence in behavioral effects between GLP-1 and GLP-1R agonists may possibly be due to their different ability to reach and activate various brain areas (Gu et al. 2013; Salinas et al. 2018), which needs to be further investigated.

Acute liraglutide treatment decreases alcohol intake and prevents the alcohol deprivation effect in rats

Before initiation of the present study, two reports found that acute pharmacological activation of GLP-1R decreases alcohol intake in the intermittent access two-bottle-choice model in rats. Both studies used the GLP-1R agonist Ex4 and showed that acute peripheral administration reduces alcohol intake in rats (Egecioglu et al. 2013c; Shirazi et al. 2013). Further support for involvement of endogenously released GLP-1 in alcohol intake is provided by the data showing that blockade of GLP-1R results in increased alcohol intake in rats (Shirazi et al. 2013). In the present study we obtained similar effects as we showed that acute administration of liraglutide reduces alcohol intake in rats (Figure 5A). The reduction of alcohol intake was compensated by an increase (not significant) of water intake, which might be the reason why there was no alternation in total fluid intake.
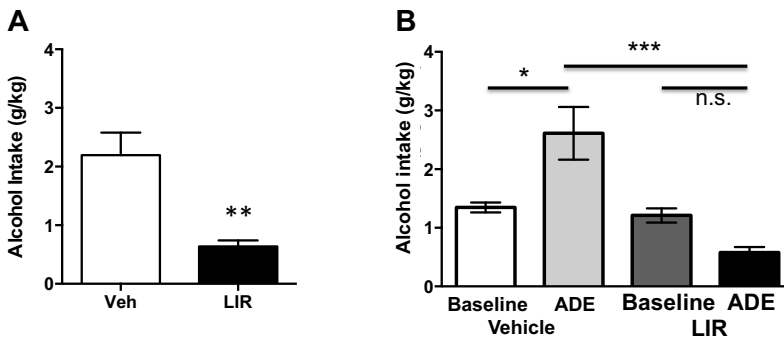
Figure 5. (A) Acute administration of liraglutide decreases alcohol intake in outbred rats. (B) Acute administration of liraglutide prevents the alcohol deprivation effect in outbred rats. There is an alcohol deprivation effect (ADE) in vehicle treated rats but not in rats treated with liraglutide, compared to the corresponding baselines.

Additionally, acute treatment with liraglutide blocks the alcohol deprivation effect in rats (Figure 5B). Relapse drinking, which is measured in the alcohol deprivation test, is seen in dependent patients that have been abstinent to alcohol and then relapse to a larger consumption (Burish et al. 1981). It has been shown in both clinical and preclinical studies that craving causes this relapse and increases alcohol intake. This gives the alcohol deprivation model face validity for alcohol relapse drinking (Spanagel 2000; Vengeliene et al. 2009). Supporting results regarding GLP-1 in relapse drinking are mice data showing that GLP-1R agonists, Ex4 and AC3174, prevent the deprivation-induced increase in alcohol intake (Suchankova et al. 2015; Thomsen et al. 2017).

Repeated liraglutide treatment decreases alcohol intake in outbred rats and reduces operant alcohol self-administration in sP rats

An important clinical aspect of treatment could be the effect of repeated liraglutide treatment on alcohol intake. In the present study we found that repeated liraglutide treatment reduces alcohol intake in outbred rats (Figure 6A) as well as decreases the motivation to obtain alcohol (Figure 6B) and the amount of alcohol consumed (Figure 6C) in the operant self-administration model in sP rats. Important to mention is that the first two to three days after treatment discontinuation, the number of lever responses for alcohol and amount of self-administered alcohol, in the liraglutide (0.1mg/kg) treated group, remained reduced in comparison with controls.
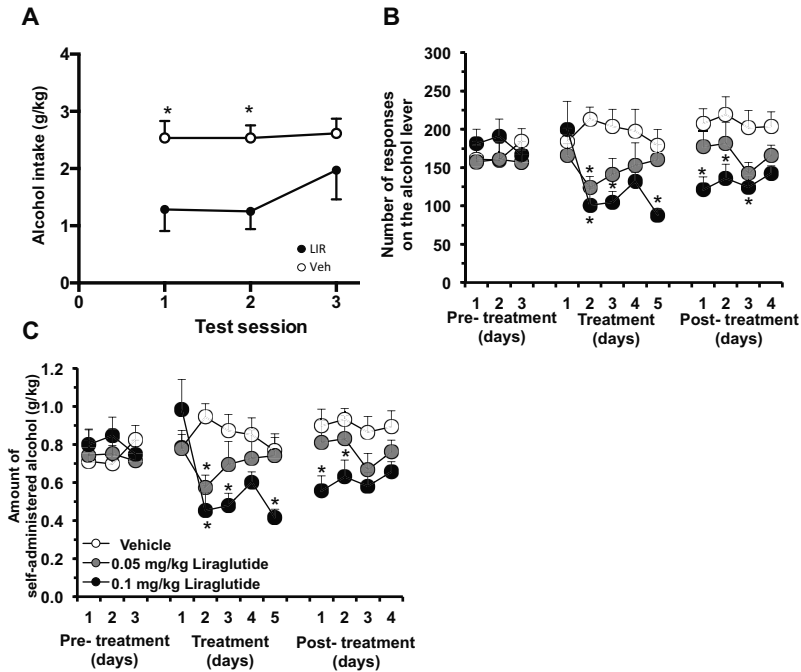
Figure 6. (A) Repeated administration of liraglutide decreases alcohol intake in outbred rats at test sessions 1 and 2. (B) Repeated treatment of liraglutide reduces numbers of lever responses for alcohol as well as (C) the amount of self-administered alcohol during the five-day treatment phase in sP rats. After treatment discontinuation, the number of lever responses for alcohol and amount of self-administered alcohol are lower in the rat group treated with liraglutide in comparison with control values.

Published data shows that repeated treatment with the GLP-1R agonist Ex4 keeps a sustained effect of reduced alcohol intake after alcohol deprivation in mice (Thomsen et al. 2017), and support a modulatory role of GLP-1R agonists on continued drinking after treatment discontinuation. Additionally, Ex4 treatment protracts latency to the first alcohol drink as well as decreases the number of drinking bouts (Thomsen et al. 2017). Another GLP-1R agonist, AC3174, reduces alcohol drinking only after repeated administration, and not on the first day of drinking, in mice (Suchankova et al. 2015). Recent interesting data from alcohol-preferring male African vervet monkeys shows similar effects of repeated liraglutide treatment. Hence, liraglutide reduces voluntary alcohol drinking in non-human primates and the reduction in alcohol intake is without causing any signs of nausea or other side effects (Thomsen et al. 2018).

Lastly, in the present study we showed that repeated liraglutide treatment reduces the operant self-administration of alcohol in sP rats. The sP rat is a rat line selectively bred for high alcohol preference and intake, and their alcohol-seeking and alcohol-taking behaviors have been proposed to model several aspects of excessive alcohol consumption in humans (Colombo et al. 2006). Acute pharmacological activation of GLP-1R by Ex4 in rats shows similar results as it prevents alcohol-seeking behavior as measured by the progressive ratio test in the operant self-administration model (Egecioglu et al. 2013c). Moreover, studies show reduction of intravenous self-administration of alcohol seen in mice treated with a high dose of Ex4 (Sorensen et al. 2016).

In conclusion, we show that pre-treatment with the GLP-1R agonist liraglutide attenuates the alcohol-induced accumbal dopamine release and CPP in mice. In rats, both acute and repeated liraglutide treatment reduces alcohol intake in the intermittent access two-bottle-choice model and acute treatment also blocks the alcohol deprivation effect. Moreover, repeated administration of liraglutide reduces alcohol intake and decreases the rewarding properties of alcohol in sP rats. Collectively, we provide evidence that the GLP-1R agonist, liraglutide, has an important role in regulating alcohol-mediated behaviors in rodents.

Paper II

Albeit previous studies, including paper I, focused on the peripheral effects of a GLP-1R agonist on alcohol-mediated behaviors, the role of GLP-1R in brain circuitries linked to alcohol reward, such as the cholinergic-dopaminergic reward link (Larsson and Engel 2004), remains to be investigated.

Ex4 infusion into NAc shell attenuates alcohol-mediated behaviors in rodents

The present study shows that local and bilateral administration of Ex4 into NAc shell prevents alcohol-induced locomotor stimulation (Figure 7A). In addition, Ex4 attenuates alcohol reward-dependent memory retrieval in the CPP model in mice (Figure 7B). These findings are consistent with previous studies reporting that systemic administration of GLP-1 and Ex4, at post-conditioning, also attenuates the ability of alcohol to cause reward-dependent memory retrieval (Egecioglu et al. 2013c; Shirazi et al. 2013). Ex4 crosses the

BBB after peripheral injection (Kastin et al. 2002; Kastin and Akerstrom 2003). Therefore, the present study could suggest that the effect on reward-dependent memory retrieval is conducted via GLP-1R within the NAc shell (Merchenthaler et al. 1999). Additionally, Ex4 into NAc shell reduces alcohol intake in rats (Figure 7C).
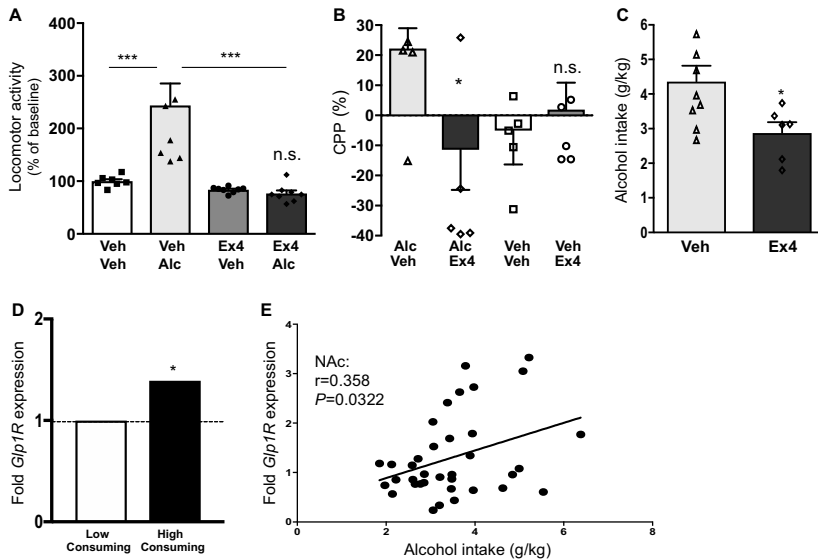

Figure 7. (A) Ex4 into NAc shell, at a dose with no effect *per se*, blocks alcohol-induced locomotor stimulation. (B) NAc-Ex4 blocks the alcohol-dependent reward of memory retrieval in the CPP test in mice. (C) In rats, Ex4 into NAc shell reduces 24-hour alcohol intake (g/kg). (D) Expression of the *Glp1R* in NAc is increased in high compared to low alcohol-consuming rats. (I) Expression of *Glp1R* in the NAc is positively correlated to alcohol intake.

The effects of local administration of Ex4 Into NAc are supported by the findings that systemic treatment with Ex4 attenuates the ability of cocaine to increase the expression of *c-Fos*, an indicator of neuronal activation, in striatum (Sorensen et al. 2015). Also, local infusion of Ex4 into the NAc reduces cocaine self-administration (Hernandez et al. 2017) strengthens the involvement of NAc GLP-1Rs.

The results also show that *Glp1R* expression is elevated in rats consuming high amounts of alcohol for 12 weeks as compared to low consumers (Figure 7D). Additional findings show significant positive correlation between *Glp1R* expression in the NAc and alcohol intake (Figure 7E). The differences in the *Glp1R* expression could suggestively be a result of long-term alcohol consumption, thus contributing to increased vulnerability in the reward

system which can be further associated with the development of AUD. Further support for a suppressive effect of activation accumbal GLP-1R, on alcohol-related behaviors, are revealed by the data showing that Ex4 into NAc shell decreases alcohol intake in female rats (Abtahi et al. 2018). Also, data shows that GLP-1R activation in NAc shell decreases cocaine self-administration (Hernandez et al. 2017), as well as reduces motivation for sucrose in the operant self-administration model (Dickson et al. 2012).

Effects of Ex4 infusion into the anterior or the posterior VTA on alcohol-mediated behaviors in rodents

In the studies of the aVTA we did not find any attenuating effects of bilateral Ex4 administration on either alcohol-induced locomotor stimulation (Figure 8A), alcohol reward-dependent memory retrieval in the CPP model (Figure 8B) in mice, or any effect on alcohol intake in rats (Figure 8C).
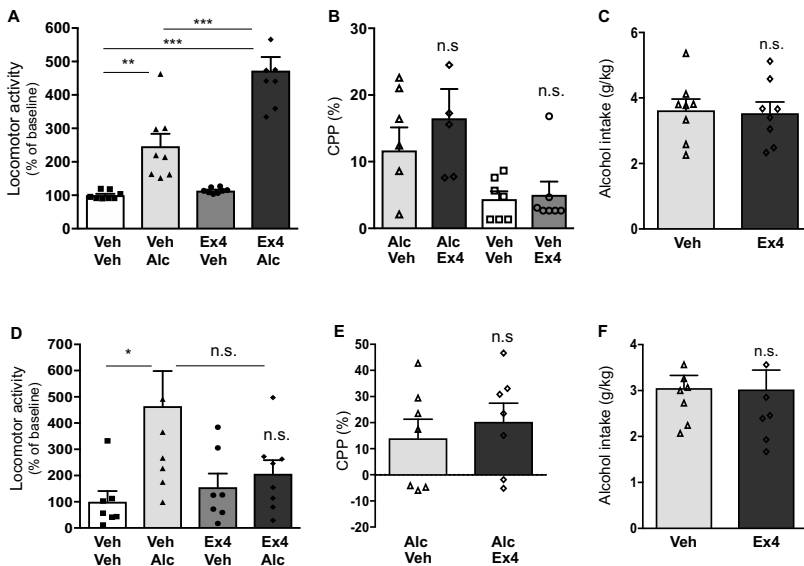
Figure 8. (A) Ex4 into aVTA, at a dose with no effect *per se*, does not block the alcohol-induced locomotor stimulation in mice. (B) Ex4 does not affect the alcohol reward-dependent memory retrieval in the CPP test in mice. (C) Ex4 into aVTA has no effect on alcohol intake (g/kg) in rats. (D) Alcohol-induced locomotor stimulation is reduced by Ex4 into pVTA. Locomotor response is lower in Ex4 compared to vehicle treated mice, but there is no significant difference between the groups. (E) Ex4 does not block alcohol reward-dependent memory retrieval in the CPP test in mice. (F) Ex4 into pVTA has no effect on alcohol intake (g/kg) in rats.

On the contrary to aVTA, Ex4 into the pVTA attenuates alcohol induced locomotor stimulation (Figure 8D). However, no effect on alcohol reward-dependent memory retrieval in the CPP model (Figure 8E) or alcohol intake (Figure 8F) was found. Regarding the dose of Ex4 in pVTA, similar doses to the present study have been found to reduce intake of sucrose, chow and high fat diet, cause significant decrease in body weight (Alhadeff et al. 2012; Mietlicki-Baase et al. 2013; X. F. Wang et al. 2015) and prevent cocaine self-administration and seeking (Hernandez et al. 2018; Schmidt et al. 2016) when infused into the pVTA. One tentative explanation on the obtained results might be that GLP-1R are not expressed on dopaminergic cells within the VTA and hence only regulate some, but not all reward-mediated behaviors.

VTA is, as mentioned earlier, a heterogeneous brain area (Holly et al. 2016; Lammel et al. 2012; Menegas et al. 2017) involving the aVTA and pVTA that play different roles on alcohol reward (Ding et al. 2009; Hauser et al. 2011; Jerlhag et al. 2006; Jerlhag and Engel 2014; Larsson et al. 2002; Lof et al. 2007). Moreover, studies have identified -5.5 mm, relative to bregma, as the boundary between the anterior and posterior regions of the VTA in rats (for review see (Sanchez-Catalan et al. 2014)). The results within the present study are contradictory to previous studies showing that infusion of a high dose of Ex4 into VTA (coordinates -5.7 mm) reduces alcohol and water intake in rats (Shirazi et al. 2013). However, in the present study we used the coordinates -5.3 mm for aVTA and -6.8 mm for pVTA. The possibility should be considered that the discrepancies of the results, are due to the site of action within VTA, and it could be implied that GLP-1R within different parts of VTA regulates different behaviors.

Effects of intra-LDTg infusion of Ex4 on alcohol-induced behaviors in rodents

Intra-LDTg administration of Ex4 inhibits the alcohol-induced increase in locomotor activity (Figure 9A). However, as opposed to intra-NAc or systemic Ex4 (Egecioglu et al. 2013c), activation of GLP-1R in LDTg by Ex4 does not alter alcohol reward-dependent memory retrieval in the CPP model (Figure 9B). Additionally, intra-LDTg administration of Ex4 reduces alcohol intake sin rats (Figure 9C).
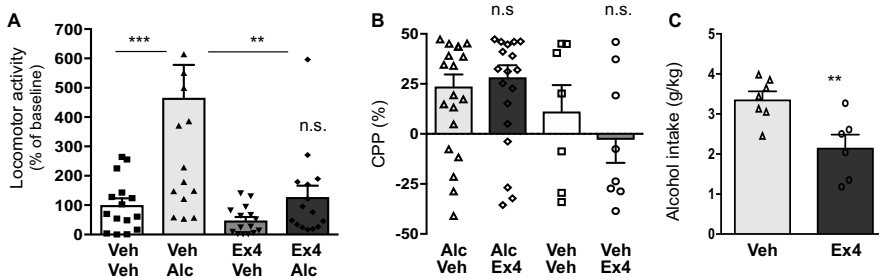
Figure 9. A) Ex4 into LDTg, at a dose with no effect *per se*, blocks alcohol-induced locomotor stimulation in mice. (B) Ex4 does not block alcohol-dependent reward of memory retrieval in the CPP test in mice. (C) Ex4 into LDTg reduces alcohol intake (g/kg) in rats.

A role of GLP-1 signaling in LDTg is supported by studies reporting that GLP-1Rs are expressed in this area (Merchenthaler et al. 1999). LDTg is anatomically connected to areas of importance for alcohol reward, including the NAc (Dautan et al. 2014) and VTA (Cornwall et al. 1990). We therefore hypothesized that Ex4 into LDTg attenuates alcohol-mediated behaviors, possibly via inhibition of the cholinergic-projection to dopamine neurons in the NAc or VTA (Larsson et al. 2005). Surprisingly, Ex4 into LDTg blocks alcohol-induced locomotor stimulation and reduces alcohol intake, but fails to attenuate alcohol reward-dependent memory retrieval in the CPP model. We hypothesize that Ex4 into LDTg, would attenuate the rewarding properties of alcohol in the CPP model, since optogenetic activation of LDTg induces a reward CPP (Steidl et al. 2017b; Steidl et al. 2017a). In such experiments LDTg-Ex4 is co-administered with alcohol during conditioning, but due to ethical limitations such experiment cannot be conducted. As of fact, daily repeated local injection would inflict too much trauma for the animals.

In summary, these data provide additional knowledge of the functional role of GLP-1R in reward-related areas. Obtained data suggests that GLP-1R within NAc and LDTg, to be more important than those in aVTA and pVTA, for alcohol-mediated behaviors.

Paper III

In paper III, activation of GLP-1R within the NTS was further assessed as a possible GLP-1R dependent neurocircuitry modulating alcohol-mediated behaviors.

Ex4 infusion into NTS attenuates rewarding properties of alcohol in mice

In the present study we show that infusion of Ex4 into the NTS blocks alcohol-induced locomotor stimulation (Figure 10A) and accumbal dopamine release in mice (Figure 10B), which is in agreement with previous studies reporting that peripheral administration of Ex4 attenuates such behaviors induced by alcohol, amphetamine, cocaine and nicotine (Egecioglu et al. 2013c; Egecioglu et al. 2013b, 2013a). Similarly, data from paper I demonstrate that peripheral injection of liraglutide blocks accumbal dopamine release caused by alcohol injection (Vallof et al. 2016)(paper I). The findings from paper III extend these initial studies by demonstrating that GLP-1R in NTS of the caudal brainstem drive modulation of alcohol-mediated behaviors in rodents. In support for a role of GLP-1R in reward regulation are previous data showing that Ex4 into the NTS reduces food intake and food reward (Alhadeff et al. ; Hayes et al. 2009; McMahon and Wellman 1998; Richard et al. 2015; Tang-Christensen et al. 1996) as well as a decreases nicotine intake in rats (Tuesta et al. 2017). Further connections to rewarding aspects are the findings that PPG neurons identified in the NTS, projects to the NAc, VTA and LDTg (Alhadeff et al. 2012; Jin et al. 1988; Merchenthaler et al. 1999; Reiner et al. 2018; Rinaman 2010), which are areas of importance for alcohol mediated behaviors (Doyon et al. 2003; Ericson et al. 2003; Larsson et al. 2005). Findings from paper II demonstrate that GLP-1R, within specific brain regions throughout the cholinergic-dopaminergic reward link, modulate alcohol-mediated behaviors. This further suggests that GLP-1R dependent connectivity from the NTS to NAc, VTA or LDTg could constitute important pathways for the ability of alcohol to express its reinforcing properties.
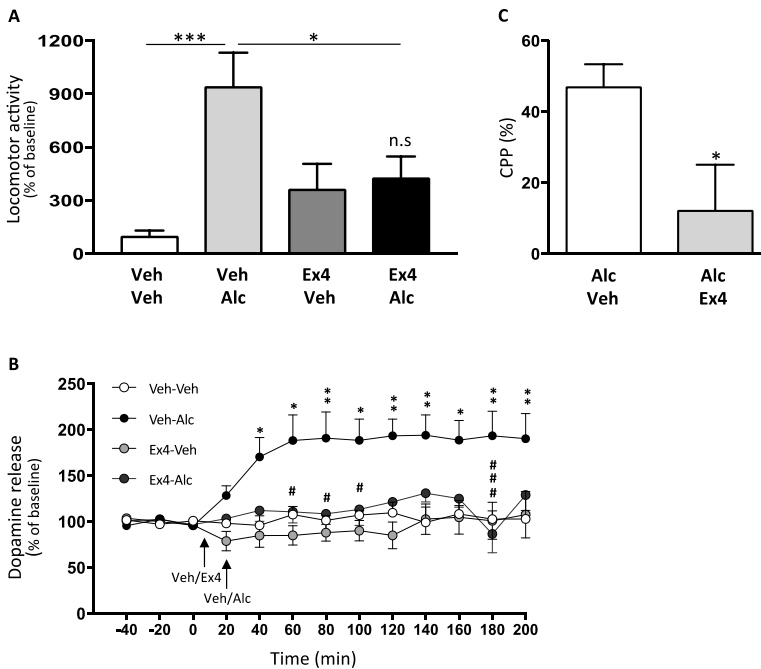
Figure 10. (A) Alcohol induced locomotor stimulation is inhibited by NTS injection of Ex4. (B) Ex4 into the NTS blocks the alcohol-induced increase in accumbal dopamine release. There is no difference in response between vehicle treated mice and those treated with Ex4 and alcohol. (C) NTS-Ex4 attenuates the alcohol reward-dependent memory retrieval in the CPP model.

As further revealed in paper III, Ex4 administration into the NTS blocks alcohol reward-dependent memory retrieval in the CPP test in mice (Figure 10C). A regulatory role of GLP-1R in the NTS for memory reward is strengthened by the findings that injections of Ex4 into the NTS blocks memory consolidation of food reward in the CPP model (Alhadeff and Grill 2014; Richard et al. 2015) and that systemic administration of Ex4 or GLP-1 attenuates alcohol reward-dependent memory retrieval in the CPP model (Egecioglu et al. 2013c; Shirazi et al. 2013).

Ex4 infusion into NTS decreases alcohol intake in rats

Data from paper III further demonstrate that Ex4 into the NTS dose-dependently reduces alcohol intake in rats. This effect is immediate and reduces alcohol intake already after 1-hour (Figure 11A). The effect is maintained and continuous which results in lower alcohol intake at 4-hour

(Figure 11B) and 24-hour time points (Figure 11C). This confirms previous studies reporting that acute peripheral administration of Ex4 resulted in reduction of alcohol intake in rats (Egecioglu et al. 2013c; Shirazi et al. 2013) and that acute or repeated systemic liraglutide treatment decreases alcohol intake in rats (Vallof et al. 2016)(Paper I).
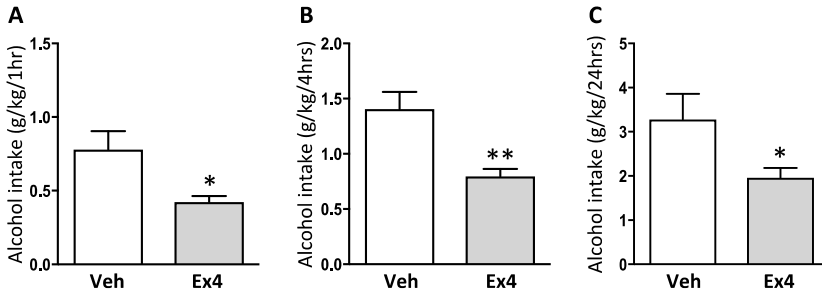

Figure 11. In comparison to vehicle, Ex4 injection into the NTS reduces alcohol intake (g/kg) in rats at (A) 1-hour, (B) 4-hour and (C) 24-hour time points.

Additional data from paper III show that Ex4-NTS significantly reduces alcohol intake without altering other parameters such as food intake or body weight. These results differ from earlier findings where direct local injection of Ex4 into the NTS causes a reduction in chow intake (Alhadeff and Grill 2014; Reiner et al. 2016) as well as palatable high fat diet (Alhadeff and Grill 2014) and where knockdown of GLP-1R within the NTS results in increased chow intake (Alhadeff et al. 2017). The findings that intra-NTS Ex4 treatment reduces intake of palatable food but not chow in rats that were given a choice between palatable food and chow (Richard et al. 2015), raises the possibility that activation of GLP-1R-NTS reduces the most rewarding substrate when exposed to a choice situation. In agreement, chow intake and body weight were reduced by Ex4 treatment, if chow was the only option provided (Richard et al. 2015).

Given that GLP-1R are not expressed directly on the PPG neurons (Hisadome et al. 2010), the possibility that Ex4 regulates alcohol-mediated behaviors via additional mechanisms should be taken into consideration. Hence, activation of presynaptic GLP-1R on astrocytes (Reiner et al. 2016), vagus (for review see (Hayes et al. 2010)), or collaterals of the PPG neurons that branch and possible projects to more than one target site (Vrang et al. 2007), could be tentative routes of action.

In conclusion, paper III provides a critical input of the neural circuits through which GLP-1 controls alcohol-mediated behaviors in rodents and collectively suggests an important role of GLP-1R-NTS activation for reward processes, both natural as well as chemical.

Paper IV

As previous studies have established that acute as well as short term GLP-1R agonist treatment reduces alcohol intake in male rats, experiments in paper IV was undertaken to investigate the ability of long-term treatment. The study was therefore conducted with dulaglutide (Barrington et al. 2011b; Barrington et al. 2011a; Glaesner et al. 2010), a GLP-1R agonist with protracted pharmacokinetics allowing once-weekly administration, to evaluate alcohol intake in male as well as female rats. Adding studies in female rats are of interest since most pre-clinical and clinical studies only include male subjects.

Repeated treatment of dulaglutide for nine weeks reduces alcohol intake in male and female rats

Treatment with once weekly dulaglutide for nine consecutive weeks shows a maintained lower level of alcohol intake compared to vehicle treated male rats (Figure 12A). Alcohol intake after discontinuation of treatment was continuously lower in the dulaglutide treated male rats for the following two weeks after completed treatment. Regarding female rats, repeated treatment of dulaglutide decreases alcohol intake (Figure 12B). However, alcohol intake after discontinuation of treatment returns to baseline for the female rats.
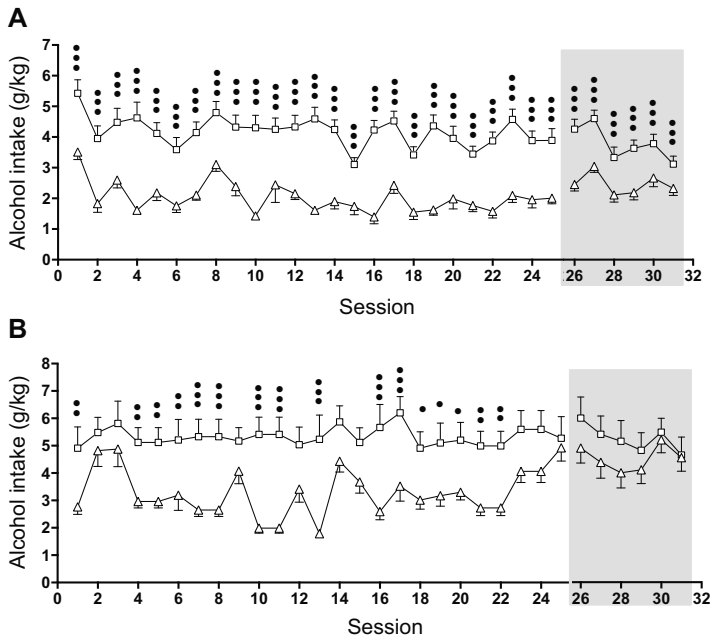
Figure 12. Compared to vehicle (square), dulaglutide (triangle) treatment for nine weeks (sessions 1-25) persistently reduces alcohol intake in male rats. After treatment discontinuation (sessions 26-31, area marked with grey), alcohol intake is lower in the previously dulaglutide-treated group compared to vehicle. (B) Compared to vehicle, repeated dulaglutide treatment decreases alcohol intake in female rats during active treatment (session 1-25). After treatment discontinuation (session 26-31) alcohol intake does not differ between the dulaglutide and vehicle groups.

In the present study, no sign of tolerance was seen over the treatment period in either male or female rats. Literature provides divided contradictory evidence regarding this aspect. In an earlier study from our group we saw a small effect of tolerance with treatment of the GLP-1R agonist liraglutide (Vallof et al. 2016)(Paper I). This tolerance effect was however not seen when repeated liraglutide treatment lowered alcohol intake in alcohol-preferring male African vervet monkeys (Thomsen et al. 2018) or repeated treatment of Ex4 protracted latency to the first alcohol drink as well as decreased the number of drinking bouts in mice (Thomsen et al. 2017). It may be argued that the different of tolerance development lies between the use of mice or rats, or that the different GLP-1 agonists have an altered effect regarding this matter. This long-term reduction in alcohol intake, without tolerance induction, is of greatest interest for the clinical use of dulaglutide when treating AUD patients.

Repeated treatment of dulaglutide for five weeks reduces alcohol intake in male and female rat

To evaluate possible effect of dulaglutide on alcohol intake, following discontinuation of treatment, two separate experiments were set up. The experiments revealed that five weeks of dulaglutide treatment reduce alcohol intake in male (Figure 13A) and female (Figure 13B) rats. These results are in line with those of nine weeks of treatment with dulaglutide.
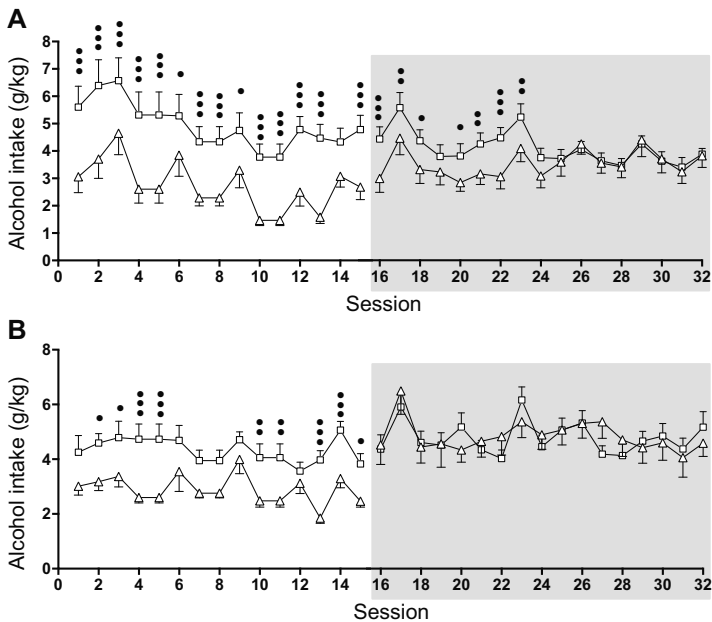

Figure 13. (A) Repeated dulaglutide treatment (triangle) decreases alcohol intake in male rats from the first treatment day to the end of treatment on session 15, compared to vehicle (square). After treatment discontinuation (session 16-32, area marked with grey) alcohol intake is lower in the previously dulaglutide-treated group, compared to vehicle. (B) Compared to vehicle, repeated dulaglutide treatment reduces alcohol intake in female rats during active treatment (sessions 1-15). The alcohol consumption is not lower in the previously dulaglutide-treated group, compared to vehicle (session 16-32).

The reducing effect of dulaglutide for nine- as well as five-weeks on alcohol consumption is more pronounced in male (52% respectively 39%) compared to female (44% respectively 33%) rats. It should be taken into consideration that dulaglutide response could be influenced by variation in the pharmacokinetic properties between male and female rats, and that therefore a higher dose could have been used in females. Dose effects of Ex4 between the two sexes have been seen before where a less potent effect was

seen in female, compared to male rats (Lopez-Ferreras et al. 2018), on the ability to reduce food intake and operant conditioning for sucrose in rats.

These data provide further knowledge on GLP-1, alcohol intake, gender and collectively constitute important information when testing such compounds clinically in patients with AUD.

GENERAL DISCUSSION

Presented within this thesis are data supporting a modulatory role of GLP-1R agonists on alcohol consumption in alcohol drinking rats. Indeed, nine as well as five weeks of treatment with dulaglutide, decreases alcohol intake in both male and female rats. To the best of our knowledge, these are the first alcohol consumption experiments in rats exposed to GLP-1R agonists for a longer period of time. Also, for the first time, a reduction in alcohol intake following peripheral treatment with a GLP-1R agonist in female rats is reported. Acute treatment with liraglutide reduces alcohol intake and prevents the alcohol deprivation effect in male rats. The alcohol deprivation effect in rodents is thought to model aspects of human drinking behavior, as seen from relapse drinking that occurs after a period of abstinence (Spanagel 2000). In addition, repeated treatment of liraglutide decreases alcohol intake in outbred rats as well as reduces the motivation to consume alcohol in selectively bred sP rats. Both the operant alcohol self-administration and the sP rat have strong translational value (Colombo et al. 2006), providing further evidence on liraglutide's ability to attenuate the reinforcing properties of alcohol, an effect that could be valuable in humans. In fact, preliminary data supports an effect of liraglutide on alcohol consumption, as treatment reduces alcohol intake in patients with type II diabetes (Kalra S 2011). Data herein also provides information on GLP-1Rs in brain specific regions and their ability to reduce alcohol intake alcohol-consuming male rats, after local injection of Ex4, into NAc shell, LDTg and NTS. These data adds further knowledge on the functional roles of GLP-1R, in some areas that are implicated in reward and food intake, and pinpoints these regions to be of importance for alcohol-consumption.

Additionally, data within the present thesis demonstrates the ability of GLP-1R agonists to attenuate alcohol-induced activation of the mesolimbic dopamine system. Indeed, acute peripheral treatment with liraglutide suppresses the well-documented effects of alcohol on the mesolimbic dopamine system as seen on alcohol-induced accumbal dopamine release and CPP in mice (Engel et al. 1988; Sanchis-Segura and Spanagel 2006). Moreover, GLP-1R activation after local injection of Ex4, into NAc shell, pVTA, LDTg and NTS attenuates alcohol-induced locomotor stimulation. Ex4 into NAc shell and NTS also attenuates the retrieval of alcohol reward memory in

the CPP model. Lastly, Ex4 into NTS blocks the ability of alcohol to induce accumbal dopamine release.

This thesis supports the importance of pre-clinical studies for development, or even elucidation of already existing medications, of new pharmacological targets for AUD. This is exemplified by studies with the partial nAChR agonist, varenicline, a Food and Drug Administration-approved agent on smoking cessation, which prevents the alcohol-induced activation of the mesolimbic dopamine system and reduces alcohol consumption in rodents (Ericson et al. 2009). Taken into the clinical setting, varenicline decreases the alcohol specific biomarker phosphatidylethanol, craving and the alcohol use disorders Identification test (also known as AUDIT) score in human patients with AUD (de Bejczy et al. 2015). Moreover, pre-clinical studies with the anti-epileptic drug topiramate, have shown effects on alcohol withdrawal and alcohol-conditioned behaviors in mice (Farook et al. 2007), and reduce drinking in alcohol-dependent patients (Johnson et al. 2003). Also, the GABA$_B$ receptor agonist baclofen has shown to suppress motivation to consume alcohol (Colombo et al. 2003b), reduce alcohol intake (Colombo et al. 2000) and suppress the alcohol-induced deprivation effect (Colombo et al. 2003a) in rats. In the clinical setting, baclofen suppresses alcohol cravings (de Beaurepaire 2012). Therefore pre-clinical and clinical research on GLP-1 and alcohol-mediated behaviors may contribute to the identification of GLP-1 and GLP-1R as potential novel pharmacological targets for the treatment of AUD.

Baseline alcohol intake between the studies in this thesis differs to some extent and may be seen as an overall limitation. For example, the baseline intake varies between 2.9 g/kg (paper II), 3.4 g/kg (paper I), 5.9 g/kg (paper III) and 6.8 g/kg (paper IV). The rat strain, Rcc Han Wistar, used in all papers has been shown to have higher voluntary alcohol intake and alcohol preference than other Wistar rats (Palm et al. 2011). Animal handling, housing, dark/light cycle and possibly other animals in the same housing room are factors that may impact the amount of alcohol consumed. These are important aspects, and different rooms have been used for different studies. However, same conditioning has been applied in every experiment conducted. If strength on the varying difference on baseline alcohol should be drawn, it should be on the fact that we show attenuation of alcohol intake in both low and high alcohol-consuming rats. Ex4 into NTS (paper III) and peripheral dulaglutide (paper IV) reduces alcohol intake in male rats consuming higher amounts of alcohol. However, liraglutide (paper I) and Ex4 infused into NAc

as well as LDTg, but not aVTA or pVTA (paper II) reduces alcohol intake in rats with lower baseline intake, indicating that baseline drinking does not influence the obtained results. Other gut brain peptides show similar effects. Fore example, NMU reduces alcohol intake in high, but not low, alcohol-consuming rats (Vallof et al. 2017) and the amylin agonist salmon calcitonin attenuates alcohol intake in low alcohol-consuming rats, but with a more robust decrease in high alcohol consumers (Kalafateli et al. 2018). Moreover, the ghrelin receptor antagonist GHS-R1A, reduces alcohol intake in high, but not low, alcohol-consuming rats (Suchankova et al. 2013).

Alcohol causes locomotor stimulation in rodents, a result of the enhanced extracellular concentration of accumbal dopamine (Engel et al. 1988). Peripherally administered liraglutide and local injection of Ex4 into NAc, pVTA and NTS attenuates alcohol-induced locomotor stimulation. As previously discussed, this attenuation may be as a result from attenuation of accumbal dopamine increase. However, other mechanisms may lie behind the attenuated locomotion. For instance, GLP-1R agonists enhance glucose-dependent insulin secretion (Holst and Seino 2009; Kreymann et al. 1987). Insulin levels are proposed to interfere with effects of psychomotor stimulant drugs in rodents (for review see (Daws et al. 2011)), which raise a possibility that the effects of the GLP-1R agonist on alcohol-induced locomotor stimulation could be insulin-dependent.

Systemic administration of GLP-1 is known to reduce food intake, with an accompanied decrease in body weight, in humans (Flint et al. 1998; Gutzwiller et al. 1999a; Gutzwiller et al. 1999b; Verdich et al. 2001) as well as in rodents (Abbott et al. 2005; Chelikani et al. 2005; Tang-Christensen et al. 1996; Turton et al. 1996). Alcohol can be a source of calories and it should be noted that GLP-1 might potentially reduce caloric intake rather than alcohol consumption. Reduction of food intake and body weight is present in paper I and IV where no signs of change on those parameters are seen in paper II and III. It should be considered that in paper I and IV, the GLP-1R agonists liraglutide and dulaglutide are administered peripherally and Ex4 in paper II and III is administered locally, which might lead to the different results in food intake and body weight. The possibility should be considered that liraglutide and dulaglutide may increase energy expenditure or decrease adiposity, as shown by other appetite-regulatory peptides such as ghrelin (Egecioglu et al. 2011). However, it appears less likely that treatment induces increase in energy expenditure, since the dose of liraglutide used here has no effect *per se* on locomotor activity. In addition, activation of GLP-1R prevents

reward expression of other addictive drugs without caloric content, such as amphetamine, cocaine and nicotine (Egecioglu et al. 2013b, 2013a; Erreger et al. 2012; Graham et al. 2013; Hernandez et al. 2017; Hernandez et al. 2018; Sorensen et al. 2015).

Central GLP-1 is known to produce nausea and to induce taste aversion (Kanoski et al. 2012). The GLP-1R agonists used in the present thesis, could attenuate alcohol-mediated behaviors as a result of these side effects. However, we show that neither acute nor repeated liraglutide treatment has an effect *per se* on CPP (paper I). Also, the noted increase in water intake does not suggest nausea or malaise. Similarly, all the doses used in NAc, VTA, LDTg and NTS (paper II and III) have no effect on CPP, locomotor activity or water intake. Ex4 into specific brain regions seems to not induce aversion as supported by studies in NAc (Hernandez et al. 2017), VTA (Hernandez et al. 2018), LDTg (Reiner et al. 2018) and NTS (Alhadeff and Grill 2014; Richard et al. 2015). Regarding dulaglutide treatment, the increase or no difference in water intake indicates that potential aversion or nausea from the treatment is possibly not present.

Experimental techniques and their execution are crucial steps in obtaining data and their interpretation. Possible limitations influencing the obtained data should therefore be taken into consideration. As in all studies involving intracranial injections (study II and III), potential adverse effects from cannula implantations, tissue lesions or altered physiological response after surgeries cannot be overlooked. Also, undesirable diffusion of Ex4 into surrounding brain areas other than the targeted should be considered, even with the low infusion volume of 0.5 µl. However, animals with misplaced guides, as opposed to guides targeting the correct area, no effect of Ex4 was observed.

In paper II and III, we have focused on GLP-1R within the cholinergic-dopaminergic reward link and NTS of the caudal brainstem, which is highly interconnected with the reward system (Alhadeff et al. 2012; Alvarez et al. 1996; Merchenthaler et al. 1999). However, It should be considered that GLP-1R outside of these systems might contribute to the regulation of alcohol-mediated behaviors. GLP-1Rs in brain areas such the arcurate nucleus and insula (Hayes et al. 2009; McMahon and Wellman 1998; Secher et al. 2014; Tang-Christensen et al. 1996; van Bloemendaal et al. 2014) have been attributed the anorexigenic properties of GLP-1, raising the possibility that GLP-1 signaling within other brain areas, then the aforementioned areas,

could be of importance. Moreover, cocaine-induced locomotor stimulation and CPP are reduced after genetic ablation of GLP-1R in the lateral septum, as a result of increased excitability of the GLP-1 containing neurons (Harasta et al. 2015). Furthermore, activation of GLP-1R via systemically administered Ex4 attenuates cocaine-induced dopamine release in the lateral septum (Reddy et al. 2016) and those receptors regulate the activity of VTA dopamine neurons to reduce stress-induced drug relapse (Highfield et al. 2000; Luo et al. 2011). Additionally, GLP-1R stimulation after Ex4 injection into the interpeduncular nucleus abolishes nicotine reward in mice (Tuesta et al. 2017). Collectively, the above-mentioned findings indicate a few possible brain areas, which may regulate reward-related behaviors via GLP-1 signaling in rodents, which could be of interest in upcoming studies.

Concluding remarks

Alcohol use disorder represents one of the most central psychiatric disorders in the world and it negatively affects the individual and their families and is a great economical burden for the society (Ferrari et al. 2014; Grant et al. 2015). Despite the big burden of AUD, there are today three approved medications in the United States and four in Europe. However, these pharmacotherapies have shown limited efficacy (Anton et al. 2006), and patients respond differently to treatments, possibly due to the complexity and heterogeneity of the disease (Heilig and Egli 2006). Thus, the need for new pharmacological treatments is eminent.

GLP-1R agonists are approved for treatment of type II diabetes (Holst 2004) and they also decrease body weight, an effect that has led to their approval for the treatment of obesity in humans (for review see (Srivastava and Apovian 2018)). Therefore, upcoming treatments with GLP-1R agonists on alcohol consumption would be better suited for overweight patients and for those with symptoms of type II diabetes.

The results presented in this thesis suggest that GLP-1R agonists administered both peripherally and centrally, attenuates alcohol-mediated behaviors in rodents. Moreover, activation of GLP1-Rs attenuates alcohol's ability to activate the mesolimbic dopamine system, reduce alcohol intake, reduce motivation to consume alcohol and prevent relapse drinking in rodents. With these promising results, we argue that GLP-1 and its receptor

plays a role in the pathophysiology of alcohol-mediated behaviors and that GLP-1R agonists deserve to be evaluated as potential pharmacological treatment for AUD.

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