The gut-brain axis and alcohol-mediated behaviours: the amylin story

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Cover illustration: Artistic interpretation of visualised brain slice showing salmon calcitonin (green) in the brain, among neuronal nuclei (cyan) and dendrites (purple).

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Στον πατέρα μου

To my father

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ABSTRACT

Alcohol use disorder (AUD) is a complex neuropsychiatric disorder with high rates of mortality and morbidity. The currently available pharmacotherapies show varied efficacy, leading to the investigation of new neurochemical targets for alcohol. Recently, gut-brain hormones involved in appetite regulation have been shown to modulate alcohol-mediated behaviours. However, the role of the anorexigenic gut-brain hormone amylin in such behaviours was until recently unknown. Therefore, this thesis aims at identifying how amylin signalling regulates behavioural responses to alcohol and suggests the underlying mechanisms of this modulation.

The studies in this thesis present novel data that, firstly, amylin receptor (AMYR) activation by the amylin analogue salmon calcitonin (sCT) attenuates the established acute effects of alcohol to increase locomotion and dopamine release in the nucleus accumbens (NAc) in mice. Secondly, acute sCT administration decreases alcohol consumption and alcohol relapse drinking in rats chronically exposed to alcohol. Notably, the gene expression of the AMYR components is different in the NAc of high, compared to low alcohol-consuming rats. In selectively bred Sardinian alcohol-preferring rats, sCT decreases the number of lever presses for alcohol reward in an operant self-administration paradigm. Thirdly, sCT crosses the blood-brain barrier and reaches reward-

related areas, including the laterodorsal tegmental area, the ventral tegmental area and the NAc, whereby activates local AMYRs to decrease acute alcohol behaviours in mice and chronic in rats. Fourthly, repeated sCT treatment decreases alcohol-induced locomotion even after discontinuation of sCT administration and alters the levels of neurotransmitters in reward-related areas. Lastly, a selective AMYR synthetic amylin analogue decreases alcohol consumption in both male and female rats and alters monoamine levels in reward-related brain areas in both sexes.

The thesis attributes an entire new role to the amylin signalling, that of the regulator of alcohol-mediated behaviours. The commercial availability of amylin analogues for the treatment of other disorders could set the ground for the development of targeted pharmacotherapies for AUD and potentially for other addictive disorders.

Keywords: reward, mesolimbic dopamine system, addiction, calcitonin, IAPP

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SAMMANFATTNING PÅ SVENSKA

”Påverkan av den aptitminskande peptiden amylin på alkoholmedierade beteenden hos gnagare”

Alkoholberoende är ett stort samhällsproblem, förknippat med hög dödlighet och en komplex sjukdomsbild. I denna neuropsykiatriska sjukdom är kroniskt intag av alkohol, sug, återfall och kontrollförslut centrala symptom. Med hjälp av flertalet djurmodeller kan forskare studera vilka mekanismer som är centrala för alkoholens emotionella upplevelse som driver det höga intaget av alkohol.

I dagsläget finns det fyra läkemedel som används vid behandling av alkoholberoende. Vid behandling varierar den kliniska effekten, och det finns därför ett stort behov av nya farmakologiska behandlingsstrategier. De mekanismer som har påverkan vid alkoholberoende är komplexa, och nyligen har studier visat att de hormoner som reglerar aptit också är av yttersta vikt för modulering av belöning och beroende.

Ett av dessa aptitreglerande hormoner är amylin. Amylin bildas i bukspottkörteln och har en viktig funktion vid reglering av blodglukosnivåer, vilket har lett till godkännande av läkemedlet för behandling av diabetes typ 2. Dessutom påverkar amylin andra viktiga fysiologiska funktioner, såsom aptitminskning och minskat födointag. Eftersom amylin har en kort halveringstid och därmed snabbt försvinner från kroppen, används ofta andra substanser som aktiverar amylinreceptorerna vid studier av amylins påverkan av funktioner i kroppen. Dessa amylinreceptoraktiverare inkluderar sCT och AM1213. Förmågan av amylin att minska födointag och aptit involverar amylinreceptorer i hjärnan. Dock har deras roll i förmedling av belöning och beroendeutveckling inte studerats. Denna avhandling syftar därför till att, med hjälp av etablerade djurmodeller, studera hur aktivering av amylinreceptorer påverkar alkohol-medierade beteenden, samt identifiera mekanismer som påverkas av denna aktivering.

Först visade vi att aktivering av amylinreceptorer med hjälp av sCT minskar den belönande upplevelsen av alkohol i möss. Dessutom minskar sCT råttors konsumtion av alkohol, utan att påverka intag av belönande mat, i detta fall jordnötssmör. I vidare råttstudier visade vi att sCT minskar de förstärkande effekterna av att dricka alkohol samt förhindrar återfallsdrickandet. Dessutom minskar en upprepad behandling med sCT inte bara alkoholintag, utan minskar också födointag och kroppsvikt. Tvärtom påverkar sCT inte motivationen att konsumera belönande chokladmjölk. Utöver detta är uttrycket av amylinreceptorer,

i ett belöningsrelaterat område, förändrat hos råttor som dricker mycket alkohol, jämfört med de som inte dricker mycket alkohol. I den tredje studien identifierar vi att sCTs förmåga att minska dessa alkohol-medierade beteenden involverar områden i hjärnan som är kopplade till belöning. Vi visade att aktivering av amylinreceptorer i flera belöningsrelaterade områden påverkar hur gnagare upplever alkohol belönande, samt minskar alkoholintag. I den fjärde studien visar vi att tidigare tillförsel av sCT, minskar den stimulerande egenskapen hos alkohol, trots att sCT inte längre finns i kroppen. I den slutliga studien visar vi att aktivering av amylinreceptorer, med hjälp av AM1213, minskar intag av alkohol hos både hon- och hanråttor, samt att detta är kopplat till förändrad neurotransmission i belöningsrelaterade områden

Sammanfattningsvis visar dessa studier för första gången att aktivering av amylinreceptorer förhindrar de belönade egenskaperna hos alkohol och därmed minskar alkoholkonsumtionen. Dessutom påvisar vi att detta moduleras via hjärnområden av vikt för upplevelse av belöning och beroendeutveckling. Eftersom de läkemedel som aktiverar amylinreceptorer är säkra och tolererbara hos patienter med typ 2 diabetes, anser vi att dessa bör testas kliniskt på patienter med alkoholberoende.

ΠΕΡΙΛΗΨΗ ΔΙΑΤΡΙΒΗΣ ΣΤΑ ΕΛΛΗΝΙΚΑ

"Επίδραση της αμυλίνης, μιας ορμόνης που μειώνει την όρεξη, σε συμπεριφορές που προκαλούνται από την κατανάλωση αλκοόλ στα τρωκτικά".

Η εξάρτηση από το αλκοόλ είναι μια πολύ περίπλοκη ασθένεια και ένα σημαντικό κοινωνικό πρόβλημα με υψηλή θνησιμότητα. Η χρόνια πρόσληψη της ουσίας, η υποτροπή και η απώλεια ελέγχου είναι κεντρικά μέρη της νευροψυχιατρικής αυτής ασθένειας. Με τη βοήθεια των ζωικών μοντέλων, εμείς οι ερευνητές μπορούμε να μελετήσουμε τους μηχανισμούς που είναι σημαντικοί για το πώς βιώνουμε το αλκοόλ και οδηγούμαστε στην υψηλή και συνεχή πρόσληψη του.

Επί του παρόντος, υπάρχουν τέσσερα φάρμακα που χρησιμοποιούνται στη θεραπεία της εξάρτησης από το αλκοόλ. Ωστόσο, η κλινική επίδραση αυτών είναι περιορισμένη και, συνεπώς, υπάρχει μεγάλη ανάγκη για νέες στρατηγικές φαρμακολογικής θεραπείας. Οι μηχανισμοί που είναι σημαντικοί στην εξάρτηση από το αλκοόλ είναι πολύπλοκοι και πρόσφατες μελέτες έχουν δείξει ότι οι ορμόνες που ρυθμίζουν την όρεξη είναι επίσης εξαιρετικά σημαντικές για τη ρύθμιση της ευφορίας και του εθισμού.

Μία από αυτές τις ορμόνες ρύθμισης της όρεξης είναι η αμυλίνη. Η αμυλίνη παράγεται στο πάγκρεας και μια σημαντική λειτουργία της είναι η ρύθμιση των επιπέδων γλυκόζης στο αίμα, η οποία έχει οδηγήσει στην έγκριση φαρμάκων για τη θεραπεία του διαβήτη τύπου 2. Ωστόσο, έχει αποδειχθεί ότι η αμυλίνη έχει άλλες σημαντικές φυσιολογικές λειτουργίες, όπως μείωση της όρεξης και της πρόσληψης τροφής. Η ικανότητα της αμυλίνης να μειώνει την πρόσληψη τροφής και την όρεξη εξαρτάται από υποδοχείς αμυλίνης στον εγκέφαλο, σε περιοχές οι οποίες εμπλέκονται στη δημιουργία εξάρτησης και ενεργοποιούνται όταν το αλκοόλ φτάσει στον εγκέφαλο. Ωστόσο, ο ρόλος της αμυλίνης στην ευφορία και την εξάρτηση από το αλκοόλ δεν έχει μελετηθεί ακόμα.

Η διατριβή αυτή στοχεύει συνεπώς στο να μελετήσει πώς 1) η ενεργοποίηση των υποδοχέων αμυλίνης στα εγκεφαλικά συστήματα ανταμοιβής επηρεάζει τις συμπεριφορές που προκαλούνται από το αλκοόλ, με τη βοήθεια καθιερωμένων ζωικών μοντέλων και 2) να εντοπίσει μηχανισμούς που είναι σημαντικοί για τον έλεγχο αυτών των συμπεριφορών.

Πρώτον, δείξαμε ότι η ενεργοποίηση υποδοχέων αμυλίνης από την καλσιτονίνη σολωμού (sCT), η οποία έχει τις ίδιες λειτουργίες με την αμυλίνη, μειώνει το αίσθημα ευφορίας που προκαλεί το αλκοόλ στα ποντίκια. Επιπλέον, η

κατανάλωση αλκοόλ μειώνεται στους αρουραίους που χορηγούνται sCT. Σε άλλες αρχικές μελέτες, δείξαμε ότι το sCT μειώνει το κίνητρο για κατανάλωση αλκοόλ και αποτρέπει την επανεμφάνιση της κατανάλωσης αλκοόλ ύστερα από κάποια περίοδο αποχής, που σημειωτέον είναι πολύ αυξημένη στους αλκοολικούς. Επίσης, η επαναλαμβανόμενη θεραπεία με sCT, όχι μόνο μειώνει την κατανάλωση αλκοόλ, αλλά επίσης μειώνει την πρόσληψη τροφής και το σωματικό βάρος. Αντίθετα, το sCT δεν επηρεάζει το κίνητρο για κατανάλωση σοκολατούχου γάλακτος, το οποίο επίσης ενεργοποιεί το σύστημα ανταμοιβής στους αρουραίους κατά παραπλήσιο τρόπο με το αλκοόλ.

Επιπλέον, η ύπαρξη υποδοχέων αμυλίνης σε περιοχές του συστήματος ανταμοιβής μεταβάλλεται σε αρουραίους που πίνουν πολύ αλκοόλ, σε σύγκριση με εκείνους που δεν πίνουν πολύ. Επίσης, δείξαμε ότι η ενεργοποίηση των υποδοχέων αμυλίνης σε πολλές περιοχές ανταμοιβής επηρεάζουν τον τρόπο που τα ζώα αντιλαμβάνονται το αλκοόλ, κατά τέτοιο τρόπο που η λήψη του δεν είναι πλέον τόσο ικανοποιητική και κατά συνέπεια αυτό μειώνει την κατανάλωσή του. Σε περαιτέρω μελέτες, δείξαμε ότι η χορήγηση του sCT μειώνει τη διεγερτική ιδιότητα του αλκοόλ ακόμα και όταν το sCT δεν είναι παρόν στο σώμα, αλλά έχει χορηγηθεί κάποιες μέρες πριν. Στις τελικές μελέτες, δείχνουμε ότι η ενεργοποίηση των υποδοχέων αμυλίνης μειώνει την πρόσληψη αλκοόλ τόσο σε θηλυκούς όσο και σε αρσενικούς αρουραίους και ότι αυτό συνδέεται με τη μεταβολή της νευροδιαβίβασης σε περιοχές του συστήματος ανταμοιβής.

Συνοπτικά, αυτές οι μελέτες δείχνουν για πρώτη φορά ότι η ενεργοποίηση των υποδοχέων αμυλίνης παρεμποδίζει τις ευφορικές ιδιότητες του αλκοόλ και μειώνει την κατανάλωσή του. Επιπλέον, αποδεικνύουμε ότι αυτό διαμορφώνεται μέσω εγκεφαλικών περιοχών που είναι σημαντικές για την ανταμοιβή και την εξάρτηση από ουσίες. Επειδή τα φάρμακα που ενεργοποιούν τους υποδοχείς αμυλίνης είναι ασφαλή και ανεκτά σε ασθενείς με διαβήτη τύπου 2, πιστεύουμε ότι αυτά πρέπει να εξεταστούν κλινικά σε ασθενείς με εξάρτηση από το αλκοόλ αλλά και από άλλες ουσίες.

LIST OF PAPERS

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

I. **Kalafateli AL**, Vallöf D, Jerlhag E. Activation of amylin receptors attenuates alcohol-mediated behaviours in rodents. **Addiction Biology** 2019; 24(3)**:** 388-402

II. **Kalafateli AL**, Vallöf D, Colombo G, Lorrai I, Maccioni P, Jerlhag E. An amylin analogue attenuates alcohol-related behaviours in various animal models of alcohol use disorder. **Neuropsychopharmacology** 2019; 44(6)**:** 1093-1102

III. **Kalafateli AL**, Satir TM, Vallöf D, Zetterberg H, Jerlhag E. Behavioural responses to alcohol involve amylin receptor signalling within brain areas processing reward. **Submitted**

IV. **Kalafateli AL**, Aranäs C, Jerlhag E. Effects of sub-chronic amylin receptor activation on alcohol-induced locomotor stimulation and monoamine levels in mice. **Submitted**

V. **Kalafateli AL**, Vestlund J, Raun K, Egecioglu E, Jerlhag E. Effects of a selective long-acting amylin receptor agonist on alcohol consumption, food intake and body weight in male and female rats. **Manuscript**

CONTENTS

ABBREVIATIONS

3-MT	3-methoxytyramine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
AMYR	amylin receptor
AM1213	NNC0174-1213 (synthetic amylin analogue)
ANOVA	analysis of variance
AP	area postrema
AUD	alcohol use disorder
CALCR	calcitonin receptor gene
COX-IV	cytochrome c oxidase IV
CPP	conditioned place preference
CTR	calcitonin receptor
DA	dopamine
DAPI	4′,6-diamidino-2-phenylindole
DOPAC	3,4-dihydroxyphenylacetic acid
DSM	diagnostic and statistical manual of mental disorders
EC	electrochemical
EDTA	ethylenediaminetetraacetic acid
Eu	europium
FAM	fluorescein amidite
FR	fixed ratio
GABA	γ-aminobutyric acid
GLP-1(R)	glucagon-like peptide-1 (receptor)
GOI	gene of interest
HPLC	high performance liquid chromatography
HVA	homovanillic acid
ICV	intracerebroventricular

IP	intraperitoneal
LDTg	laterodorsal tegmental area
MAP2	microtubule-associated protein 2
NA	noradrenaline
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptors
NeuN	neuronal nuclei
NMDA	N-methyl-D-aspartate
NMU	neuromedin U
PFC	prefrontal cortex
PR	progressive ratio
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative real-time polymerase chain reaction
RAMP	receptor activity-modifying protein
RG	reference gene
SC	subcutaneous
sCT	salmon calcitonin
SUD	substance use disorder
TBS	tris-buffered saline
VTA	ventral tegmental area

INTRODUCTION

"Was and will make me ill,
I take a gram and only am"

A. Huxley, Brave New World

Addiction

Addiction is a broad term describing a chronic and relapsing brain disorder (Hunt et al., 1971), characterized by compulsive drug-seeking and loss of control (Koob et al., 2001). Addiction, often referred to as substance use disorder (SUD) nowadays, is often associated with uncontrolled substance abuse, which affected individuals continue despite the faced negative consequences.

Nevertheless, the term addiction has been proposed to also describe other compulsive behaviours and is not limited to substance abuse. Other common behaviours occurring in daily life can become compulsive and can be considered addictive, including overeating, shopping, gambling and having sex (Holden, 2001). Importantly, studies have suggested that all the aforementioned addictive behaviours alter the same reward areas in the human brain, including limbic structures and the prefrontal cortex (PFC) (Grant et al., 2006; Potenza et al., 2003; Volkow et al., 2004b). These behaviours share similar characteristics, such as tolerance, withdrawal and loss of control among others (Griffiths, 2005).

Providing the complex nature of addiction, two main theories have described its causes in the course of time. On one hand, the drug-centred hypothesis suggests that chronic use of a substance, or committing to a compulsive behaviour, causes molecular changes in the brain, especially in regards to the dopamine system, which turns an individual from healthy to addicted (Berke et al., 2000; Deroche-Gamonet et al., 2004; Nestler, 2001). On the other hand, the individual-centred approach suggests an inherited genetic predisposition in the reward system (Wolfe et al., 2000), for example resulting in a dopaminergic hypo- and/or hyper-function, further leading to reward system deficits (Balldin et al., 1993; Balldin et al., 1992; Bowirrat et al., 2005; Volkow et al., 1996). Nevertheless, providing that addiction is a complex heterogeneous disorder also affected by environmental conditions, these two theories alone may not be sufficient to explain the development of addiction and more factors could be involved.

A general proposed model for the addiction stages represents addiction as a three-stage cycle (Koob et al., 2010). An initial rewarding stimulus causes euphoric feelings that are reinforcing, leading to increased incentive salience of the consumed substance. That, in turn, leads to binge consumption of the substance and intoxication caused by consuming increasingly higher amounts. Consequently, the withdrawal from the rewarding substance following the binge stage, leads to emotional and physical pain. The last loop of this cycle is characterized by relapse, when the individual turns to the substance for relieving the pain, and the initial impulsivity towards the stimulus, now turns into compulsivity.

Undoubtedly, abused substances (Robinson et al., 2004) as well as addictive behaviours (Koehler et al., 2013) alter the neurocircuits and neurochemistry of the brain. In order to pinpoint the neurocircuits and neurotransmitters involved in addiction, extensive research involves preclinical addiction models, which constitute a fundamental part of the initial work.

The reward system

The reward system is the part of the brain that processes the incentive salience (motivation and desire for a reward) and the associative learning (positive reinforcing and conditioning) of rewarding stimuli (Berridge et al., 2015; Schultz et al., 1997). Drugs of abuse and addictive behaviours activate the parts of the brain that process reward (Chen et al., 2010). Since the beginning of their exploration, these systems have been characterized as evolutionarily stable and relatively similar across species (Glickman et al., 1967).

Rewards are attractive and motivational and can be classified in two big categories, intrinsic and extrinsic. Intrinsic rewards are unconditioned rewards that are inherently pleasurable, whereas extrinsic rewards occur from a learned association and are not inherent (for review (Schultz, 2015)). Simple examples of intrinsic rewards are food, water and sex, whereas examples of extrinsic rewards are addictive drugs, gambling and compulsive overeating. It is also suggested that extrinsic rewards, drugs for instance, may have a more profound effect on the reward system of the brain rather than intrinsic rewards, like food or exercise (Wise et al., 1989).

One of the main neurotransmitters involved in the reward system is dopamine. Dopamine has been suggested to be involved in reward processing and to be responsible for the hedonic response of reward (Cador et al., 1991; Engel, 1977; Robinson et al., 1993). It is also suggested that it plays an important role

in the processing of motivated behaviours for reward (Berridge et al., 2015; Berridge et al., 1998). All addictive drugs, such as alcohol, acutely increase the dopamine levels in reward-related areas (for review (Jayaram‐Lindström et al., 2016). Interestingly, human studies in individuals addicted to drugs like alcohol and cocaine, show decreased dopamine D2 receptors, as well as reduced dopamine release (Volkow et al., 2002; Volkow et al., 1996). Similar findings extend to other compulsive behaviours, as for example reduced number of dopamine D2 receptors were identified in reward-related areas in compulsively overeating individuals (Wang et al., 2004).

The mesocorticolimbic dopamine system

The mesocorticolimbic dopamine system has been suggested as an important part of the reward system in the brain and its role has been established in processing intrinsic (Hansen et al., 1991), as well as extrinsic rewards, including alcohol (for review (Everitt et al., 2008)). This system is suggested to also modulate addictive behaviours in addition to processing reward stimuli (Kelley et al., 2002).

One of the main regions in this system is the ventral tegmental area (VTA), which contains the highest number of dopaminergic neurons in the brain (for review (Kalivas, 1993)). The VTA is not an organized nucleus, but appears to be anatomically heterogeneous, with different inputs to each of its subregions (Lammel et al., 2012).

The cortical part of this system (including projections from the VTA to the PFC) is mainly associated with the motivational and emotional aspects of reward (Russo et al., 2013). The limbic part (i.e. the mesolimbic dopamine system), includes projections from the VTA to the nucleus accumbens (NAc), amygdala, and hippocampus. The limbic part can be further subdivided into the mesoaccumbal dopamine system, which includes VTA dopaminergic projections to NAc (***Figure* 1**). This part is the focus of the present studies and is responsible for pleasure, euphoria and reinforcement (Koob, 1992a; Wise, 1987) and is an essential centre for dopamine neurotransmission (Koob, 1992a; Nestler, 2001). NAc is separated into two anatomically and functionally distinct areas, the NAc core (central) and the NAc shell (surrounding the core) (Zahm, 1999; Zahm et al., 1992).

The cholinergic-dopaminergic reward link

The activity of the mesoaccumbal dopamine system is regulated by various inputs, including the cholinergic projections from the laterodorsal tegmental area (LDTg) (***Figure* 1**). Activation of the cholinergic neurons of the LDTg causes activation of nicotinic acetylcholine receptors (nAChR) in the VTA dopaminergic neurons (Blaha et al., 1996), which project to the NAc and cause local dopamine release (Forster et al., 2000). Direct activation of nAChRs in the VTA by nicotine increases dopamine release in the rat NAc (Brazell et al., 1990). Optogenetic activation of the cholinergic projections in the LDTg causes conditioned place preference (CPP) expression in mice (Steidl et al., 2017) and induces operant responding for optical stimulation in the LDTg in rats (Steidl et al., 2015), further demonstrating the importance of this system in reward processing.

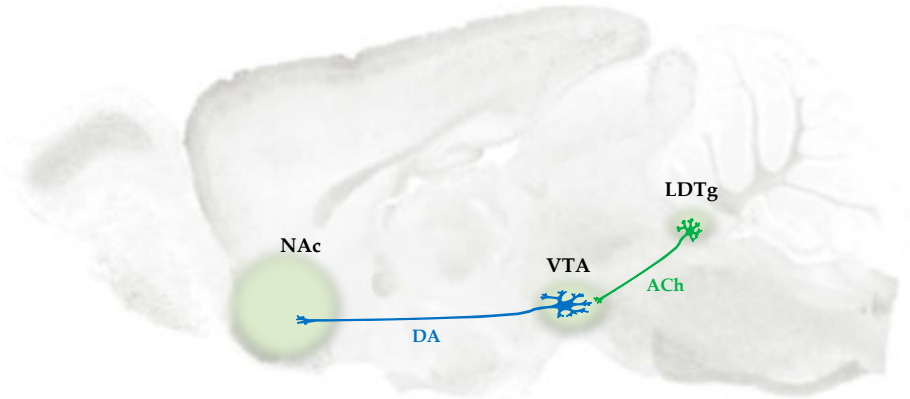

***Figure* 1. Simplified schematic representation of the mesoaccumbal dopamine and cholinergic-dopaminergic systems.** Cholinergic neurons in the LDTg project to the VTA and released ACh activates receptors on local dopaminergic neurons. Dopaminergic neurons originating in the VTA project to the NAc whereby the neurotransmitter released is DA. (**LDTg**: laterodorsal tegmental area, **NAc**: nucleus accumbens, **VTA**: ventral tegmental area, **ACh**: acetylcholine, **DA**: dopamine)

Alcohol in the brain

The substance in alcoholic drinks that causes the feeling of euphoria and the rewarding substance in alcohol dependence is ethanol (simplified as alcohol throughout this thesis). It is a small molecule quickly absorbed when ingested and evenly distributed throughout tissues in the heart, muscles and the brain (for review (Paton, 2005)). Alcohol can cross the blood-brain barrier and makes its way into the central nervous system (Snider et al., 1991). In the

brain, alcohol induces various pharmacodynamic responses and affects many neurochemical pathways, a number of which are presented in ***Figure* 2**. In lower doses, alcohol is rewarding and stimulating, but in higher doses it induces anxiolysis and sedation (Engel et al., 1992; Sutker et al., 1983). These behavioural responses involve numerous neurotransmitters, hormones and neuropeptides (for review (Engel et al., 1988)).

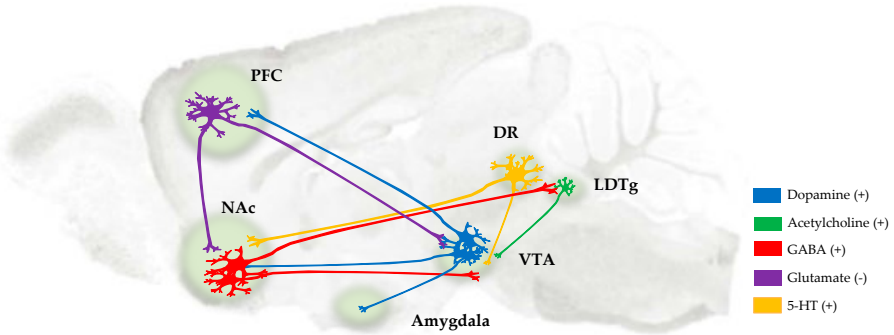

***Figure* 2. Schematic representation of brain areas and neurotransmitters activated by alcohol.** (**DR**: dorsal raphe, **LDTg**: laterodorsal tegmental area, **NAc**: nucleus accumbens, **PFC**: prefrontal cortex**, VTA**: ventral tegmental area, **+**: increase, **–**: decrease).

Contrary to other drugs of abuse, alcohol does not bind to any identified neurotransmitter or neurotransmitter transporter in the brain (for review (Gilpin et al., 2008). More specifically, it is suggested to act as an allosteric modulator of ligand-gated ion channels, like the 5-HT3 serotonin receptor subtype (Lovinger et al., 1998; Weight et al., 1991; Zhou et al., 1998), the γ-aminobutyric acid $(GABA)_A$ receptor, and the N-methyl-D-aspartate (NMDA) glutamate receptor (Lovinger et al., 1989; Weight et al., 1991). Furthermore, alcohol is proposed to act as an allosteric modulator of glycine receptors, (Mascia et al., 1996; Soderpalm et al., 2017), as well as nACh receptors (Blomqvist et al., 1992).

Acute effects of alcohol

The acute rewarding properties of alcohol, at least in part, involves dopamine release in the NAc shell. This was initially implied in rodent and human studies revealing that inhibition of dopamine synthesis reduces the stimulatory properties of alcohol in rodents (Engel et al., 1974), as well as the stimulatory, euphoric experience in humans (Ahlenius et al., 1973). Furthermore, alcohol administration (Engel et al., 1988), and anticipation for alcohol in rodents (Melendez et al., 2002) increases dopamine release in the NAc shell and volun-

tary alcohol consumption increases dopamine release in the same area, in a dose-dependent manner (Doyon et al., 2003; Engel et al., 1988; Larsson et al., 2005; Weiss et al., 1993).

Moreover, activation of mesoaccumbal dopamine neurons by alcohol is further evident when intravenous administration of alcohol evokes dose-dependent firing in dopamine neurons in the VTA (Gessa et al., 1985). This dopamine release is specific to the shell of NAc, as the same response is not observed in the core region of the NAc (Bassareo et al., 2003; Cadoni et al., 2000). In support of animal studies, alcohol consumption increases dopamine in the human striatum (Boileau et al., 2003; Urban et al., 2010) and is associated with self-reported euphoria and alcohol craving in the clinical setting (Ramchandani et al., 2011; Yoder et al., 2007).

It has been shown that alcohol acts at the level of LDTg, VTA and NAc (for review (Jayaram‐Lindström et al., 2016)) to cause dopamine release in the NAc, which is linked to euphoria. Alcohol doses given directly into the anterior part of the VTA in rats have the same effect of increasing dopamine release in the NAc shell (Ding et al., 2009; Jerlhag et al., 2013) and alcohol in the posterior part of the VTA leads to alcohol seeking in operant-chamber settings (Hauser et al., 2011). Moreover, rats self-administer alcohol directly into the posterior part of the VTA (Rodd et al., 2004a; Rodd et al., 2004b; Rodd-Henricks et al., 2000) rendering this heterogeneous brain area an important site for the processing of alcohol's rewarding effects.

Other studies show that alcohol also acts on the level of the NAc shell, as local perfusion increases dopamine release in the same area (Ericson et al., 2008). This effect is diminished by blocking the nAchRs located in the anterior VTA (Ericson et al., 2008), suggesting that ethanol in the NAc enhances acetylcholine release in the anterior VTA which in turn increases dopamine in the NAc (Blomqvist et al., 1997; Larsson et al., 2005). The proposed mechanism for these events includes GABAergic projections from the NAc to the VTA (Walaas et al., 1980). The hypothesis is that alcohol in the NAc inhibits the GABAergic projections to the anterior VTA neurons, which in turn enhances dopamine release in the NAc shell and that these events include glycine receptors (for review (Soderpalm et al., 2009)).

Aside from the NAc shell and VTA, the LDTg may also be a key player in alcohol reward expression. Alcohol is suggested to excite the cholinergic afferents in the LDTg causing acetylcholine release in the VTA, which in turn activates the mesolimbic dopamine system (for review (Larsson et al., 2004)). In

fact, alcohol intake in high alcohol-consuming rats causes concomitant VTA-acetylcholine and NAc-dopamine release (Larsson et al., 2005).

Chronic effects of alcohol

Chronic consumption of alcohol causes continuous activation of the mesolimbic dopamine system. This can alter neuronal circuits through neuroadaptation, which consequently contributes to sensitization, tolerance, withdrawal and dependence (Gilpin et al., 2008). Chronic exposure to alcohol is associated with downregulation of the dopamine D2 receptor gene (Jonsson et al., 2014) and long-term alcohol consumption reduces mRNA levels of the long dopamine D2 receptor isoform in the NAc in rats (Feltmann et al., 2018). Long-term voluntary alcohol consumption decreases dopamine release in the NAc in rats (Feltmann et al., 2017) and the VTA of alcohol-preferring rats is more sensitive to the reinforcing properties of alcohol after chronic consumption (Rodd et al., 2005). Rats chronically consuming high amounts of alcohol have a lower dopamine tone in the NAc compared to rats consuming lower amounts (Ericson et al., 2019), whereas chronic alcohol consumption lowers baseline dopamine levels, but dopamine increase in response to alcohol intake is still present (Diana et al., 1993).

In humans, chronic alcohol consumption is associated with decreased dopamine D2 receptors in the striatum of addicted individuals (Balldin et al., 1993; Volkow et al., 1996) and it plays a significant role in the cravings for alcohol (Heinz et al., 2004). Further functional magnetic resonance imaging studies in humans show activation of the VTA and NAc in high-risk, but not in low-risk drinkers, after exposure to alcohol-associated cues (Kareken et al., 2004).

Alcohol use disorder

Alcohol use disorder (AUD) is a heterogeneous, chronic and relapsing brain disorder (Grant et al., 2015), causing high rates of mortality and morbidity (Lim et al., 2012). It is a burden both for society and the individual and is ranked as one of the leading causes of mental illness (Ferrari et al., 2014). AUD is a recognized psychiatric disorder with main characteristics of compulsive, heavy alcohol use and loss of control (for review (Carvalho et al., 2019)). The following 11 diagnostic criteria for AUD are updated in the Diagnostic and

Statistical Manual of Mental Disorders-V (DSM-V) (American Psychiatric Society, 2013):

1. Drinking more or longer than intended?
2. Wanted to cut down or stop drinking, or tried to, but couldn't?
3. Spent a lot time drinking or being sick?
4. Experienced craving?
5. Drinking interfered with normal responsibilities and social activities?
6. Continued drinking even if it was causing trouble with family and friends?
7. Given up or cut down on important or interesting activities?
8. Gotten into situations while or after drinking that increased your chances to get hurt, more than once?
9. Continued drinking even after causing health problems?
10. Had to increase drinking to get the desired effect?
11. Presented withdrawal symptoms?

Out of these criteria, one has to fulfil two or more within a 12-month period in order to be diagnosed with AUD. The severity of the disorder is defined as mild (2-3 symptoms), moderate (4-5 symptoms) or severe (>6 symptoms).

Disease impact

AUD is a disorder affecting a large number of people across the world. Globally, 237 million men and 46 million women are affected by AUD (World Health Organization, 2018). In the United States, 14.4 million people over the age of 12 were diagnosed with AUD and 75.2% among those with a SUD struggled with alcohol (Substance Abuse and Mental Health Services Administration, 2017).

Interestingly, the European Union is the most heavy-drinking region in the world, with more than 20% of the population aged 15 years and older reporting heavy episodic drinking at least once a week (World Health Organization, 2018). In Sweden alone, the prevalence for the development of AUD in ages 15 years and older was estimated at 11% and the prevalence for heavy episodic drinking in the general population at 28 % in 2016 (World Health Organization, 2018).

Alcohol, not only affects the brain causing addiction, it also has a great negative impact on the rest of the body, causing organ damage and eventually failure after excessive or chronic consumption. Long-term consumption can lead to heart problems (Fogle et al., 2010), hypertension and eventually stroke (Kawano, 2010). The liver is one of the most affected organs, as its role in al-

cohol detoxification process is pivotal. Most common conditions developed due to liver damage include steatosis, hepatitis, fibrosis and cirrhosis (Gao et al., 2011). Moreover, there may be an association between alcohol consumption and some forms of cancer like colon, rectum and mouth cancer as shown by epidemiological data (Bagnardi et al., 2001). Importantly, moderate social drinking has also been suggested to increase the risk factor for some types of cancer like breast cancer in non-smoking women (Chen et al., 2011).

Along with the personal health impact of AUD, there is a greatly harmful impact on the society. An estimate of 0.9 million injury deaths globally are assigned to alcohol-related injuries and 90.000 deaths were caused by violence related to alcohol (World Health Organization, 2018). Notably, 187.000 road deaths that were attributed to alcohol, involved people other than drivers (World Health Organization, 2018). Importantly, AUD has detrimental consequences on the family environment, as it increases the occurrence of domestic violence, physical aggression and childhood abuse and neglect, among others (Hutchinson et al., 2014).

Sex differences

AUD is prevalent in both men and women, with some differences per sex, as currently emerging studies show. Males have a 36% rate of lifetime prevalence for AUD, whereas women have 22.7% (Grant et al., 2015). Both sexes develop brain atrophy after chronic alcohol abuse, but women have similarly high levels of atrophy even if they have been addicted for a shorter period of time (Mann et al., 2005).

Although historically the rate of men developing AUD has been higher than women, recent data suggest that this gap is closing (Colell et al., 2013; Keyes et al., 2008; White et al., 2015). In the last decade, the rates of AUD in women have increased by 84% compared to a 35% increase in men (Grant et al., 2017). Additionally, over the past 16 years, alcohol use and binge drinking prevalence has increased in women compared to men (Grucza et al., 2018). Women with AUD have higher risks for developing alcohol-caused liver diseases (Agabio et al., 2017; Szabo, 2018) as well as cardiovascular complications (Agabio et al., 2016). In Sweden, the alcohol-related deaths among women had increased by 16% between 1979 and 2006 (Swedish Council for Information on Alcohol and Other Drugs, 2017).

Importantly, women with AUD face health issues appeared only in females, such as increased risk for breast cancer (Chen et al., 2011), pregnancy and perinatal complications (Andersen et al., 2012). Notably, one of the major im-

pacts of excessive alcohol consumption during pregnancy is the fetal alcohol spectrum disorder, characterized by dysmorphia, growth restriction and neurodevelopmental abnormalities in the offspring (for review (Sokol et al., 2003)).

Despite the differences of AUD characteristics and prevalence between males and females, the latter remain substantially understudied, especially in preclinical neuroscience research (Zucker et al., 2010). For that reason, recent attempts have been made for the inclusion of both sexes, by introducing sex as biological variable in preclinical animal alcohol research (Clayton, 2018; Guizzetti et al., 2016).

Development of AUD

AUD follows the "vicious cycle" similar to any other addiction disorder; the individual starting with recreational alcohol consumption receives positive reinforcement cues, which associated with external stimuli, create the substrate for further motivational use (Brown et al., 1980). Continuing alcohol consumption can result in heavy/binge drinking, which in turn is followed by abstinence and in order to alleviate negative reinforcement, it ultimately leads to dependence (for review (Koob, 1992b)). The shift between recreational to compulsive alcohol use is proposed to include neuronal circuits processing motivational behaviours, along with alterations in a number of neurotransmitter signalling systems (for review (Gilpin et al., 2008)).

The different neurochemical, genetic, environmental and social factors contributing to the development of AUD, rank it as one of the most complex neuropsychiatric disorders. Twin studies have confirmed that AUD is heritable, indicating that genetic factors are of importance in the development of the disorder (Cloninger et al., 1981; Prescott et al., 1999). A recent genome-wide study in a mixed population sample identified 18 genetic loci that are associated with alcohol consumption and AUD (Kranzler et al., 2019).

Other environmental factors such as stressful life events, family environment and parental support and warmth are suggested to contribute to the heritability of the disorder (Kendler et al., 2007). A meta-analysis of twin and adoption studies assessing the impact of sex, assessment method and study design of earlier studies, identified that AUD is approximately 50% heritable (Verhulst et al., 2015). The availability of alcohol in the social context along with alcohol drinking norms in the family and social context are also identified as risk factors.

Additional risk factors that influence the development of AUD include individual personality traits, like novelty seeking, harm avoidance and high re-

ward-sensitivity among others (Chartier et al., 2010; Oreland et al., 2018). Furthermore, sex, age and hormonal status contribute to the risk of developing AUD and other addictive disorders (Engel, 1985).

Existing pharmacotherapies

Currently, four pharmacological agents have been approved and are commercially available for the treatment of AUD, namely disulfiram, acamprosate, naltrexone and nalmefene. Disulfiram acts by inhibiting the enzyme aldehyde dehydrogenase, which causes an accumulation of acetaldehyde, a metabolite that causes an unpleasant feeling (Barth et al., 2010). Acamprosate's mechanism of action is proposed to affect a number of neurotransmitters and receptors in the brain, in particular glutamate NMDA receptors, and is believed to repair the balance between excitatory glutamate and inhibitory GABA neurotransmission (Plosker, 2015). It is also suggested that acamprosate controls extracellular dopamine levels in the NAc, via glycine receptors in that area and nACh in the VTA (Chau et al., 2010); however, the complete mechanisms of action need to be further explored. Naltrexone targets the opioid system and is an opioid receptor antagonist, which decreases the reinforcing properties of alcohol, thus decreasing alcohol drinking (Pettinati et al., 2006). Finally, nalmefene's mechanism of action and effects are very similar to naltrexone, but it antagonises and partially agonises the opioid system (Swift, 2013).

Importantly, these agents can be combined with each other or with psychosocial interventions for a better clinical outcome (Anton et al., 2006; Pettinati et al., 2005). Additionally, compounds that are not approved for the treatment of AUD like varenicline, a smoking cessation compound (for review (de Bejczy et al., 2015) and baclofen, a $GABA_B$ receptor agonist (for review (Agabio et al., 2018), appear to have a therapeutic effect on the disorder.

Although the therapeutic approach to AUD has changed over the years, the existing pharmacotherapy shows variable efficacy between patient groups (for review (Hillemacher et al., 2015)). This might be linked to the fact that AUD is a very heterogeneous disorder. For instance, individuals with a genetic alteration in the μ-opioid receptor gene have a better clinical outcome for naltrexone (Anton et al., 2008). The different typologies of AUD patients (for instance Lesch or Cloninger (Cloninger et al., 1988; Lesch et al., 1996)) may contribute to the improvement of treatment prescriptions (for review (Leggio et al., 2009)). All the aforementioned factors indicate the substantial need for the development of additional medications. Therefore, the growing knowledge on

the neurochemical pathways involved in alcohol dependence is of considerable importance.

Gut-brain peptides and reward-related behaviours

Growing evidence supports that reward and food intake behaviours share common perplexing neurobiological mechanisms mainly mediated through the mesolimbic dopamine system (Abizaid et al., 2006; Edwards et al., 2016; Egecioglu et al., 2010). This neurochemical overlap of reward and food intake systems (Thiele et al., 1998; Thiele et al., 2004; Volkow et al., 2012b), involves gut-brain peptides. These are usually produced in the digestive system and act on the central nervous system, and their physiological role is to control appetite and feeding. Some of them, like ghrelin, increase appetite, whereas others like amylin, glucagon-like peptide-1 (GLP-1) and neuromedin U (NMU), inhibit food intake (for review (Ahima et al., 2008)).

There are numerous studies demonstrating another role for those peptides as regulators of natural and substance rewards (for review (Jerlhag, 2019b)). Particularly, the orexigenic ghrelin activates the mesolimbic system (Jerlhag et al., 2006) and increases alcohol-mediated behaviours (Jerlhag et al., 2009), as well as nicotine-, cocaine- and amphetamine-induced behaviours in rodents (Jerlhag et al., 2010; Jerlhag et al., 2011a). On the contrary, GLP-1 and NMU decrease alcohol- (Vallöf et al., 2019; Vallöf et al., 2019; Vallöf et al., 2016a; Vallöf et al., 2016b), as well as cocaine and amphetamine-mediated behaviours in rodents (Egecioglu et al., 2013a). This evident association between gut-brain peptides and reward-related behaviours is an important topic for further investigation, especially by exploring other gut-brain peptides with central action.

The amylin signalling

The established common neural background of appetite and reward regulation has shed light upon amylin, an important regulator of food intake and appetite, as well as glucose homeostasis (for review (Hay et al., 2015)). Amylin is a hormone of 37 amino acids and is secreted by the β-cells of pancreas together with insulin (Westermark et al., 2011) after certain stimuli, like meal initiation, nutrient signals and neural activation among others (Butler et al., 1990; Cooper, 1994).

This hormone was identified in 1987, as a main component of diabetes type II-associated islet amyloid deposits (Cooper et al., 1987; Westermark et al., 1987) and it is suggested to act synergistically with insulin to control glucose disposal (Rink et al., 1993). Physiologically, amylin inhibits glucagon secretion and delays gastric emptying (Clementi et al., 1996). These functions have led to the synthesis of amylin analogues, like pramlintide, for the treatment of type 1 and 2 diabetes mellitus (Edelman et al., 2008).

Amylin also signals meal satiation (Lutz et al., 1995; Young et al., 1998), a function that categorizes it among the anorexigenic hormones, those that inhibit appetite and reduce food intake. Studies on amylin have implicated this hormone in the regulation of both homeostatic and hedonic feeding as well as in body weight modulation (for review (Boyle et al., 2018)), roles that will be further discussed below.

Endogenous circulating amylin increases after meal initiation and exogenous administered amylin reduces food intake shortly after administration (Lutz et al., 1995). Preclinical data show that amylin decreases food intake in mice, independent of their energy balance status (food-deprived or non-deprived) (Morley et al., 1991) and reduces food intake in obese and diabetic obese mouse models (Morley et al., 1994). Similarly, central administration of amylin in the brain, decreases 24-hour food intake values in rats (Rushing et al., 2000). Chronic administration of amylin inhibits eating by decreasing meal size in rats (Lutz et al., 2001a; Mack et al., 2007), without inducing taste aversion (Lutz et al., 1995; Morley et al., 1997; Naeve et al., 2005).

Amylin has also been identified to act as an adiposity signal, which means that circulating levels of the hormone are proportional to body adiposity and consequently can regulate body weight (Woods et al., 2000). Indeed, higher endogenous amylin plasma levels are associated with obesity (Enoki et al., 1992; Leckström et al., 1999; Martin et al., 2010), however the direct link between plasma amylin levels and body adiposity has not yet been fully outlined. In the preclinical setting, rat studies have identified that chronic administration of amylin, both peripheral and central, decreases body weight and fat gain in rats (Mack et al., 2007; Roth et al., 2007; Rushing et al., 2001). Amylin levels seem to be higher in obese rats (Pieber et al., 1994) and obese rats fed with high-fat diet (Boyle et al., 2017), compared to their respective controls. Furthermore, amylin reduces 24-hour body weight values in outbred (Rushing et al., 2000; Shah et al., 1984), as well as in obese rats (Feigh et al., 2011), and centrally acting amylin reduces adiposity and body weight in rats (Rushing et al., 2001; Wielinga et al., 2010). Supportively, rat studies with centrally infused amylin antagonists show increased adiposity (Rushing et al., 2001).

Additionally, amylin is suggested to physiologically regulate energy homeostasis, by increasing energy expenditure (for review (Lutz, 2010)). Acute and chronic administration of exogenous amylin and amylin analogues increases energy expenditure additionally to body weight in mice and rats (Isaksson et al., 2005; Osaka et al., 2008; Roth et al., 2006). Acute systemic administration of amylin does not affect energy expenditure, but central activation of amylin receptors (AMYRs) enhances it by approximately 25% (Wielinga et al., 2007).

Amylin shares common properties with other peptides, found across species, leading to the development of compounds imitating its activity by agonizing AMYRs. One such compound is salmon calcitonin (sCT), an amylin (and calcitonin) receptor agonist derived from salmon (Epand et al., 1986). A large number of studies has showed that sCT mimics amylin's action in the regulation of food intake (Bello et al., 2008; Chelikani et al., 2007; Eiden et al., 2002; Lutz et al., 2000), body weight (Chelikani et al., 2007; Feigh et al., 2011; Lutz et al., 2001b) and energy expenditure (Wielinga et al., 2007). sCT binds potently to AMYRs in order to exert its anorexigenic properties (Lutz et al., 2000), making it a valuable candidate for the research of amylinergic pathways. This has opened the way to the synthesis of other analogues, selectively binding to AMYRs with protracted affinity, like the new recombinant amylin analogue NNC0174-1213 (AM1213), facilitating more thorough investigation of the role of AMYRs. Additionally, for further identification of the role of those receptors, the antagonist AC187 has been extensively used (Mollet et al., 2004; Reidelberger et al., 2004).

Based on the above characteristics, the agonists sCT and AM1213 and the antagonist AC187 were chosen as the tools to investigate the role of the amylinergic pathway in the present studies.

Amylin receptors

AMYRs are comprised of the core calcitonin receptor (CTR), which is a G protein-coupled receptor, and one of the receptor activity-modifying proteins (RAMP) 1, 2, or 3 (Hay et al., 2015), as shown in ***Figure* 3**. The CTR belongs to the secretin family of G protein-coupled receptors (for review (Barwell et al., 2012)) and has been proposed to have two distinct isoforms namely CTRa and CTRb, both present in humans and rodents (Poyner et al., 2002). The role of the RAMPs is to modify the activity of the CTR in order to modulate signalling for amylin and calcitonin (Hay et al., 2016). Since the CTRb isoform presents decreased affinity for calcitonin ligands (Poyner et al., 2002), the most studied

and characterized AMYRs include the CTRa/RAMP1, 2 and 3 complex, comprising the AMY1, 2 and 3 receptor respectively (for review (Bower et al., 2016)).

AMYRs have high affinity for the agonists amylin and sCT and for the antagonist AC187 among others (Gingell et al., 2014; Hay et al., 2015). All the above compounds bind to calcitonin and AMYR subtypes with different potency, altering receptor pharmacology. In general, sCT binds with higher affinity to the CTR alone and the AMYR subtypes 1 and 3, compared to subtype 2 (Alexander et al., 2013; Alexander et al., 2015; Gingell et al., 2014).

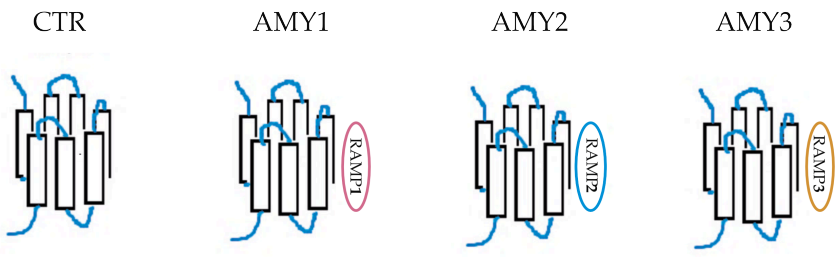

***Figure* 3. The three types of AMYRs:** Combination of the core CTR receptor with one of the RAMP1, 2 and 3 forms the respective AMY1, 2, and 3 receptors. (**RAMP:** receptor activity modifying protein)

Signalling in the central nervous system

The AMYR components are located in many brain areas, including the nucleus of the solitary tract, area postrema (AP), NAc, hypothalamus and dorsal raphe among others (Becskei et al., 2004; Sexton et al., 1994; Ueda et al., 2001). Studies support that amylin crosses the blood-brain barrier (Banks et al., 1998; Banks et al., 1995), however, whether physiologically sufficient amounts of amylin reach the brain is yet not fully elucidated. Additionally, the recent evidence that amylin is expressed in the lateral hypothalamus in the brain (Li et al., 2015) could further explain the presence of local binding sites. It is generally hypothesized that amylin's satiety effects are expressed through central mechanisms in areas involved in feeding control (***Figure* 4**), like the nucleus of the solitary tract and the AP (Braegger et al., 2014; Lutz et al., 2001a; Potes et al., 2010). The physiological role of activation of AMYRs in other brain areas, like the parabrachial and lateral parabrachial nucleus, has also been reported (Lutz et al., 2018; Whiting et al., 2017).

Recently, AMYRs in reward-related areas like the LDTg, VTA and NAc have been shown to also mediate the amylin-related effects on energy balance and

food reward *(see below)* (Mietlicki-Baase et al., 2014; Mietlicki-Baase et al., 2015a; Reiner et al., 2017a).

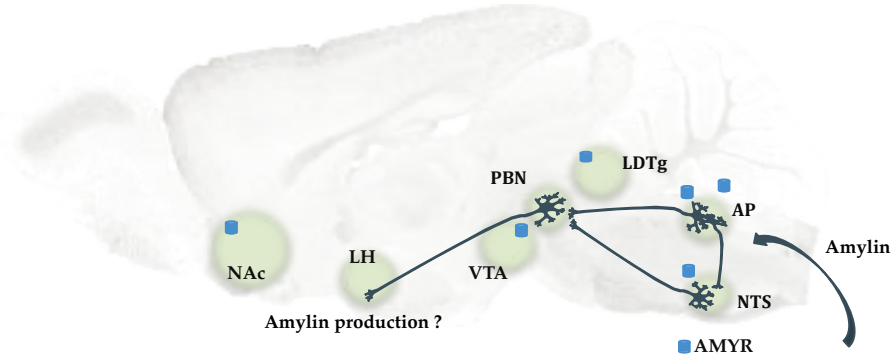

***Figure* 4. The main studied brain pathway for the expression of amylin's effects:** Peripheral amylin enters the brain through the brainstem and subsequently activates areas of the midbrain, by acting on local AMYRs. (**AP**: area postrema, **LDTg**: laterodorsal tegmental area, **LH**: lateral hypothalamus, **NAc**: nucleus accumbens, **NTS**: nucleus of the solitary tract, **PBN**: parabranchial nucleus, **VTA**: ventral tegmental area, **AMYR**: amylin receptors)

Role in food intake and reward

Most studies have focused on the involvement of the AP as the site of action for peripheral amylin (Braegger et al., 2014). Amylin administered in the AP reduces food intake, while the AMYR antagonist AC187 in the AP blocks the ability of peripheral amylin to decrease food intake (Mollet et al., 2004). More recent studies showed that AMYRs in the LDTg and VTA are important for the expression of the anorectic effects of sCT. Intra-LDTg sCT administration decreases food intake and body weight, and supresses meal size in rats and these effects are suggestively mediated through AMYRs on GABAergic neurons (Reiner et al., 2017a). Moreover, sCT administration into the VTA decreases chow intake in rats (Mietlicki-Baase et al., 2013). The involvement of the NAc core is also implied, as dopamine receptor activation in that area decreases the inhibitory food intake and body weight effects of intra-VTA sCT in rats (Mietlicki-Baase et al., 2015b).

Despite the involvement of reward-related areas in the expression of the anorectic effects of amylin and sCT, their implication on food reward has not yet been extensively studied. sCT administered into the VTA reduces food-induced phasic dopamine release in the NAc core and attenuates palatable high-fat food intake (Mietlicki-Baase et al., 2015b), palatable sucrose solution intake and sucrose self-administration in rats (Mietlicki-Baase et al., 2013).

Interestingly, dopamine D2 receptors are implicated in the expression of peripheral amylin's satiety effects (Lutz et al., 2001c) and AMYRs on the VTA are located on dopaminergic neurons (Mietlicki-Baase et al., 2015b), indicating the importance of the dopamine system in amylinergic reward regulation. Similarly to the VTA, administration of sCT into the LDTg attenuates the motivation to consume sucrose solution in an operant self-administration paradigm in rats (Reiner et al., 2017a).

Data about the effects of AMYR activation on substance reward are more limited. Two preclinical studies reported that sCT administered into the third ventricle blocks amphetamine-induced locomotor stimulation in rats (Twery et al., 1986) and that peripheral calcitonin administration reduces voluntary alcohol drinking in rats (Laitinen et al., 1992). Only one recent clinical study shows associations between intravenous cocaine administration and plasma amylin levels in cocaine users (Bouhlal et al., 2017).

Rodent studies have established that amylin signalling regulates energy balance and adiposity and is implicated in the regulation of food reward. Importantly, amylin regulates the above behaviours by, at least in part, acting on central AMYRs located in the reward system of the brain. Nevertheless, the possible involvement of amylin pathways in the regulation of addictive drugs still remains in the background. Given the need for personalised treatments for AUD, the exploration of the amylin signalling presents a promising potential in uncovering new pharmacological targets.

AIMS OF THE THESIS

This thesis attempts to shed light on the link between central amylinergic pathways and substance reward. The overall objective is to explore the role of the amylinergic pathway in alcohol-mediated behaviours, by means of studies in rodents.

Specifically, the present studies aim to evaluate the role of amylin signalling in acute and chronic alcohol-induced behaviours (**Papers I-II**) and to identify the brain regions (**Paper III**), the underlying molecular mechanisms (**Paper IV**) and the sex differences (**Paper V**) involved.

MATERIALS AND METHODS

Animals

Relevance of animal models in addiction research

The study of addiction largely depends on the animal models that have been established in order to identify potential novel targets for pharmacotherapy (Sanchis-Segura et al., 2006). The complexity of AUD is an obstacle in modelling this condition in animals; however, animal models provide a valuable tool in better understanding behavioural and neurobiological mechanisms underlying the development and progression of AUD.

In the present studies, both mice and rats were used, since both species show similar responses to gut-brain peptides in regards to alcohol-mediated behaviours (for review (Jerlhag, 2019a). In addition, mice and rats respond similarly to alcohol and other addictive drugs in the used animal models (for review (Spanagel, 2000; Tabakoff et al., 2000).

The methods used for this thesis include models that reflect reward, primarily measured by the ability of drugs, like alcohol, to activate the mesolimbic dopamine system (Soderpalm et al., 2013). Therefore, some of the paradigms used here in mice, like locomotor activity, CPP and microdialysis experiments give the first insight on how a substance acts on the reward-processing brain areas. The rat models, such as the intermittent alcohol access paradigm and operant self-administration that have previously been established and used here, reflect behaviours that are seen in patients diagnosed with AUD. They thus provide a valuable input towards the development of new pharmacological agents for the treatment of AUD (Spanagel, 2000; Tabakoff et al., 2000).

Conditions and ethical considerations

All animals were kept group-housed (except for the intermittent alcohol access paradigm rats and operated mice and rats that were kept single-housed) in the animal facilities under stable temperature and humidity conditions (20°C and 50% respectively) and were allowed to acclimatize for one week after arrival. Food (Normal chow, Teklad diet, Envigo; Madison, WI, USA) and water was supplied *ad libitum*, unless stated otherwise. In the Italian laboratory, food

(Normal chow, Mucedola; Settimo Milanese, Italy) and water supply was different during the initial training phase of the self-administration experiments.

A separate set of animals was used for each experiment, unless otherwise specified. The Swedish ethical Committee on Animal research in Gothenburg approved all animal experiments conducted in Sweden. The animal experiments at the Italian laboratory were approved by the Ethical committee at the University of Cagliari and were conducted in accordance with the Italian law on the "Protection of animals used for experimental and other scientific purposes". All efforts were made to minimize animal number and suffering and all predetermined endpoints were taken into consideration.

Mice

For all the mice studies described in this thesis (**Papers I, III** and **IV**), age-matched male NMRI mice (8-12 weeks old and 20-25 g body weight at the time of arrival, Charles River; Susfeldt, Germany) were used. The strain was selected for the experiments of locomotor activity (including dose-response studies), microdialysis, CPP, palatable food intake, blood alcohol concentration and corticosterone levels. This mouse strain shows robust stimulatory response in alcohol-induced behaviours (Jerlhag et al., 2009), making it a valuable tool for alcohol research. Moreover, this is an outbred strain representing genetic variation, which reflects clinical conditions more closely than inbred or knock-out strains.

Rats

For the intermittent alcohol access paradigm (**Papers I, II**, **III** and **V)**, the gene expression analysis (**Paper II**), the biochemical analysis (**Paper V**) and the immunohistochemistry experiments (**Paper IV**), age-matched male outbred RccHan Wistar rats (200-250 g body weight at the time of arrival, Envigo; Horst, Netherlands) were used. This strain was chosen as it displays high and stable alcohol intake with relevant blood-alcohol concentration levels (Simms et al., 2008). The studies in **Paper V** included also females of the same strain (180-200 g body weight at the time of arrival, Envigo; Horst, Netherlands). Females were included in an attempt to identify different responses between sexes in alcohol intake after pharmacological manipulation, as females tend to be understudied in preclinical behavioural research.

For the operant alcohol self-administration experiments in **Paper II,** selectively bred Sardinian alcohol-preferring (sP) rats were used. These rats are

characterized by high alcohol intake and high motivation to receive alcohol in operant self-administration paradigms (Colombo et al., 2006). For the operant chocolate drink self-administration experiments in the same paper, Wistar rats (Harlan Laboratories; San Pietro al Natisone, Italy) were used, a commonly used strain in operant paradigms. The above self-administration experiments were conducted in the CNR Neuroscience Institute, Monserrato, Cagliari, Italy, in collaboration with Prof. Giancarlo Colombo.

Drugs

Alcohol

For peripheral alcohol administration (**Papers I, III** and **IV**) alcohol of 95% v/v (Solveco AB; Stockholm, Sweden) diluted in 0.9% sodium chloride (resepective vehicle solution) was in all cases administered intraperiotaneally (IP) at the dose of 1.75 g/kg, 5 minutes prior to the initiation of the experiment (this timeline is needed for the exertion of its stimulatory effects). This dose has previously been shown to activate the mesolimbic dopamine system as measured by locomotor stimulation, accumbal dopamine release and CPP paradigms (Egecioglu et al., 2013b; Jerlhag et al., 2011b). For the voluntary alcohol intake in the intermitent alcohol access paradigm (**Papers I, II, III** and **V**) 95 % alochol was diluted to reach 20% v/v with tap water, as a standard protocol used for this model (Simms et al., 2008). For the alcohol-self administration experiments in sP rats (**Paper II**), alcohol was presented to a dilution of 15 % v/v as per standard experimental protocol (*see Operant alcohol self-administration*) (Colombo et al., 2006).

sCT

The amylin (and calcitonin) receptor agonist sCT (Tocris Bioscience; Bristol, United Kingdom) was in all cases administered IP, 30 minutes prior to the initiation of the experiment, since this timeline was established in pilot experiments as the needed time for sCT to block alcohol's stimulatory effects. In the initial experiments, the effects of a lower dose (1 µg/kg; **Papers I** and **II**) on alcohol behavioural responses was tested, and in the studies the standard dose of 5 µg/kg (**Papers I-IV**) was given. The latter dose has an inhibitory effect on food intake and body weight and has been extensively used in the literature

(Braegger et al., 2014; Lutz et al., 2000; Mietlicki-Baase et al., 2013; Pecile et al., 1987).

In **Paper III**, sCT was administered locally and bilaterally at a delivery volume of 0.5 µl/side into the LDTg (0.005 µg per side), VTA (0.4 µg per side) and NAc shell (0.02 µg per side), diluted in Ringer solution (NaCl 140 mM, CaCl2 1.2 mM, KCl 3.0 mM and MgCl2 1.0 mM; respective vehicle). All doses were selected based on data from our dose response studies that showed no effect *per se* on baseline locomotor activity and gross behavioural observation of the animals.

In **Paper III**, FAM-labelled sCT (custom made, Phoenix Pharmaceuticals Inc, Burlingame, CA, USA) was used for the immunohistochemistry experiments. This was diluted in 10% DMSO in PBS buffer (pH 7.5) and was further diluted in 0.9% sodium chloride for a 5µg/kg IP and in Ringer solution for a 2 µg intracerebroventricular (ICV) dose. The respective vehicle solutions for that experiment were 75% of 0.9% sodium chloride (IP) and 25% of 10% DMSO in PBS (ICV).

AC187

The amylin receptor antagonist AC187 (Tocris Bioscience; Bristol, United Kingdom) was administered IP (**Paper II**) at a dose of 250 µg/kg, diluted in 0.9% sodium chloride (respective vehicle), 5 min prior to experiment initiation in order to compensate for the drug's short half-life and bioavailability. The dose selected was based on previous studies showing increased food intake after administration of AC187 (Reidelberger et al., 2004) and observations that this dose does not cause gross behavioural effects on rats.

AM1213

The synthetic amylin analogue AM1213 (Novo Nordisk A/S; Måløv, Denmark) used in **Paper V** was tested as a selective and long-acting amylin receptor agonist, in order to identify its effects on chronic alcohol-induced behaviours in male and female rats. It was diluted in vehicle sodium acetate, glycerol and sterile water solution of pH 4.00±0.05 (respective vehicle) and administered subcutaneously (SC) at the dose of 0.3 mg/kg, 60 minutes prior to experiment initiation. The dose was selected as the one with no effect *per se*, after a behavioural test battery (Irwin test) performed at the Novo Nordisk laboratories, and the time frame of 60 minutes was selected due to the drug's protracted half-life.

Surgical procedures

Guide and probe implantation

To facilitate intracranial injections into specific brain areas (**Paper III**) and to allow for dopamine collection via a microdialysis probe (**Papers I** and **III**), the same surgical procedures were used in both mice and rats. The rodent was anesthetized with isoflurane (Baxter International; Apoteket AB, Gothenburg, Sweden) using a pump (Univentor 400 Anaesthesia Unit, Univentor Ldt.; Zejtun, Malta) and was kept on a heating pad to prevent hypothermia and placed on a stereotaxic frame (David Kopf Instruments; Tujunga, CA, USA).

Two drops of local anaesthetic (mix of 10 mg/ml Xylocaine® and 5 µg/ml adrenaline, Astra Zeneca; Apoteket AB, Gothenburg, Sweden) was applied locally on the incision surface and the analgesic carprofen (Rimadyl® (5 mg/kg), Astra Zeneca; Apoteket AB, Gothenburg, Sweden) was administered SC prior to the operation. Eye drops gel (Viscotears®, Théa; Kronans Apotek) was applied on the eyes to avoid dryness and a saline injection (0.9% sodium chloride) was administered SC to avoid dehydration.

The skull bone was then exposed and holes for the bilateral guides or the custom-made dialysis probe, as well as a hole for the anchoring screw were drilled. The guides/probe were anchored to the screw and the skull bone with dental cement (DENTALON® plus, Agntho's AB; Lidingö, Sweden). The coordinates targeting the LDTg, VTA and NAc shell and ICV in mice and rats are presented in ***Table* 1**. For the intracranial injections in **Paper III**, a dummy cannula was inserted through the guide, in order to remove clotted blood and damper spreading depression. Vehicle or sCT was infused over 60 seconds using an injector cannula, which was retracted after an additional 60 seconds.

	AP (Relative to bregma)	ML (Relative to midline)	DV (Relative to skull bone)
		Mouse	
LDTg	–5.00	±0.50	–3.20
VTA	–3.40	±0.50	–4.30
NAc shell	+1.40	±0.60	–4.70
		Rat	
LDTg	–8.80	±1.00	–7.00
VTA	–5.20	±1.00	–8.30
NAc shell	+1.85	±1.00	–7.80
ICV	–1.40	0.00	–4.70

***Table* 1. Stereotaxic surgery coordinates in mm.**

The infusion/probe location sites were subsequently verified following the termination of the experiment by slicing the brain in a vibratome (Vibroslice, Campden Instruments Ltd; Loughborough, UK) and the position of the cannule/probe was grossly observed. Only animals with correct placements were included in the statistical analysis.

Behavioural procedures

Locomotor activity in mice

Locomotor activity experiments are conducted as a first assessment of the effect of a drug on the mesolimbic dopamine system. Drugs of abuse cause locomotor stimulation, which is considered a first indication of enhanced extracellular dopamine release in the NAc (Blomqvist et al., 1992). Locomotor activity was registered in six locomotor boxes (Open Field Activity System, Med Associates Inc; Georgia, Vermont, USA). The system registers the distance travelled (cm per 5 minutes) of each mouse during the entire time defined by the protocol. During all the locomotor activity experiments the mice were allowed

to habituate to the boxes for one hour prior to the first injection (i.e. initiation of the experiment).

sCT dose-response (Papers I and III). Mice were administered various sCT doses or vehicle) and the cumulative locomotor activity was registered for 90 minutes following injections.

Acute sCT administration (Papers I and III). Mice received sCT or vehicle and alcohol or vehicle IP injection and the cumulative locomotor activity was registered for 60 minutes.

Repeated sCT administration (Paper IV). Mice were injected daily with sCT or vehicle IP for 5 days After two days without injections, the mice received an alcohol or vehicle injection and cumulative locomotor activity was registered for 60 minutes.

Repeated sCT and alcohol co-administration (Paper IV). Mice were injected daily with sCT or vehicle IP together with an alcohol or vehicle injection IP for 5 days. Each day, the mice were tested in the locomotor activity boxes and the cumulative locomotor activity was registered for 30 minutes.

CPP in mice

This paradigm has been extensively used to assess the ability of a drug to induce a CPP in rodents (Bardo et al., 2000). Two distinct CPP experiments were conducted for attesting the rewarding properties of alcohol or retrieval of alcohol reward-dependent memory. This experiment utilizes two interconnected chambers with different visual and tactile cues (custom made boxes), which the mice learn to associate with the presence or absence of reward. All sessions were 20 minutes long and the expression of CPP was calculated as the percentage of difference of the time spent in the drug-paired (i.e. least preferred) compartment during post- and pre-conditioning.

CPP of acute alcohol reward. In this paradigm, we evaluated effects of sCT on the ability of alcohol to create a CPP response in mice (**Paper I**). The mice were allowed to explore both chambers on the first pre-conditioning day and their chamber preference was then evaluated. During the conditioning (days 2-5), each mouse received one sCT (or vehicle) plus one alcohol injection (morning or afternoon) and were placed in their less preferred chamber and a vehicle

(plus vehicle) injection (afternoon or morning) paired to their preferred chamber in a balanced design throughout the week. Two treatment groups were thus created: vehicle-alcohol and sCT-alcohol. During the post-conditioning day (day 6), the mice were allowed to freely explore both chambers and their preference was again evaluated.

CPP of alcohol reward-dependent memory retrieval. In this paradigm, we evaluated effects of sCT on the retrieval of previously formed alcohol reward-dependent memory as assessed by expression of the CPP (**Paper I** and **III**). On the first pre-conditioning, day the mice were given a vehicle injection and were allowed to explore both chambers and their preference was then evaluated. During the conditioning (days 2-5), each mouse received one alcohol injection (morning or afternoon) and was placed in the less preferred chamber and one vehicle injection (afternoon or morning) paired to its preferred chamber. During the post-conditioning day, the mice received a single sCT or vehicle injection in a randomized balanced design, were then allowed to freely explore both chambers and their preference was again evaluated. Two treatment groups were thus created: vehicle-alcohol and sCT-alcohol. In **Paper III,** this is the only CPP paradigm used as daily intracranial injections are challenging and cause discomfort to the animals.

Microdialysis in freely moving mice

In vivo microdialysis was conducted in freely moving mice, in order to assess extracellular dopamine levels in the NAc shell at different time intervals and after pharmacological manipulations (**Papers I** and **III**). In all experiments, the probes targeted the NAc shell as this area presents a more robust dopamine release as a response to alcohol (Ding et al., 2009) and is anatomically and functionally distinct from the core region (Bassareo et al., 2003). In general, after one hour of habituation to the microdialysis set-up, perfusion samples were collected from the mice in 20-minute intervals during the entire experimental protocol. The baseline dopamine levels were defined as the average of three consecutive samples (-40 to 0 minutes). The collected dialysates were further analysed in a HPLC/EC system *(see HPLC/EC detection)*.

In **Paper I**, an initial alcohol injection was given prior to sCT administration in order to establish the dopamine increase after the alcohol challenge. However, as in our hands alcohol injections always cause a robust and established dopamine elevation and in order to avoid potential enhanced response to alcohol, this initial injection is omitted from the experimental setups of **Paper III**.

Intermittent alcohol access paradigm in rats

This drinking paradigm induces high alcohol consumption in outbred rats and gives a valuable insight into the alcohol consumption behaviour seen in humans (Spanagel, 2000). The model of intermittent access to alcohol displays pharmacologically relevant blood-alcohol concentration levels and has been extensively used in preclinical animal research (Simms et al., 2008). In general, the rats were given free access to one bottle of 20% alcohol and one bottle of water during three 24-hour sessions per week (Mondays, Wednesdays and Fridays). The rats had unlimited access to two bottles of water between the alcohol-access periods (Tuesdays, Thursdays and weekends). This process was repeated for 10-12 weeks (per individual experiment) and all bottles and food were weighed daily (***Figure* 5**).

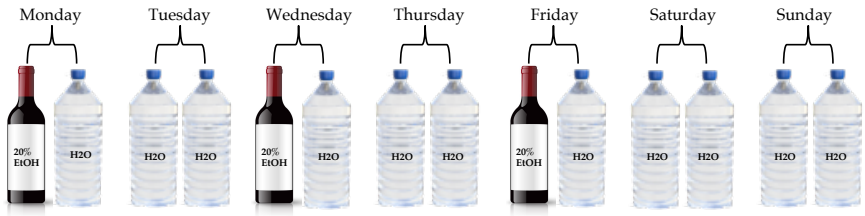

***Figure* 5. Schematic representation of the weekly schedule of the intermittent alcohol access paradigm in rats.** The rats were given a choice of a 20% alcohol containing bottle and a water bottle. The alcohol bottle was alternated with water every other day, except for the weekends were rats had access only to water. This weekly procedure lasted for 10-12 weeks depending on the experiment.

Acute sCT or AC187 administration. For **Paper I**, rats were separated in low and high alcohol consuming (low consumers: <average 3.5 g/kg/24hrs alcohol intake, high consumers: >average 3.5 g/kg/24hrs alcohol intake). The rats were administered a single injection of sCT (IP for **Paper I** and locally for **Paper III**) or vehicle and **AC187** (IP for **Paper II**) or vehicle, on one of the alcohol-drinking days (Monday or Wednesday). The studies were a within-subjects balanced design, so that all rats alternately received both sCT and vehicle injections, one each day (***Figure* 5**). Alcohol intake, alcohol preference, water intake, total fluid intake and food intake values were obtained at 1 and 24 hours after bottle presentation for the sCT experiments in **Paper I** and **III** and at 1, 4 and 24 hours for the AC187 experiments in **Paper II**. Body weight was always reg-

istered at 24 hours. In this experiment two separate group of rats (one per drug administration) were used.

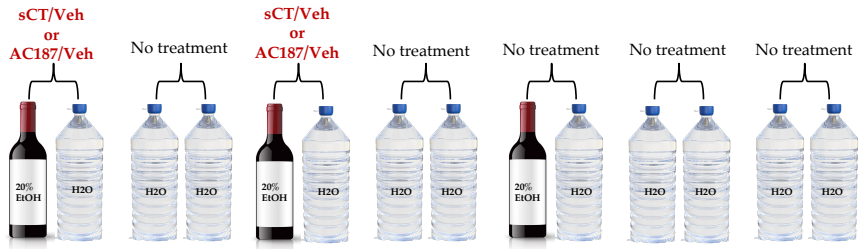

***Figure* 6. Timeline for the acute sCT (or AC187) administration experiment in rats.** Injections were given on Monday and Wednesday. Each group of rats received both sCT (or AC187) and vehicle injections and each animal served as its own control. Two separate groups of rats (one per drug administration) were used for this experiment (**Veh**: vehicle).

Repeated sCT administration. Daily, rats were given a single injection of sCT or vehicle IP on three subsequent alcohol-drinking days (Monday, Wednesday, and Friday) in a between-subjects balanced design (**Paper II, *Figure* 7**). Alcohol intake, alcohol preference, water intake, total fluid intake and food intake values were obtained at 1 and 24 hours after bottle presentation and body weight was registered at 24 hours.

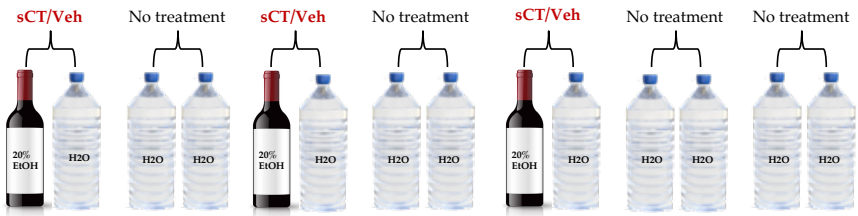

***Figure* 7. Timeline for the repeated sCT administration experiment in rats.** Injections were given on Monday, Wednesday and Friday. Each group of rats received either sCT or vehicle injections (**Veh**: vehicle).

Two-week AM1213 administration. Male and female rats were daily injected SC with AM1213 or vehicle, Monday through Saturday for two continuous weeks. Measurements of bottles, food intake and body weight were done daily (**Paper V, *Figure* 8**). On alcohol days (Monday, Wednesday and Friday), alcohol intake, alcohol preference, water intake, total fluid intake and food intake values were obtained at 1, 4 and 24 hours after bottle presentation and body weight was registered only at 24 hours. After this two-week intervention, all values were registered every 24 hours for an additional two-week washout period without administration of AM1213.

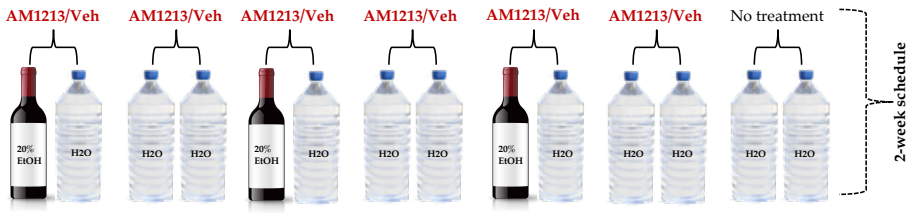

***Figure* 8. Timeline for the two-week AM1213 administration experiment in rats.** Injections were given Monday-through Saturday for two continuous weeks. Each group of rats received either AM1213 or vehicle injections (**Veh**: vehicle).

Alcohol deprivation effect (ADE) model in rats. The ADE is a behaviour characterized by increased alcohol intake in rats after forced abstinence from alcohol for a period of time. This behaviour is also present in alcohol dependent individuals, making this model a valid representation of AUD phenotypes in preclinical research (Spanagel, 2000). In the experiments conducted in **Paper II**, rats were deprived of alcohol for 10 days, after 10 weeks under the intermittent alcohol access paradigm. The rats were then given a single sCT or vehicle IP injection prior to alcohol bottle presentation and their 24-hour alcohol intake was registered.

Operant self-administration in rats

This paradigm reflects the motivation of the rats to obtain a reward. Rats can be trained to lever press in order to receive a rewarding substance (e.g. alcohol, palatable drink, food reward, etc.). Two different operant paradigms can be applied here. In the fixed ratio (FR) schedule of reinforcement the rats learn to press the lever for a set amount of times in order to get the reward; while in the progressive ratio (PR) the number of times that the rats need to press the lever progressively increases. The last successfully completed ratio that results in the reward presentation, is defined as the breakpoint. The FR reflects the reinforcement of a reward, as it shows how many times a rat is willing to press the lever in order to obtain the reward. The PR is considered as a measure of motivation, as the rat needs to increasingly "work" harder for the reward.

Operant alcohol self-administration. These experiments were conducted at the Italian laboratory (CNR Neuroscience Institute, Monserrato, Cagliari, Sar-

dinia, Italy) following a previously established protocol for this task (Maccioni et al., 2012). The sP rats used in this experiment were first exposed to the intermittent alcohol access paradigm where they were given a 10% v/v alcohol bottle for 24 hours for total 10 days. Afterwards, the rats were trained to a FR alcohol self-administration paradigm in operant chambers (Med Associates; St Albans, VT, USA) with two retractable levers and two stimulus lights. In the last training sessions (sessions 9 and 10), the alcohol concentration was increased to 15% v/v. After 20 sessions of maintenance in 15% v/v alcohol, where the rats showed a stable response to the task, the effect of sCT on the alcohol lever pressing and amount of alcohol administered was evaluated (**Paper II**) during a 30-minute session.

Operant chocolate self-administration. These experiments, also conducted at the Italian laboratory, were performed under the PR schedule of reinforcement (Maccioni et al., 2017). The outbred rats used in this study were first trained under a FR for responding to a single lever delivering a chocolate beverage (Nesquik®, Nestlè; Milan, Italy; chocolate powder in tap water). The operant boxes used in this study were equipped with one lever and one stimulus light above the lever. After reaching the response requirement for the FR, sCT was administered and its effect on the motivation to obtain the palatable chocolate beverage was evaluated under the PR, where the rats had to progressively increase their lever presses up to the achievement of a breakpoint. The effect of sCT on the chocolate beverage lever presses and breakpoint (**Paper II**) was registered during a 60-minutes test session.

Palatable peanut butter intake in mice

These experiments were conducted in **Paper I** to initially assess the effect of sCT on a palatable and rewarding food in mice, since peanut butter has been previously used in studies as a measurement of food reward (Egecioglu et al., 2010). In our experiments, all mice were allowed to familiarize with the peanut butter (Crunchy peanut butter with no added sugar, Green Choice; The Netherlands) taste in their home cage for 7 days before the test. At the test day, sCT or vehicle was administered 30 minutes prior to peanut butter exposure and peanut butter intake was measured at the time point of 1 hour in an empty cage.

Biochemical and molecular procedures

Tissue isolation

For the execution of the biochemical and molecular experiments in the present thesis, brain tissue was isolated by the means of either punches of microdissection of fresh tissue or frozen tissue punches. Depending on the brain areas that needed to be isolated at times, either technique was used.

Fresh tissue microdissection. For the experiments in **Paper II** (gene expression analysis) and **IV** (*ex vivo* biochemical analysis), after euthanisation of the animals, the whole brain was rapidly removed and immediately placed on a cold glass plate. The desired brain regions were rapidly dissected with the help of microdissection instruments, transferred into a labelled plastic tube, immediately placed in dry ice and stored at -80ºC until further processing.

Frozen tissue punches. For the studies in **Paper IV** (Western Blot) and **V** (*ex vivo* biochemical analysis), after euthanisation of the animals, the whole brain was rapidly isolated and placed into plastic tubes and snap frozen at -80ºC. Further dissection of the brain regions of interest was performed by punching them from frozen tissue. Specifically, brains were placed in a cold mouse brain matrix (Zivic instruments; Pittsburgh, PA, USA) and coronally sectioned in 1 mm slices according to the brain atlas (Franklin et al., 1996). The desired section was placed under a stereoscope on a very cold glass plate (mix of dry ice and regular ice) to avoid tissue degradation and the areas were isolated bilaterally, using a tissue biopsy punch (Zivic instruments; Pittsburgh, PA, USA).

HPLC/EC detection

Detection of the neurotransmitters and their metabolites is essential in understanding the mechanisms and systems involved in amylinergic regulation of alcohol-induced behaviours. In all such experiments, both in vivo and ex vivo levels of neurotransmitters were measured through electrochemical detection in an HPLC apparatus as described previously (Mefford, 1981).

Microdialysis experiments. For the detection of dopamine (**Papers I** and **III**), two HPLC apparatuses were used. In the first apparatus, 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 consisted 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. The dopamine currents were detected using the Dionex Chromeleon software package (Dionex; Sunnyvale, CA, USA) and were identified based on external dopamine standards.

Levels of monoamines and metabolites. In **Paper IV** and **V**, dissected brain tissue samples were homogenized by ultrasound homogenization (Sonifier Cell Disruptor B30, Branson Sonic Power Co; Danbury, CT, USA). The centrifuged supernatant was collected and analysed for noradrenaline (NA), dopamine (DA) and its metabolites 3-Methoxytyramine (3-MT), homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), as well as serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), using a split fraction HPLC-EC system. The currents were recorded with the Dionex Chromeleon software package (Dionex; Sunnyvale, CA, USA) and the concentration of monoamines and acids was calculated based on external standards.

Gene expression analysis

Real time qPCR was performed to identify whether chronic alcohol exposure changes the expression levels of the amylin receptor in rat brain areas. Therefore, the main components of the amylin receptor *CALCR*, *RAMP1* and *RAMP3* (genes of interest, GOI) were investigated **(Paper II)**. The TaqMan® Gene Expression Essays used for screening in these experiments were as follows Rn01427056_m1 (*RAMP1* rat), Rn00571815_m1 (*RAMP3* rat), Rn00587525_m1 (*CALCR* rat) and Rn01775763_g1 (*GAPDH* rat) (Thermo Fisher Scientific; Waltham, MA, USA).

The total RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen) and samples were loaded on the QIAcubeTM (Qiagen; Hilden, Germany) for automated RNA extraction, following the manufacturer's protocols. The quantitative real-time PCR (qRT-PCR) analysis was performed in the facilities

of TATAA Biocenter AB Gothenburg, Sweden. The corrected C_T values raw data were analysed using the comparative CT method as previously described (Livak et al., 2001). The low alcohol-consuming rats were set as the internal calibrator and the individual ΔC_T values were calculated as: $C_{T\ (GOI)} - C_{T\ (RG)}$.

Immunohistochemistry

Immunohistochemistry experiments were performed in **Paper III** in order to identify whether sCT penetrates the brain after peripheral injection. 30 minutes following a FAM-sCT (IP or ICV) or vehicle injection (IP), the rats were perfused and the brains were post-fixed for 24 h in Histofix and then immersed to 30% sucrose solution in PO4. One hemisphere was sagitally cut into 40 µm sections in a sliding microtome (Leica SM2000R, Leica Microsystems; Nussloch, Germany).

Immunohistochemistry was performed on free-floating sagittal brain sections containing all the brain areas of interest. The sections were incubated overnight at 4ºC with the following primary antibodies: Rabbit Anti-NeuN conjugated with Alexa Fluor® 647 (1:500, ab190565, Abcam; Cambridge, UK) and Chicken Anti-MAP2 (1:500, ab5392, Abcam; Cambridge, UK). Goat Anti-Chicken IgY H+L Alexa Fluor® 568 (1:500, ab175477, Abcam; Cambridge UK) was used as secondary antibody for MAP2 and was incubated in room temperature for 1 hour. In between the last washing steps, DAPI dye (600 uM, D1306, ThermoFisher Scientific; Waltham, MA, USA) was incubated for 15 minutes. The sections were mounted on adhesion glass slides (SuperFrost Plus™, ThermoFisher Scientific; Waltham, MA, USA) and cover slips were applied using a few drops of mounting media (ProLong™ Gold Antifade; Life Technologies Europe BV).

For the slide analysis a Zeiss LSM 800 confocal microscope with a 63x oil-immersed objective were used. Z-stacks (0.5 µm in-between) were merged with maximum intensity using the ImageJ software (public domain software, NIH; Bethesda, MD, USA). Further analysis of the images included uniform brightness adjustments and montage pictures of the separate channels using the Fiji ImageJ software (public domain software, NIH; Bethesda, MD, USA).

Western Blot

The Western Blot experiments in **Paper IV** were designed to explore differences in the levels of the CTR, after the mice were given sub-chronic sCT and

acute alcohol administration and were subjected to the locomotor activity paradigm.

The isolated tissue was homogenized and protein concentration was determined. The electrophoresis took place in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad; Hercules, CA, USA) and the gel was then transferred on a PVDF membrane using the Trans-Blot® Turbo™ Mini PVDF Transfer Packs (Bio-Rad; Hercules, CA, USA). The membrane was incubated with the following primary antibody solutions: Anti-CTR (recognizing two isoforms of the CTR, namely CTRa and CTRb; ID: ab11042, Abcam; Cambridge, UK) was diluted 1:1000 in TBS-T solution and the reference protein Anti-COX IV (ID: ab14744, Abcam; Cambridge, UK) was diluted 1:1000 in TBS-T+5% non-fat dry milk solution. The membrane was incubated with secondary antibody solutions the next day for one hour in room temperature: 1:5000 in TBS-T of Eu-Labelled Goat Anti-Mouse ScanLater™ (Molecular Devices; San Jose, CA, USA) was used against a-COXIV and 1:5000 in TBS-T of Eu-Labelled Goat Anti-Rabbit ScanLater™ (Molecular Devices; San Jose, CA, USA) was used against a-CTR.

Blood alcohol concentration in mice

Blood alcohol concentration measurements aid in distinguishing whether our pharmacological manipulations affect alcohol's rewarding or caloric properties. Mice trunk blood was collected in microtubes (Vacuette, Greiner Bio-one; Florence, Italy) and the blood-alcohol concentration analysis was outsourced to Sahlgrenska University Hospital (Gothenburg, Sweden).

Plasma corticosterone levels in mice

Measurement of plasma corticosterone levels is a reliable indication for identifying stress levels in rodents, as corticosterone is proposed to be present in high levels after stressful events (De Souza et al., 1982). Capillary blood from the tail was collected in microtubes (Microvette®, Sarstedt; Helsingborg, Sweden), was centrifuged and corticosterone thereafter measured in serum with an Enzo Corticosterone ELISA kit (AH Diagnostic; Stockholm, Sweden).

Statistical analyses

A fundamental step for the interpretation of the obtained results is accurate statistical analysis. This section describes the statistical approaches taken for the different experimental analyses. All analyses were conducted in and all graphs were generated with the GraphPad Prism Software (GraphPad Software Inc; CA, USA) and crosschecked for confirmation with the SPPS Statistics Software (IBM Corporation; NY, USA). The Western Blot membrane bands were firstly quantified using the ImageJ software (public domain software, NIH; MD, USA) before further statistical analysis.

Ordinary one-way ANOVA was used for the analysis of all dose-response studies in the locomotor activity tests (**Papers I** and **III**), the peanut butter intake experiments in **Paper I** and the operant alcohol and chocolate self-administration experiments in **Paper II** (including body weight analysis). Ordinary two-way ANOVA was performed for the analysis of all the locomotor activity experiments (**Papers I**, **III** and **IV**) and for the overall effect of acute treatment and time on ADE experiments in **Paper II**. Repeated measures two-way ANOVA was used for the analysis of the microdialysis experiments (**Papers I** and **III**) and the alcohol intake data in **Paper V**.

For Post hoc ANOVA analysis, Tukey's test was used for all pairwise comparisons between the means (all one-way and two-way ANOVA analyses) and Bonferroni's post hoc test was used for further analysis of planned comparisons (repeated measures two-way ANOVA and peanut butter intake experiments in **Paper I**).

Unpaired two-tailed t-test was used for the analysis of all the CPP experiments (**Papers I** and **III**), the gene expression (**Paper II**), the biochemical analysis (**Papers IV** and **V**), the baseline and between-treatment values of the ADE experiment (**Paper II**) and the blood-alcohol concentration and corticosterone data (**Paper I**). Paired two-tailed t-test was used for the analysis of all the alcohol and food intake data in **Papers I** and **II** and the analysis of baseline and ADE values within the treatment groups in **Paper II**.

The Kruskal-Wallis non-parametric test was used for the analysis of the Western blot data in **Paper IV**, as the analysed ratio values violated the assumption of normality and parametric tests were thus not appropriate.

RESULTS

Paper I

In this initial paper, we tested the effects of a single sCT injection on the acute responses to alcohol in mice and on alcohol intake in rats chronically exposed to alcohol. These studies identified a preliminary role of the activation of AMYRs in the regulation of alcohol-mediated behaviours in rodents.

In these experiments, we found that sCT given before an acute alcohol injection in mice blocked the locomotor stimulatory effect of alcohol. Pretreatment with sCT blocked the dopamine release in NAc shell in mice, which is induced by acute alcohol administration and decreased alcohol-induced reward in the CPP paradigm in mice. Additionally, a single sCT injection decreased alcohol intake, both in high and low alcohol-consuming rats, with a more profound reduction in the high consumers group. ***Figure* 9** summarizes the main findings in **Paper I**.

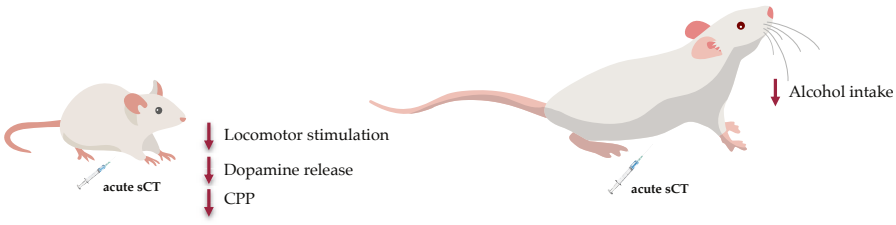

***Figure* 9. Schematic representation of the main findings in Paper I.** Acute sCT treatment attenuates acute alcohol-mediated behaviours in mice and chronic alcohol behaviours in rats. (**CPP**: conditioned place preference**, sCT**: salmon calcitonin)

We also found a reducing effect on food intake and body weight in rats, which interestingly followed the same differential pattern as alcohol intake. The same dose of sCT decreased food intake without effects on body weight in low alcohol-consuming rats, but robustly decreased food intake and body weight in high alcohol-consuming rats. Of note, sCT did not affect peanut butter consumption in mice, a food that is considered rewarding (Egecioglu et al., 2010). Lastly, our results showed that sCT did not affect blood-alcohol concentration and corticosterone levels in mice.

Paper II

In this paper, we investigated further how repeated sCT affects alcohol intake in rats. We also identified that AMYR activation modulates complex alcohol consumption behaviours in rats, which model different aspects of AUD in humans.

In these studies, we found that sCT administered for three days decreased alcohol intake in rats for the first two treatment days, while the intake returned to baseline on the third. This effect was accompanied by a similar decrease in alcohol preference. Additionally, we found for the first time, that acute administration of sCT prevented the development of ADE in alcohol-deprived rats. Acute sCT administered in two doses reduced the lever responses for alcohol self-administration in sP rats in a dose-dependent way, accompanied by reduction in amount of alcohol self-administered. ***Figure* 10** summarises the main findings in **Paper II**.

***Figure* 10. Schematic representation of the main findings in Paper II.** Repeated sCT treatment attenuates chronic alcohol-induced behaviours in rats. (**sCT**: salmon calcitonin, **ADE**: alcohol deprivation effect)

Chronic alcohol consumption decreased the *CALCR* gene expression in the NAc in high compared to low alcohol-consuming rats, while it increased *RAMP1* gene expression in the same area in the high consumption group.

We found a robust decrease in food intake and body weight in rats after repeated sCT treatment. Notably, we did not detect a tolerance effect in these parameters, as they were present throughout the treatment days. The robust decrease in body weight was present in both self-administration paradigms for alcohol and chocolate beverage. We additionally found that acute sCT, administered in two doses, did not affect lever pressing or breakpoint for a palatable chocolate beverage in rats.

In order to explore the effects of AMYR inhibition, we found that the AMYR antagonist AC187 increased only short-term alcohol intake, which is in line

with the drug's short-term effect on food intake increase (Rushing et al., 2001). Notably, administration of the AMYR antagonist did not affect food intake or body weight.

Paper III

The previous papers established the effects of peripheral sCT on alcohol-mediated behaviours. **Paper III** focused on the role of the brain areas processing reward, namely the LDTg, VTA and NAc shell, in the amylinergic regulation of acute alcohol behaviours in mice and chronic in rats.

We showed that systemically administered sCT, labelled with a FAM fluorescent tag, penetrates the brain and is visualized in the rat LDTg, VTA and NAc. Furthermore, local and bilateral administration of sCT into the LDTg decreased alcohol-induced locomotor stimulation and dopamine release in the NAc shell in mice and decreased alcohol intake in rats.

Similarly to LDTg, local infusions of sCT into the VTA blocked alcohol-induced locomotion and dopamine release in the NAc shell in mice, as well as decreased alcohol intake in rats. sCT into the NAc shell blocked alcohol-induced locomotor stimulation in mice and tended to decrease alcohol intake in rats.

sCT into the LDTg, VTA or NAc shell did not affect alcohol reward-dependent memory retrieval in the CPP. Of note, activation of AMYRs in the LDTg tended to reduce food intake, but sCT in the VTA or NAc shell did not affect food intake or rat body weight. ***Figure* 11** summarises the main findings in **Paper III**.

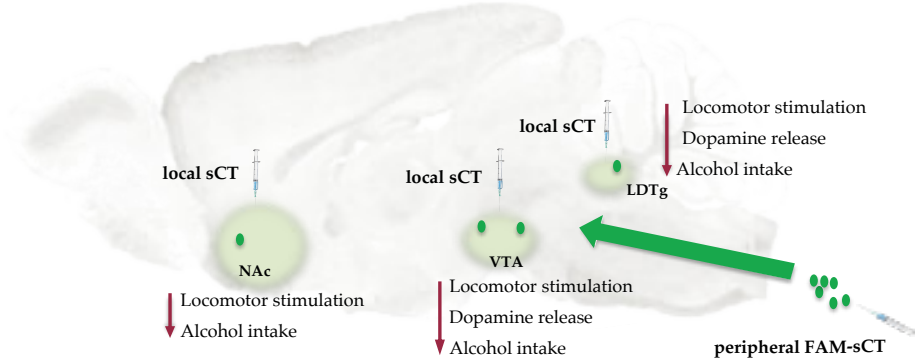

***Figure* 11. Schematic representation of main findings in Paper III.** FAM-labelled sCT is detected in the LDTg, VTA and NAc. Locally injected sCT decreases alcohol-mediated behaviours in both mice and rats. (**sCT**: salmon calcitonin, **LDTg**: laterodorsal tegmental area, **VTA**: ventral tegmental area, **NAc**: nucleus accumbens)

Paper IV

In this paper we explored the less studied effects of sub-chronic sCT administration on both behavioural and molecular level. We explored how it affects alcohol-induced locomotor activity and whether these changes are associated with the protein levels of the CTR *per se* and with monoamine concentration.

Mice that had previously been treated with sCT for five days showed decreased alcohol-induced locomotion, despite the absence of sCT treatment at the time of testing. The analysis of the LDTg, VTA and NAc of the mice from the above behavioural experiment did not show differences in the levels of CTR *per se*.

Our *ex vivo* biochemical data showed that sub-chronic sCT administration for five days altered the levels of monoamines and their metabolites in mice, implying that these changes might be driving the observed behavioural effects. In particular, we found that sCT increased the 5-HIAA/5-HT ratio and decreased the DOPAC/DA ratio in the VTA. ***Figure* 12** summarises the main findings in **Paper IV**.

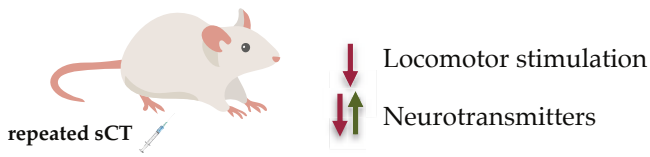

***Figure* 12. Schematic representation of main findings in Paper IV.** Repeated sCT pre-administration for five days decreases locomotor stimulation after an acute alcohol challenge in mice, despite the absence of sCT. Five-day sCT administration alters the levels of monoamines and their metabolites in reward-related areas in the mouse brain.

Notably, when sCT was co-administered with alcohol, it did not have an effect on locomotor stimulation induced by alcohol in the three first days, but there was an increase of locomotion in the sCT pre-treated group on the fourth day.

Paper V

Apart from AMYRs, sCT also activates the sole CTR (Lin et al., 1991); therefore, in this paper we used a selective and long-acting AMYR agonist, AM1213,

to explore its effects on alcohol intake in male and female rats. In order to explore the underlying neurochemical mechanisms, we also examined the effects of short- and long-term AM1213 treatment on monoamines and their metabolites in reward-related areas in rats.

Two-week administration of AM1213 decreased voluntary alcohol intake in both male and female rats. The initial reduction in alcohol intake, was followed by a tolerance effect of AM1213 in both sexes on the third alcohol session in males and fourth in females. An interesting finding is the differential response to AM1213 between male and female rats on the later sessions. The compound increased alcohol intake on the fourth session in male rats and the intake remained elevated throughout the experiment, while the intake in females returned to and remained at baseline levels on that session.

Our *ex vivo* biochemical findings showed that three-day administration of AM1213 increased dopamine and serotonin in the VTA and serotonin in the NAc in male rats. In females, short-term AM1213 administration increased serotonin in both the LDTg and the VTA and reduced the dopamine turnover in the NAc. In the VTA of male rats, long-term AM1213 administration had the opposite effect on the monoamine levels than short-term administration, tentatively explaining the increase in alcohol intake noted in the later sessions in males. In females, long-term administration of AM1213 did not have an effect on monoamines or their metabolites, a finding that could correlate to the return to baseline alcohol drinking.

Notably, repeated administration of AM1213 regulated food intake in a biphasic manner between sexes. Initially, the compound decreased food intake in both sexes. Food intake returned to baseline on the last session in males, whereas in females, this return to baseline was noted on the third session. A robust effect of the compound was noted on the body weight, as AM1213 decreased body weight in both sexes throughout the treatment period, although this decrease was more profound in male rats. ***Figure* 13** summarises the main findings in **Paper V.**

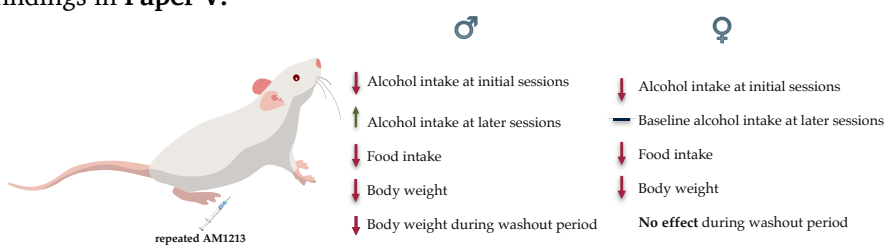

***Figure* 13. Schematic representation of main findings in Paper V.** Repeated AM1213 administration differentially regulates alcohol intake, food intake and body weight between male and female rats.

During the two-week washout period and after treatment discontinuation, no sustained effect of AM1213 on alcohol intake in either male or female rats was observed. Notably, male rats showed a sustained body weight reduction on the first four days following treatment discontinuation, an effect that was not noted in females. This effect in males suggested that body weight reduction does not reflect sole differences in food intake, but perhaps a more long-term regulation after AMYR activation.

DISCUSSION

The role of the amylinergic pathway had exclusively been assigned as the regulation of food intake and energy balance (for review (Boyle et al., 2018)). The studies demonstrated in this thesis reveal a new role for this pathway, where AMYR activation regulates alcohol-mediated behaviours. Importantly, these results fall in line with previous research showing modulation of those alcohol-mediated behaviours by other gut-brain peptides (for review (Jerlhag, 2019a)). Collectively, AMYR activation decreases alcohol-mediated behaviours involving, at least partly, reward-related areas in the brain.

Regulation of alcohol-induced behaviours by AMYRs

Our first studies showed that AMYR activation by sCT attenuates the well-established alcohol-induced effects in mice, like locomotor stimulation and increased dopamine release in the NAc shell. Increased locomotor stimulation is associated with increases in dopamine in the NAc shell in rodents. Dopamine increase is strongly associated with increase in euphoria after alcohol consumption (Boileau et al., 2003; Volkow et al., 2004a; Volkow et al., 2012a). Thus, our results offered a first indication that the amylinergic modulation of alcohol's behavioural effects likely occurs via dopaminergic pathways.

We further found that sCT reduces voluntary alcohol intake in rats with a more profound effect in rats that consume high amounts of alcohol compared to the ones consuming lower amounts. Interestingly, GLP-1R activation in reward-related areas decreases alcohol intake differently between high and low alcohol consumers (Vallöf et al., 2019). The same effect has been seen with a ghrelin receptor antagonist, which reduces alcohol intake more robustly in rats that had been consuming alcohol for five months compared to rats that were exposed to alcohol only for two months (Landgren et al., 2012).

On that note, we found that the AMYR components, *CALCR* and *RAMP1* are differentially expressed in the NAc of high compared to low alcohol-consuming rats. Aligned with our results, *NMUR2* gene expression in the dorsal striatum is decreased in high, compared to low alcohol-consuming rats (Vallöf et al., 2019) and GLP-1R expression is positively correlated to alcohol intake in the NAc of high consumers (Vallöf et al., 2019).

Our findings can explain the different response to sCT in regards to alcohol intake between high and low consumers. A possible explanation is that chronic alcohol exposure alters AMYR expression, rendering high consumers more responsive to AMYR activation. The physiological role of the decreased *CALCR* and increased *RAMP1* expression can only be speculated. A hypothesis could be that the ratio of CTR/RAMP1 is altered in high alcohol consumers, which affects their response to receptor agonists, implying differences in the receptor subtype formation rather than changes in their amount.

Although not directly related, studies have shown that alcohol-preferring and high alcohol-drinking rats have fewer binding sites for another peptide of the calcitonin family, the calcitonin gene-related peptide, in forebrain regions (Hwang et al., 1995). This peptide has been identified in lower levels in the hippocampus and frontal cortex of alcohol-preferring rats compared to non-preferring (Ehlers et al., 1999). Given that both calcitonin and the calcitonin gene-related peptide derive from alternate splicing of the same gene in the brain (Rosenfeld et al., 1983), there might be a correlation between alcohol consumption and their expression, and even their function.

Of note, acute sCT prevented the development of ADE in rats deprived of alcohol for ten days and dose-dependently decreased the number of lever presses for alcohol self-administration in sP rats. This effect of sCT is of particular interest, as the aforementioned models represent different assets seen in clinical AUD (Spanagel, 2000).

Additional evidence of the importance of AMYRs in the modulation of alcohol's behavioural effects comes from our antagonist studies, where AC187 increased alcohol intake in rats. On a similar note, the orexigenic ghrelin increases, whereas receptor antagonism decreases alcohol intake in rats (Suchankova et al., 2013).

Tolerance effect caused by repeated AMYR activation

Repeated administration of sCT decreased voluntary alcohol intake in rats, however the intake retuned to baseline after the third sCT administration. Similar tolerance effects have been observed when sCT is infused over a long period of time, where it does not cause further food intake decrease after five days of administration (Chelikani et al., 2007). A similar tolerance pattern has been also noted in studies using repeated administration of GLP-1 analogues (Vallöf et al., 2016a).

This tolerance effect in our studies could be explained by downregulation of amylin or the calcitonin receptor, after sCT treatment, in brain areas associated with reward. Although repeated calcitonin exposure downregulates the CTR in osteoclasts both *in vitro* and *in vivo* (Samura et al., 2000; Wada et al., 1996), it remains unknown whether this is the case for brain receptors.

Implication of the central reward system

Our results that sCT crosses the blood-brain barrier and reaches the LDTg, VTA and NAc confirm our original hypothesis that peripheral sCT acts in the brain to regulate alcohol-mediated behaviours by binding on local AMYRs. AMYRs in the LDTg have been previously shown to regulate food intake (Reiner et al., 2017b), and our results show that sCT in this area decreases the acute behavioural effects of alcohol in mice and chronic behaviours in rats. Given that AMYRs are located on GABAergic neurons in the LDTg (Reiner et al., 2017b), a possible hypothesis involves increased GABAergic transmission in that area during amylinergic regulation of alcohol-mediated behaviours. However, the exact neurochemical mechanisms underlying these events are not yet known.

Similarly, our VTA results that local sCT injections decrease acute and chronic alcohol-induced behaviours in rodents, further support our initial hypothesis of the involvement of the dopaminergic system. The VTA is comprised of dopaminergic neurons, which after activation, increase dopamine transmission in the NAc (Brodie et al., 1998; Engel, 1977). Studies showing that peripheral sCT does not affect food intake in rats with knocked down CTRs in the VTA, while it does so in controls (Mietlicki-Baase et al., 2015b), strengthen the implication of VTA in the amylin signalling. Albeit not strictly related to the present studies, a general role of the dopamine system in the amylinergic pathways comes also from studies showing that inhibition of sexual behaviour after central amylin administration in rats is mediated through dopamine transmission (Clementi et al., 1999). Moreover, pharmacological antagonism of dopamine D2 receptors prior to amylin administration attenuates amylin's anorectic effect in food-deprived rats (Lutz et al., 2001c).

AMYRs have been identified on dopaminergic neurons in the VTA (Mietlicki-Baase et al., 2015b), a finding that implies stimulation of those neurons after AMYR activation. Our results, however, do not show increases in dopamine release *per se* (as shown by our microdialysis data) after AMYR activation, but rather indicate that receptor activation prior to alcohol, probably

blocks its ability to activate the downstream dopamine pathway. The relationship between AMYRs, alcohol and the dopamine system remains to be clarified. Moreover, the implication of AMYRs located on other neuronal types in the VTA cannot be disregarded given the heterogenous physiology of the area.

AMYRs have been also identified in the NAc shell (Baisley et al., 2014b; Sexton et al., 1994) and their activation decreases sucrose intake in *ad libitum* fed rats (Baisley et al., 2014a). Our previous findings that amylin receptors in the NAc are differentially expressed between low and high alcohol-consuming rats, can be linked to our behavioural findings that sCT decreases acute and chronic alcohol-mediated behaviours in rodents. All the above demonstrate that these three areas play an important role in the amylinergic regulation of alcohol's effects. Given that these areas are interconnected and part of the main reward system, their synergistic or compensatory actions need to be further elucidated; however, other areas or neuronal populations can be part of this signalling and need to be investigated.

The role of the reward system in the regulation of alcohol-mediated behaviours by gut-brain peptides is also identified in studies with ghrelin, GLP-1 and NMU. Local injections of these peptides regulate alcohol-mediated behaviours and these behaviours seem to be region-specific (Jerlhag et al., 2007; Vallöf et al., 2019; Vallöf et al., 2019).

Notably, we found no effects of AMYYR activation on peanut butter intake in mice, blood-alcohol concentration and corticosterone levels in mice. This is an indication that the amylinergic pathway, likely does not control alcohol-mediated behaviours through caloric and metabolic modulation or stress-related mechanisms. Amylin or sCT do not cause malaise or aversion (Mietlicki-Baase et al., 2013), excluding the possibility of aversive stimuli caused by AMYR activation *per se*. The above collectively suggest that, most probably, the amylinergic regulation of alcohol-induced behaviours falls under the scope of reward regulation and not under peripheral actions of the drugs administered.

Involvement of different neurochemical pathways

To further elucidate the effects of repeated sCT on the initial behavioural responses to acute alcohol, we found that repeated treatment decreased alcohol-induced locomotion in animals previously treated with sCT when the compound was not on board. This indicates that sCT probably affects some alcohol-induced behaviours in long-term and perhaps modulates AMYRs in such a way that these changes persist even when the drug is no longer present. In order to

understand whether these changes are traceable at the receptor level, we identified that these behavioural outcomes do not reflect changes in the CTR in the LDTg, VTA and NAc of the same mice. Amylin administration does not change mRNA levels of the CTRa, but affects the mRNA levels of the RAMPs in rats (Liberini et al., 2016). Therefore, the role of RAMPs in the expression of our behavioural results cannot be excluded.

Our biochemical results demonstrate that sCT administered for five days alters serotonergic and dopaminergic transmission in the VTA and NAc and noradrenergic transmission in the hippocampus. These results suggest that amylin signalling involves different neurotransmitters and brain areas. The locomotor stimulation behavioural data could be, at least partially, influenced by these changes in neurotransmission.

When sCT is co-administered with alcohol for five days, alcohol-induced locomotion in animals treated with sCT shows an interesting biphasic effect. The initial decrease in locomotor stimulation in the first three days, is shifted to an increase in locomotion on the fourth day. Markedly, similar results were presented at an earlier study, where repeated calcitonin administration initially decreased, but at later sessions increased voluntary alcohol intake in rats (Laitinen et al., 1992).

Sex-dependent effects of AMYR activation

Our primary studies included male rodents and had exclusively used sCT as an AMYR agonist, however sCT binds to the CTR and not solely to AMYRs (Young, 2005). Therefore, we tested AM1213, a selective and long-acting synthetic amylin analogue, on voluntary alcohol intake in both male and female rats. Repeated AM1213 decreased alcohol intake, similarly to sCT, in both sexes confirming the role of AMYRs in the regulation of alcohol consumption.

An interesting finding in these studies is the differential amylinergic regulation of alcohol intake between sexes. In male rats, alcohol consumption was initially decreased and increased at later sessions, while consumption in female rats returned to and remained at baseline during those later sessions. This effect in males confirms our previous results of a biphasic alcohol effect after AMYR activation, however, it provides a novel observation for the role of amylin regulation of alcohol in females.

Although the exact mechanism behind this discrepancy remains unknown, recent studies showed that female rats have higher levels of the amylin mRNA in the brain than males (Li et al., 2015), implying a variable molecular back-

ground between sexes in respect to amylin signalling. Our biochemical data could explain the discrepancies in alcohol consumption, since dopaminergic and serotonergic neurotransmission in reward-related areas essential for alcohol reward regulation (Di Chiara et al., 1986; Lovinger et al., 1998; McBride et al., 1990), is different between sexes and treatment periods (short- and long-term).

Different amylinergic modulation of alcohol-mediated behaviours and energy balance

Acute and repeated sCT administration reduced food intake and body weight in rats. An interesting finding in the repeated sCT studies is that food intake and body weight are decreased after administration, and the tolerance pattern noted in alcohol intake is not present. However, the food intake pattern after AM1213 administration follows the same tolerance pattern seen with alcohol intake. This is an essential indication that, most likely, the amylin signalling differentially affects alcohol-mediated behaviours and energy balance and that this may be regulated differently by the different compounds.

sCT did not affect palatable peanut butter intake in mice or the motivation to consume a palatable chocolate beverage in rats that had been pre-exposed to those diets. This shows that amylinergic regulation of food reward probably involves different pathways than that of alcohol reward or energy balance. Recent studies show that amylin decreases the consumption of a palatable chocolate diet in rats, but only when this rewarding food is relatively "novel" (three days of exposure); however, amylin does not affect consumption of this diet when the rats have been exposed to it for a longer period of time (three weeks of exposure) (Boyle et al., 2018). This could indicate that when the rewarding cue is strongly present, the amylinergic pathway bypasses the homeostatic regulation of energy balance and has more prominent effect on the rewarding stimulus, as the latter is a stronger cue. This could also explain our peanut butter and chocolate self-administration data, as the animals had already been exposed to the rewarding stimulus prior to sCT injections.

AM1213 had a robust effect on food intake and body weight on both male and female rats. In male rats, the effect on body weight was sustained during the washout period, when the compound was not on board. Other studies have shown that continuous amylin and leptin daily co-administration is necessary throughout the experimental setup in order to sustain body weight reduction in diet-induced obese rats (Trevaskis et al., 2010). Although these studies have a

more long-term design (4-8 weeks) than the ones presented herein, our results suggest that AM1213 has a long-lasting inhibitory effect on body weight, at least in male rats. The above support a distinct amylinergic regulation of alcohol consumption, food intake, and body weight and that this regulation also varies between sexes.

Concluding remarks

This thesis supports the hypothesis that amylin signaling decreases alcohol-mediated behaviours and that AMYR activation is essential for these effects. These outcomes appear to be regulated by altered neurotransmission in the reward system of the brain. Particularly, AMYR activation may attenuate the rewarding effects of alcohol by preventing alcohol's ability to stimulate dopamine neurons. Importantly, different amylinergic pathways may control alcohol-mediated behaviours and energy balance and these differences appear to be sex-dependent.

FUTURE DIRECTIONS

The involvement of the reward system in the amylinergic regulation of alcohol-mediated behaviours opens the way for the investigation of its role in other addictive substances. Studies on the effects of amylin analogues on the modulation of other drugs of abuse, such as cocaine and amphetamine, could be the next step in amylinergic research. Tentatively, the role of the amylin signalling in behavioural addictions could also be an interesting perspective.

This thesis has mainly focused on the behavioural outcomes of AMYR activation in respect to the effects of alcohol. Though interesting, the preliminary molecular results, need to be further investigated in more detail. A subsequent step would be the identification of AMYRs on neuronal populations implicated in reward processing, and the documentation of the neurotransmitter systems involved.

Moreover, the differences between sexes observed in our studies, set the grounds for additional investigation of all the behavioural paradigms in female rodents. Our results suggest that amylinergic regulation of alcohol consumption, food intake and body weight is substantially different between sexes. This can be proved advantageous in the development of personalised, sex-based AUD treatments.

An intriguing approach would be the investigation of the systems which act synergistically with amylin signalling, for example insulin and leptin. Amylin is co-secreted with insulin in order to control glucagon secretion (Scherbaum, 1998) and acts together with leptin, enhancing its body weight inhibitory effects (Trevaskis et al., 2010). Although one study has reported that mice pretreated with insulin show decreased alcohol-induced locomotion and behavioural sensitization (Kliethermes et al., 2011), the mechanisms of action of insulin in that setting still remain unknown.

Leptin has been studied more extensively in regards to alcohol-related behaviours in the clinical setting. Studies show that women who consume moderate amounts of alcohol have increased leptin serum levels (Roth et al., 2003), similar to abstaining alcohol-dependent women (Cardoso Fernandes Toffolo et al., 2012). Increased leptin levels are associated with withdrawal-induced alcohol craving (Jahn et al., 2001) and abstinence in patients with alcohol addiction (Kiefer et al., 2005). A possible interaction of the amylin signalling with the above-mentioned hormones could reveal more about the mechanisms of action by which amylin regulates alcohol-mediated behaviours.

An interesting perspective would be a combination treatment with AMYR agonists and other peptides that have been already investigated in regards to alcohol-mediated behaviours, for example GLP-1, NMU and ghrelin. Recent data reveal that sCT and liraglutide combination treatment causes prolonged weight loss in diet-induced obese rats (Liberini et al., 2019). The effects of such treatment combination on alcohol-mediated behaviours, could possibly overcome the tolerant effect of AMYR activation that has been noted so far.

It is still unknown whether the observed effects of sCT or AM1213 are attributed to AMYRs or CTRs. This is an intriguing question for further research and more selective AMYR and CTR agonists will shed more light on the receptor contribution to the observed effects. Ultimately, this could contribute to the identification of high specificity targets for the development of pharmacotherapies for AUD.

The identification and synthesis of GLP-1 analogues like liraglutide and dulaglutide has led to their application as treatments for diabetes and obesity (for review (Ryan et al., 2011; Thompson et al., 2015)). Amylin analogues, like pramlintide, are already approved for the treatment of diabetes (Edelman et al., 2008; Ratner et al., 2002) and sCT is used for the treatment of bone diseases (for review (Chesnut et al., 2008)). As more personalised treatments for AUD is a current necessity, the above approved agents should be applied in the clinical setting for their therapeutic efficacy on AUD and even SUD.

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