Thesis for the degree of Doctor of Medicine

REWARD-RELATED GENES AND ALCOHOL DEPENDENCE

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Cover: Schematic illustration showing a saggital section of the human brain, including the helix-shaped DNA molecule, the 28 amino acid peptide ghrelin, a nicotinic receptor subunit, as well as the chemical formula of ethanol.

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Abstract

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Introduction: The rewarding properties of alcohol are mediated by the brain reward systems, specifically by the cholinergic-dopaminergic reward link, involving both nicotinic acetylcholine receptors (nAChRs) as well as the ghrelin signalling system. The susceptibility for developing alcohol dependence is influenced by genetic factors. Therefore, the aim of this thesis is to investigate the genes encoding nAChRs as well as ghrelin and its receptor (GHS-R1A) in human genetic association studies of alcohol dependence. Furthermore, various aspects of ghrelin signalling have been investigated in rats with different alcohol preference. **Observations:** In the genetic association studies it was shown that; (1) nAChR gene variants influence alcohol consumption and body weight in alcohol-dependent individuals; (2) genetic variants of the ghrelin signalling system influence the risk of developing alcohol dependence, even though the effect size is small, and these variants might also affect body weight. The animal studies in this thesis showed that; (3) GHS-R1A antagonism reduces alcohol intake in a genetic rat model of high alcohol consumption; (4) GHS-R1A gene expression is higher in high alcohol consuming rats than in low alcohol consuming ones in reward-related brain areas; (5) alcohol counteracts the reduction of plasma ghrelin levels over time. **Conclusions:** The data presented in this thesis suggest that genetic variations of reward-related genes may be involved in the pathogenesis of alcohol dependence, although not as major susceptibility genes. Rather, they contribute to increased vulnerability in the reward systems that, in combination with environmental factors, may lead to dependence.

Keywords: alcohol – dependence – reward – smoking – body weight – gene – polymorphism – nAChR – ghrelin – GHS-R1A

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This thesis is based on the following research papers, which will be referred to in the text by their Roman numerals:

I. **Sara Landgren**, Jörgen A. Engel, Malin E. Andersson, Arturo Gonzalez-Quintela, Joaquin Campos, Staffan Nilsson, Henrik Zetterberg, Kaj Blennow, Elisabet Jerlhag. Association of nAChR gene haplotypes with heavy alcohol use and body mass. *Brain Research, 1305 Suppl: S72-9, 2009*

II. **Sara Landgren**, Elisabet Jerlhag, Henrik Zetterberg, Arturo Gonzàlez-Quintela, Joaquin Campos, Ulrica Olofsson, Staffan Nilsson, Kaj Blennow, Jörgen A. Engel. Association of pro-ghrelin and GHSR gene polymorphisms and haplotypes with heavy alcohol-use and body mass. *Alcoholism Clinical and Experimental Research, 32(12): 2054-61, 2008*

III. **Sara Landgren**, Elisabet Jerlhag, Jarmila Hallman, Lars Oreland, Lauren Lissner, Elisabeth Strandhagen, Dag S. Thelle, Henrik Zetterberg, Kaj Blennow, Jörgen A. Engel. Genetic variation of the ghrelin signalling system in severe female alcohol dependence. *In press, Alcoholism Clinical and Experimental Research, 2010*

IV. **Sara Landgren**, Jörgen A. Engel, Petri Hyytiä, Henrik Zetterberg, Kaj Blennow, Elisabet Jerlhag. Regulation of alcohol drinking by the ghrelin signalling system in rat lines selected for differential alcohol preference. *Submitted manuscript, 2010*

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LIST OF ABBREVIATIONS

5HT	serotonin (5-hydroxy-tryptamine)
$5HT_3$	serotonergic receptor 3 (protein)
5HTT	serotonin transporter (gene)
A	adenosine
AA	alko, alcohol rats
ACh	acetylcholine
ADH	alcohol dehydrogenase (gene)
ALDH	aldehyde dehydrogenase (gene)
ANA	alko, non-alcohol rats
ANKK1	ankyrin repeat and kinase domain containing 1 (gene)
BMI	body mass index (kg/m^2)
C	cytosine
cDNA	complementary deoxyribonucleicacid
CEU	central European descent, from Utah
CHB	Chinese Han, from Beijing
*CHRN**	nicotinic acetylcholine receptor (gene)
CI	confidence interval
CNS	central nervous system
CNV	copy number variation
COMT	catechol-O-methyl transferase (gene)
CpG	cytosine-phosphate-guanine
CPP	conditioned place preference
CRH	corticotrophin releasing hormone
DA	dopamine
ddNTP	dideoxynucleotide triphosphate
DNA	deoxyriobonucleic acid
dNTP	deoxynucleotide triphosphate
DRD2	dopamine receptor D_2 (gene)
DRD_2	dopamine receptor D_2 (protein)
EtOH	ethanol
G	guanine
GABA	γ-aminobutyric acid
GHRL	pro-ghrelin (gene)
GHSR	growth hormone secretagogue receptor (gene)
GHS-R1A and B	growth hormone secretagogue receptor 1A and 1B (proteins)
GWAS	genome-wide association study
HAD	high alcohol drinking rats
HapMap	the international haplotype mapping project
htSNP	haplotype tagging single nucleotide polymorphism
HUGO	human genome project
HWE	Hardy Weinberg equilibrium
ICD	international statistical classification of diseases and related health problems
JPT	Japanese, from Tokyo
LAD	low alcohol drinking rats

LD	linkage disequilibrium
LDTg	laterodorsal tegmental area
mAChR	muscarinic acetylcholine receptor (protein)
MAO_A and $_B$	monoaminoxidase A and B
MSP	methylation specific polymerase chain reaction
mRNA	messenger ribonucleic acid
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor (protein)
NMDA	N-methyl-D-aspartic acid
NP	alcohol non-preferring rats
OPRM1	opioid receptor μ_1 (gene)
OR	odds ratio
P	alcohol preferring rats
PCR	polymerase chain reaction
PFC	prefrontal cortex
qRT-PCR	quantitative real-time polymerase chain reaction
RT-PCR	reversed transcriptase polymerase chain reaction
RIA	radioimmunoassay
RNA	ribonucleic acid
siRNA	small interfering ribonucleic acid
sNP	Sardinian alcohol non-preferring rats
SNP	single nucleotide polymorphism
sP	Sardinian alcohol preferring rats
T	thymidine
TAD	TaqMan allelic discrimination
UChA	University of Chile low alcohol drinking rats
UChB	University of Chile high alcohol drinking rats
VTA	ventral tegmental area
YRI	Yoruba, from Nigeria

PREFACE

Since prehistoric times humans have produced, enjoyed and over-consumed alcoholic beverages. What is the driving force behind this behaviour and what is the mechanism for the transition from enjoyable drinking to dependence? During the 14th century, alcohol was seen as an efficient pharmacological agent for several diseases. Today alcohol is considered enjoyable, and moderate alcohol consumption is accepted in most western societies. The health effects and dangers associated with over consumption of alcohol are well-established. So why can't everybody maintain a healthy relationship with alcohol? Some people might say that it is a matter of character and moral, while, in the scientific community, the opinion has changed. As the mechanisms of action in the brain for alcohol and other drugs of abuse are being unravelled, alcohol dependence and other addictions are now considered as psychiatric conditions requiring treatment in the same way as any other disease.

Why is one individual capable of drinking alcohol with moderation, while another develops an addictive behaviour? As with any other trait or characteristic in our appearance or personality, some of the answers to this question can be found in our DNA. Even though we all carry the same amount of genetic material, small changes in our genetic code result in individual differences such as eye-colour, temperament or propensity for developing diseases. It is known that about 50% of the risk of developing alcohol dependence can be explained by genetic variation. It is however not known which genes or which genetic variants are responsible for this effect.

The aim of this thesis is to investigate the genetic basis for some of the effects of alcohol in the brain by studying both high and low alcohol consuming humans and rats.

INTRODUCTION

The brain reward systems

In the mid 50's, Olds and Milner (1954) serendipitously found that rats would work to self-administer electrical currents into some, but not into other brain regions. These brain areas were later anatomically mapped and found to mediate reward, pleasure and euphoria and are therefore called "the reward systems". These systems are well-conserved among species and have evolved as a means to increase the survival rate by stimulating and enhancing the motivation for natural behaviours such as breeding, and intake of food and water (Fisher *et al.*, 2002; Hansen *et al.*, 1991; Kelley and Berridge, 2002; Wise and Rompre, 1989). Further, humans as well as animals can learn to activate the reward systems artificially: Either with addictive drugs, such as alcohol and nicotine, or with addictive behaviours, such as compulsive shopping, compulsive gambling or compulsive overeating (Grant *et al.*, 2006; Holden, 2001). Such artificial stimulation is more powerful in activating the reward systems than natural rewards, and is hypothesized to hijack the reward systems (Wise and Rompre, 1989), leading to a loss of interest for natural rewards.

Mapping of the reward systems has identified several brain areas associated with reward, such as the prefrontal cortex (PFC), hippocampus, amygdala and nucleus accumbens (NAc) (Dahlström and Fuxe, 1964; Engel *et al.*, 1988; Koob, 1992; Ungerstedt, 1971). The core part of these reward systems is the mesolimbic dopamine (DA) system that consists of DAergic projections from the striatum (ventral tegmental area, VTA) to limbic (NAc and amygdala) and cortical areas (PFC) (Figure 1). Most drugs of abuse, such as alcohol (Di Chiara and Imperato, 1988; Engel and Carlsson, 1977; Imperato and Di Chiara, 1986), as well as natural rewards, such as food (Martel and Fantino, 1996) and sexual activity (Fisher *et al.*, 2002), increase the activity in these neurons, causing DA release in the NAc. Moreover, it has been shown that the mesolimbic DA system is important for increasing the incentive value for motivated behaviours, such as food and drug seeking (Wise, 2002).

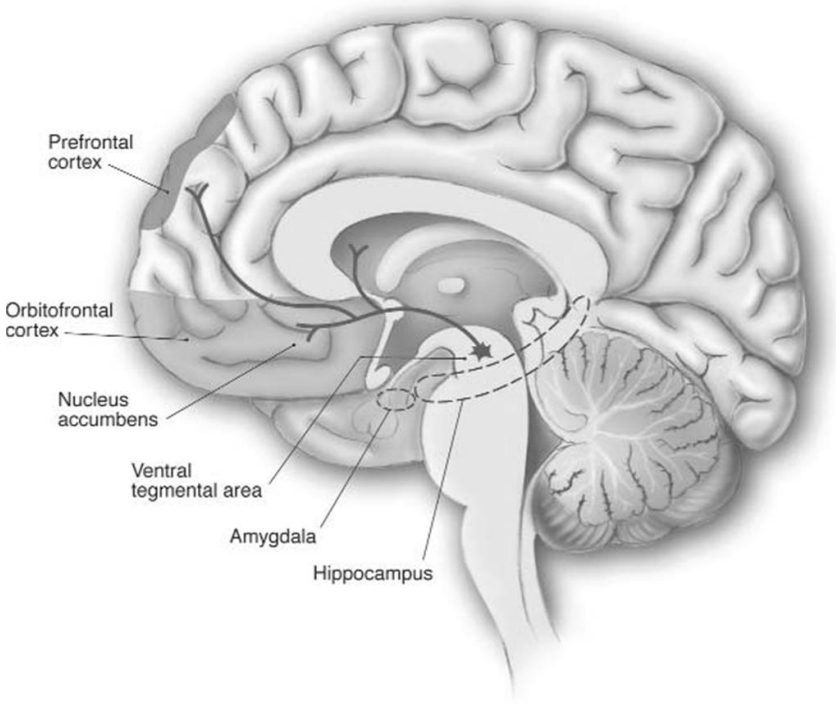

Figure 1. The mesocorticolimbic reward system. This system consists of dopaminergic projections from the striatum (ventral tegmental area, VTA) to limbic (nucleus accumbens, NAc; amygdala) and cortical (prefrontal cortex, PFC) areas (Holden, 2001).

Addiction

Addiction is a chronic relapsing brain disorder. It is characterized by a pathological, uncontrolled drug intake causing alterations in brain function and structure. Imaging studies of the human brain have shown that repeated use of dependence producing drugs as well as compulsive overeating results in changes that undermine voluntary control (Volkow and Li, 2004), and alterations in the reward systems (Volkow and Fowler, 2000). There is a difference in the concepts substance use, substance abuse and substance dependence. Substance use is a controlled drug intake for non-medical purposes, while substance abuse is a harmful, compulsive drug intake that is continued despite negative consequences for both mental and physical health. Continued drug use might lead to development of drug dependence, which is defined by the criteria used in the Diagnostic and Statistical Manual for mental disorders 4th edition (APA, 2004) (Table 1).

Table 1. Diagnostic criteria for substance dependence.

In the Diagnostic and Statistical Manual for mental disorders, substance dependence is defined as the occurrence of ≥3 of these criteria over a 12-month period (APA, 2004).

1 Tolerance, as defined by either of the following: (a) A need for markedly increased amounts of the substance to achieve intoxication or the desired effect, or (b) Markedly diminished effect with continued use of the same amount of the substance.

2 Withdrawal, as manifested by either of the following: (a) The characteristic withdrawal syndrome for the substance or (b) The same (or closely related) substance is taken to relieve or avoid withdrawal symptoms.

3 The substance is often taken in larger amounts or over a longer period than intended, *i.e.* loss of control.

4 There is a persistent desire or unsuccessful efforts to cut down or control substance use, *i.e.* craving.

5 A great deal of time is spent in activities necessary to obtain the substance, use the substance, or recover from its effects.

6 Important social, occupational, or recreational activities are given up or reduced because of substance use.

7 The substance use is continued despite knowledge of having a persistent physical or psychological problem that is likely to have been caused or exacerbated by the substance (for example, current cocaine use despite recognition of cocaine-induced depression or continued drinking despite recognition that an ulcer was made worse by alcohol consumption).

(APA, 2004)

Chemical and behavioural addictions

Besides drug addiction, the term "addictive behaviours" also includes eating disorders, such as binge eating, pathological gambling and sex addiction. Indeed, imaging studies have shown that the same brain circuits are activated, and dysfunctional in all these disorders (Grant *et al.*, 2006; Potenza *et al.*, 2003; Volkow and Li, 2004). As described above, both drugs of abuse and other addictive behaviours activate the brain reward system by DA release in the NAc, indicating that they share common neurobiological mechanisms (Grigson, 2002; Marks, 1990; Nestler, 2005). This implies that

pharmacological agents having beneficial effects on one disorder might be useful also for the others.

Mechanisms of addiction

There are different theories explaining the phenomena of dependence, *e.g.* the drug-centred and the individual-centred hypotheses. The drug-centred theory states that chronic drug-use causes molecular changes in the brain reward systems, and this in turn changes an individual's behaviour from a normal to a dependent state (Berke and Hyman, 2000; Deroche-Gamonet *et al.*, 2004; Nestler, 2001). The individual-centred theory claims that individuals prone to become dependent are born with an increased vulnerability in their reward systems such as a hypo- and/or hyper- DAergic function (Wolfe and Maisto, 2000).

The brain is a highly plastic organ, and there are apparent differences in the alcoholic brain compared to the brain of healthy individuals, *e.g.* enlargement of the ventricles (Ding *et al.*, 2004), and reduced brain weight, that is correlated with the level of alcohol consumption (Harding *et al.*, 1996), have been observed. On a molecular level, alcohol affects several neurotransmitters and receptors, such as monoamines, opioids and endocannabinoids. As an example, alcohol-dependent individuals are reported to have reduced DA receptor D_2 (DRD_2) receptor sensitivity in comparison with controls (Balldin *et al.*, 1992), which appears to be persistent after several years (7±6 years of sobriety)(Balldin *et al.*, 1993), as well as a reduced number of DRD_2 receptors (Volkow *et al.*, 2002). However, it remains to be clarified whether these changes are alcohol-induced or innate, since a vast number of processes are affected in the brain during prolonged alcohol exposure.

The neuroadaptive cycle of the addiction process can roughly be divided into three stages: Binge/intoxication, withdrawal/negative and preoccupation/ craving. Occasional drug-use is driven by impulsive actions largely associated with positive reinforcement mechanisms, *i.e.* the pleasurable feelings of alcohol lead to increased drinking. However, in addiction, impulsivity is to a large extent combined with compulsivity, associated with negative

reinforcement, *i.e.* an individual drinks to get relief from anxiety and stress during withdrawal. The neuroplasticity responsible for this transition involves various brain regions in all of the three stages of the addiction cycle. Today the research field of addiction is focused on identifying the genetic, epigenetic and molecular mechanisms mediating these changes, and thus finding the answer to why some individuals are more susceptible to developing an addiction. Both NAc and VTA are implicated in the binge/intoxication stage, as activation of the mesolimbic DA system is involved in the positive reinforcement of alcohol and other drugs of abuse. In the withdrawal/negative stage the amygdala is involved in mediating the stress and anxiety responses. The occupation/craving stage probably responsible for the high degree of relapse in addictive disorders, involves glutamatergic transmission from the PFC to other reward-related brain areas, but also hippocampus in the recognition of addictive cues (reviewed in Koob and Volkow, 2010). However, it should be noted that this is only one of the theories presented to describe the addiction process.

Alcohol dependence

Alcohol dependence is a disorder that causes great damage and suffering for the individual and their families as well as large costs to society (Garbutt *et al.*, 1999). Even though numbers vary considerably between studies, the yearly costs in Sweden attributed to alcohol dependence are estimated to between 20-100 billion SEK (CAN, 2009; Johnson, 2000), and in Sweden, around 5000-7000 deaths every year are alcohol-related (CAN, 2009). The prevalence for alcohol dependence in Western countries has been estimated to about 4-6% (Grant, 1997), which corresponds fairly well to the estimated prevalence in Sweden (Andreasson, 2002). Alcohol abuse and dependence are major health problems with about the same prevalence as depression or anxiety disorders (Hasin *et al.*, 2005), and therefore it is of great importance to find new treatment strategies for this disorder.

Drinking >20 g ethanol (EtOH)/day in women and >40 g EtOH/day in men causes liver damage, while drinking >70 g EtOH/day causes severe alcohol-related health problems (CAN, 2009). In clinical studies, the system of standard drinking units is often used to measure alcohol consumption.

One standard drink of alcohol contains about 10-12 g EtOH, and roughly corresponds to one small glass of wine (Gual *et al.*, 1999; Miller *et al.*, 1991). The limit for heavy alcohol consumption used in Papers I and II, is set to 280 g EtOH/week in men and 210 g EtOH/week in women. However, these measures all differ between countries.

Sub-grouping of alcohol-dependent individuals

Patients with alcohol dependence are often categorized into different groups, *i.e.* typologies, based on their disease progress, pattern of consumption, *etc.* since it is believed that different pathological mechanisms can cause dependence in different individuals. As alcohol dependence is such a multifaceted disorder, this sub-grouping could be advantageous when trying to individualise treatment strategies and finding new disease mechanisms. One such typology, based on heredity patterns, includes the type 1 and type 2 forms of alcohol dependence developed by Robert C. Cloninger in the early 80's (Cloninger *et al.*, 1981). Type 1 alcohol dependence develops during adulthood, and is thought to mainly rely on environmental factors, having low genetic predisposition. Type 2 alcohol dependence, on the other hand, develops during adolescence or early adulthood. This form is often accompanied with risk-taking, aggressive and criminal behaviour and is thought to have a high genetic predisposition, predominantly on the paternal side. Earlier, type 2 alcohol dependence was regarded as a predominantly male form, but has recently been shown to apply also for women (Hallman *et al.*, 2001; Traber *et al.*, 2009). Other typologies include the statistically developed type A and B forms (Babor and Caetano, 2006), and the more biologically correlated Lech's types 1-4 (Lesch *et al.*, 1990). These typologies, and other not equally used are reviewed by Leggio *et al.* (2009).

Sex differences in alcohol dependence

There are apparent differences between men and women in drug addiction in general, but also in alcohol dependence. Alcohol dependence is more prevalent in men than in women, even though the numbers are increasing at a higher rate in women. Females become addicted at a much lower alcohol

intake, and the progression from drug abuse to addiction is faster in women than in men (Diehl *et al.*, 2007; Mann *et al.*, 2005). The health consequences are more severe in women than in men, *i.e.* brain atrophy, liver and heart damage progresses more rapidly. Once dependent, women tend to find it more difficult to quit drinking than men do, and they have a higher risk of relapse (reviewed in Becker and Hu, 2008).

It is not known whether these sex differences reflect differences in neurobiology and in the vulnerability to drug abuse, or if they are a matter of dosage, since it has not been equally socially accepted for women to drink (Blume, 1990; Reed and Mowbray, 1999). However, data from animal studies suggests an actual neurobiological divergence between males and females (Devaud *et al.*, 2006). As an example, female rats have been shown to acquire drug self administration at a higher rate than male rats, they work harder for obtaining drug infusions and binge for longer periods of time. These differences are thought to, at least in part, be mediated by ovarian hormones (reviewed in Lynch *et al.*, 2002). However, to be able to better understand these neurobiological effects, female alcohol dependence needs to be further investigated.

Treatments

The available treatments for alcohol dependence are both psychosocial and pharmacological. Often, a combination of both is used (Garbutt *et al.*, 1999). The psychosocial treatments all focus on modifying maladaptive thoughts and behaviours related to alcohol. However, the form varies considerably *e.g.* concerning length, time and setting (groups or individual treatment) (Berglund *et al.*, 2003). The pharmacological treatments available today include the following drugs: Disulfiram, interfering with alcohol metabolism causing an unpleasant feeling even after low-doses of alcohol (Barth and Malcolm, 2010); acamprosate that is suggested to be a glutamatergic modulator (Cano-Cebrian *et al.*, 2003), possibly by interfering with glycine receptors (Chau *et al.*, 2010) even though the mechanism of action is not fully elucidated; and naltrexone, an opioid antagonist that affect the acute rewarding properties of alcohol, reducing alcohol-induced reinforcement (Pettinati *et al.*, 2006). As these treatments all have limited efficiency, other

pharmacological agents are under investigation. These include: DAergic agents, such as DRD_2 antagonists/partial agonists that block DA in NAc: γ-aminobutyric acid receptor B ($GABA_B$) agonists, such as baclofen, shown to reduce alcohol intake in mice; anticonvulsants facilitating $GABA_A$ receptor transmission and impeding glutamatergic transmission; the nicotinic acetylcholine receptor (nAChR) antagonist varenicline, blocking the rewarding properties of alcohol; as well as agents that interfere with the cannabinoid, corticotropin-releasing hormone (CRH) and serotonergic systems (reviewed in Garbutt, 2009; and in Kranzler, 2000). It should be noted that several other systems, not mentioned here, are also studied in relation to alcohol dependence.

Alcohol, nicotine and nicotinic acetylcholine receptors

Alcohol and nicotine dependence

Alcohol and nicotine are the most commonly abused drugs and there is a high degree of co-morbidity between alcohol and nicotine dependence (Falk *et al.*, 2006; Grucza and Bierut, 2006; Li *et al.*, 2007). This correlation may be due to a common vulnerability involving genetic and/or environmental factors pre-disposing an individual to both nicotine and alcohol abuse, (Grant, 1998), and in addition, to drug-addiction in general (Uhl *et al.*, 2009). Further, nicotine facilitates alcohol consumption and *vice versa* (Bien and Burge, 1990; DiFranza and Guerrera, 1990), and in alcohol dependence is 10 to 14 times more common among smokers than among non-smokers (Daeppen *et al.*, 2000; DiFranza and Guerrera, 1990). In older studies, approximately 90% of all alcoholics smoked (Ayers *et al.*, 1976; Batel *et al.*, 1995; Bien and Burge, 1990; Miller and Gold, 1998; Walton, 1972), while today these figures have decreased, being about 73% and 84% in the studies of this thesis (Papers I/II and III, respectively), which is still a much higher percentage than for the average population. Moreover, smoking alcohol-dependent patients use more cigarette/day than do other smokers (Dawson, 2000). Alcohol is known to potentiate the rewarding effects of nicotine, including smoking satisfaction, relief of craving for cigarettes, stimulating as well as calming effects (Rose *et al.*, 2004). Early onset smoking is strongly correlated with alcohol abuse and dependence later in life (Grant, 1998).

Furthermore, nicotine-use during pregnancy may be particularly related to problems with substance abuse in the next generation (Brennan *et al.*, 2002). In summary, these data suggest that alcohol and nicotine may share neurochemical mechanisms of action in the brain reward systems such as those that involve nAChRs (Larsson and Engel, 2004).

Alcohol, the cholinergic-dopaminergic reward link and nAChR subtypes

Even though the mechanism of action of alcohol is not fully elucidated, it is known to interact with ligand-gated ion channel receptors in the brain, including serotonergic receptor 3 ($5HT_3$), $GABA_A$, N-methyl-D-aspartic acid (NMDA) receptors (Grant, 1994), and nAChRs (Lovinger, 1997; Narahashi *et al.*, 1999). These receptors are all involved in mediating the effects of alcohol (Engel *et al.*, 1992; Engel and Liljequist, 1983; Larsson and Engel, 2004). Given the above mentioned relationship between alcohol and nicotine consumption, the nAChR could serve as one possible common denominator for alcohol-nicotine interactions.

The nAChRs consists of five subunits that form an ion channel. The subunits expressed in the central nervous system (CNS) are the α_2-α_{10} and β_2-β_4 (Lukas *et al.*, 1999). Various combinations of the subunits can form a large variety of nAChRs, *i.e.* subtypes. The subtypes have diverse distribution patterns within the brain and are characterized by significant differences in properties such as ligand pharmacology and activation (Chavez-Noriega *et al.*, 1997). Moreover, divergent functional roles may in all probability be allocated to these subtypes (for review see Nicke *et al.*, 2004).

Electrophysiological studies have suggested that alcohol acts as a co-agonist to acetylcholine (ACh) on nAChRs (Forman *et al.*, 1989; Forman and Zhou, 1999; Narahashi *et al.*, 1999; Wu and Miller, 1994; Wu *et al.*, 1994). This effect is dependent on the α-subunit (Zhou *et al.*, 2000), implying that alcohol may interact directly with nAChRs, on DAergic cell bodies in the VTA. The cholinergic input to the VTA, originates primarily in the laterodorsal tegmental area (LDTg) (Blaha *et al.*, 1996). The LDTg has been suggested to regulate the activity of the ventral tegmental DA neurons that project to the ventral striatum (*i.e.* accumbal DA) (Forster and Blaha, 2000,

2003), via activation of nAChR, muscarinic acetylcholine receptors (mAChR) as well as glutamatergic receptors in the VTA (Forster and Blaha, 2000; Forster *et al.*, 2002). Similarly, activation of nAChRs in the VTA, increases accumbal DA release (Nisell *et al.*, 1994). Hence, these cholinergic afferents to the VTA, mainly originating in LDTg, appear to be an important part of the brain reward systems (Beninato and Spencer, 1987; Larsson *et al.*, 2005; Rada *et al.*, 2000). Together with the mesolimbic DA system this network has been named the "cholinergic-DAergic reward link", important for mediating the rewarding feelings of both natural rewards, such as food, and of drugs, such as alcohol (Jerlhag *et al.*, 2006a; Jerlhag *et al.*, 2006b; Larsson and Engel, 2004; Larsson *et al.*, 2004) (Figure 2).

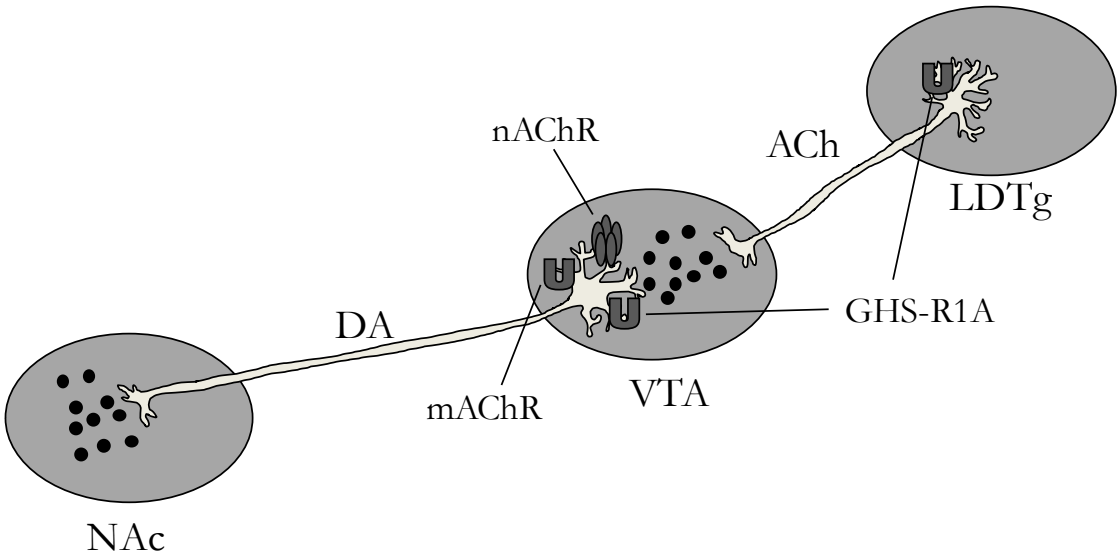

Figure 2. The cholinergic-dopaminergic reward link. This link is composed of the cholinergic projection from the laterodorsal tegmental area (LDTg) to the ventral tegmental area (VTA) and the mesolimbic dopamine (DA) system projecting from the VTA to the nucleus accumbens (NAc). Activation of the LDTg causes a release of acetylcholine (ACh) in the VTA which by interactions with nicotinic ACh receptors (nAChR) and/or muscarinic ACh receptors (mAChR) stimulate the mesolimbic DA system causing a release of DA in NAc. These areas also contains ghrelin receptors, *i.e.* growth hormone secretagogue receptors (GHS-R1A).

Chronic alcohol administration increases the number of nAChRs in reward-related brain areas (Yoshida *et al.*, 1982) and high alcohol preferring rats drinking alcohol display an enhanced ACh release in the VTA concomitantly, and almost time-locked, with accumbal DA overflow (Larsson *et al.*, 2005). Interestingly, the rats' alcohol intake was positively correlated to the increase in VTA-ACh, suggesting that alcohol activates cholinergic afferents to the VTA (Larsson *et al.*, 2005). Further, it has been demonstrated that the locomotor stimulatory, rewarding and DA enhancing effects of alcohol are mediated by nAChRs in the VTA (Blomqvist *et al.*, 1997; Ericson *et al.*, 1998; Jerlhag *et al.*, 2006b; Larsson *et al.*, 2004; Larsson *et al.*, 2002), thus indicating that alcohol activates the reward link.

More specifically, an activation of central (in the VTA) nAChRs, specifically the $\alpha_3\beta_2$*, β_3* and α_6* subtypes, mediate the locomotor stimulatory, DA-enhancing, rewarding and anticipatory effects of alcohol (Kuzmin *et al.*, 2008; Larsson and Engel, 2004; Larsson *et al.*, 2002; Löf *et al.*, 2007). Moreover, varenicline, a partial $\alpha_4\beta_2$ (Rollema *et al.*, 2007), and weak $\alpha_6\beta_2\beta_2$ antagonist (Mihalak *et al.*, 2006), decreases alcohol consumption and seeking (Steensland *et al.*, 2007), as well as attenuates alcohol and nicotine interactions in rats (Ericson *et al.*, 2009). Furthermore, varenicline reduces alcohol self-administration in smoking heavy drinking humans, in a laboratory setting (McKee *et al.*, 2009). Taken together, alcohol may, via activation of the cholinergic input to the VTA cause ACh release, and thereby, via $\alpha_3\beta_2$*, β_3* and α_6* containing nAChRs, excite the mesolimbic DA system.

Ghrelin, the brain reward systems and alcohol

The reward systems mediate the motivation for food intake and the feeling of pleasure after a good meal (*vide supra*). Further, the reward systems have been implicated in addictive behaviours such as compulsive overeating (Knutson *et al.*, 2001; Potenza *et al.*, 2003). Interestingly, growing evidence on common mechanisms involved in alcohol and food seeking behaviour has been found (Holderness *et al.*, 1994; Welch and Fairburn, 1998; Wolfe and Maisto, 2000; Volkow and Wise, 2005), and there is a co-morbidity between eating disorders and drug or alcohol abuse (Wolfe and Maisto, 2000). Not so surprisingly, that there appears to be a neurochemical overlap between the

hedonic reward systems and systems regulating energy balance (DiLeone *et al.*, 2003; Thiele *et al.*, 2003; Thiele *et al.*, 2004). The mechanisms of this overlap are now being unravelled, especially implicating peptides involved in regulating energy balance.

Ghrelin was discovered in 1999, isolated from stomach as the first endogenous ligand to the growth hormone secretagogue receptor (GHS-R1A), an orphan receptor at that time. It is a 28 amino acid peptide that depends on an octanylation at the third amino acid serine for its activity (reviewed in Kojima, 2008). Since this discovery, ghrelin has emerged as an important gut-brain signal for the control of food intake, energy balance and body weight homeostasis (Tschöp *et al.*, 2000; Wren *et al.*, 2000) as well as hunger, appetite and meal initiation (Cummings, 2006; Cummings *et al.*, 2001), by mechanisms that include direct actions in the brain (Nakazato *et al.*, 2001; Tang-Christensen *et al.*, 2004). As stated above, ghrelin acts on the GHS-R1A (Howard *et al.*, 1996; Kojima *et al.*, 1999). Besides being expressed in the hypothalamus, regulating food intake, this receptor is present in the hippocampus as well as in mesolimbic structures (Guan *et al.*, 1997) (Figure 2), implying that ghrelin's central actions extend beyond energy homeostasis, possibly also including the effects of drugs of abuse (Cummings *et al.*, 2007; Jerlhag *et al.*, 2009).

Ghrelin, reward and alcohol

Ghrelin has been shown to activate the cholinergic DAergic reward link (Jerlhag *et al.*, 2006a; Jerlhag *et al.*, 2007). It may therefore be suggested that ghrelin has incentive value for motivated behaviours such as food and drug seeking. Local administration of ghrelin into VTA or LDTg has locomotor stimulatory and DA enhancing properties; thus indicating that ghrelin, via GHS-R1A in VTA and in LDTg, activates the cholinergic-DAergic reward link similar to alcohol (Abizaid *et al.*, 2006; Jerlhag *et al.*, 2007). The unselective nAChR antagonist mecamylamine blocks these behavioural and neurochemical effects of ghrelin. Furthermore, the locomotor stimulatory and DA-enhancing effects of ghrelin (into the VTA or LDTg) are mediated via the $\alpha_3\beta_2$*, α_6* and β_3*, rather than $\alpha_4\beta_2$*, α_7*, subtypes in the VTA, indicating further analogies between ghrelin and alcohol (Jerlhag *et al.*, 2008).

As ghrelin is mainly produced in peripheral tissues, it is also important to note that ghrelin administered peripherally also activates the brain reward systems, as indicated by increasing locomotor activity and accumbal DA, as well as inducing a conditioned place preference (CPP). This suggests that the observed pre-prandial rise in plasma ghrelin might increase the incentive value of motivated behaviours such as food seeking (Jerlhag, 2008). Accordingly, ghrelin increases the intake of rewarding food in rodents (Egecioglu *et al.*, 2010).

Co-morbidity between alcohol dependence and eating disorders is well-known, and it has been implied that ghrelin has a role also in drug-seeking behaviour (Volkow *et al.*, 2002). The plasma levels of ghrelin are positively associated with the craving of alcohol-dependent patients (Addolorato *et al.*, 2006), preferably in subgroups of patients (*e.g.* Lesch's type one) (Hillemacher *et al.*, 2007). Additionally, the plasma levels of ghrelin are elevated in alcohol-dependent patients as well as in smokers in some studies (Bouros *et al.*, 2006; Kim *et al.*, 2005; Kokkinos *et al.*, 2007; Kraus *et al.*, 2005), while others have shown an acute reduction in plasma ghrelin after alcohol ingestion (Zimmermann *et al.*, 2007) and after smoking cessation (Lee *et al.*, 2006). Further, an association between elevated plasma levels of ghrelin and cocaine-seeking behaviours in rats as well as locomotor stimulation and CPP for cocaine has been established (Tessari *et al.*, 2007; Wellman *et al.*, 2005; Wellman *et al.*, 2008). The plasma levels are also altered in several eating disorders (Monteleone *et al.*, 2005; Monteleone *et al.*, 2003; Tanaka *et al.*, 2002; Tanaka *et al.*, 2003). Given that hyperghrelinemia is associated with certain forms of compulsive overeating (Chanoine, 2005; Couce *et al.*, 2006) and also with alcohol dependence (Kim *et al.*, 2005; Kraus *et al.*, 2005) it may be hypothesized that the ghrelin signalling system is involved in the pathophysiology of these conditions.

In support of this contention, intracerebroventricular administration of ghrelin increases alcohol intake in mice (Jerlhag *et al.*, 2009). Moreover, alcohol-induced locomotor stimulation, accumbal DA release and CPP are consistently abolished in models of suppressed central ghrelin signalling, *i.e.* in GHS-R1A knockout mice and mice treated with two different GHS-R1A antagonists (Jerlhag *et al.*, 2009), showing that central ghrelin signalling is required for the rewarding properties of alcohol. Basically, the same pattern

can be seen in ghrelin knockout mice, even though the effects are more prominent in the GHS-R1A knockouts (unpublished data). This raises important questions regarding the physiological role of ghrelin, that not only influences hunger but clearly also has a broader role in the search for rewarding substances such as alcohol and possibly in other addictive behaviours, indicating that ghrelin receptor antagonists could be an efficient treatment strategy for addictive behaviours.

Molecular genetics

The genetic code

Almost every single cell in our body carries the blueprints to the human being, *i.e.* our genome. The human genome is divided on 23 entities, called chromosomes, of which we have two copies. One half of each chromosome pair is inherited from the mother and the other half from the father. The chromosomes consist of deoxyribonucleic acid (DNA), composed of 4 different nucleotide bases; adenine (A), cytosine (C), guanine (G) and thymine (T) assembled in a certain order that constitutes the genetic code. This code is based on triplets of nucleotides called codons, each representing one amino acid.

The unique double helix structure of DNA and the basis of the genetic code were discovered in the 50's by Watson and Crick (1953). They found that A only binds to T and G only binds to C, which makes the two strands of DNA in the double helix perfectly complementary to each other, allowing the DNA to replicate itself using one of the strands as a template. This phenomenon is also taken advantage of in several of the molecular genetic techniques used in this thesis.

The human genome can be divided into segments called genes, each containing information on how to produce proteins. The number of human genes was first estimated to about 100 000, while that number was reduced to about 35 000 when the Human Genome project (HUGO) was finished. Recent estimates are considerably lower, about 20 000 (Pennisi, 2003). At the same time the concept of a gene as a single protein coding element has been challenged, as the number of splice variants of proteins increases, genes

within genes are discovered, and the importance of non-coding ribonucleic acid (RNA) is unravelled (Brosius, 2009). A gene, in its classic form comprises a range of elements each with a different function; the regulatory promoter region, the amino acid coding exons, and the non-coding introns. When a protein is being produced, the gene is first activated on the DNA level, and transcribed into single stranded RNA. In this process all non-coding regions are cleaved off resulting in a sequence called messenger RNA (mRNA), which in turn is used as a template translated into a protein (Figure 3).

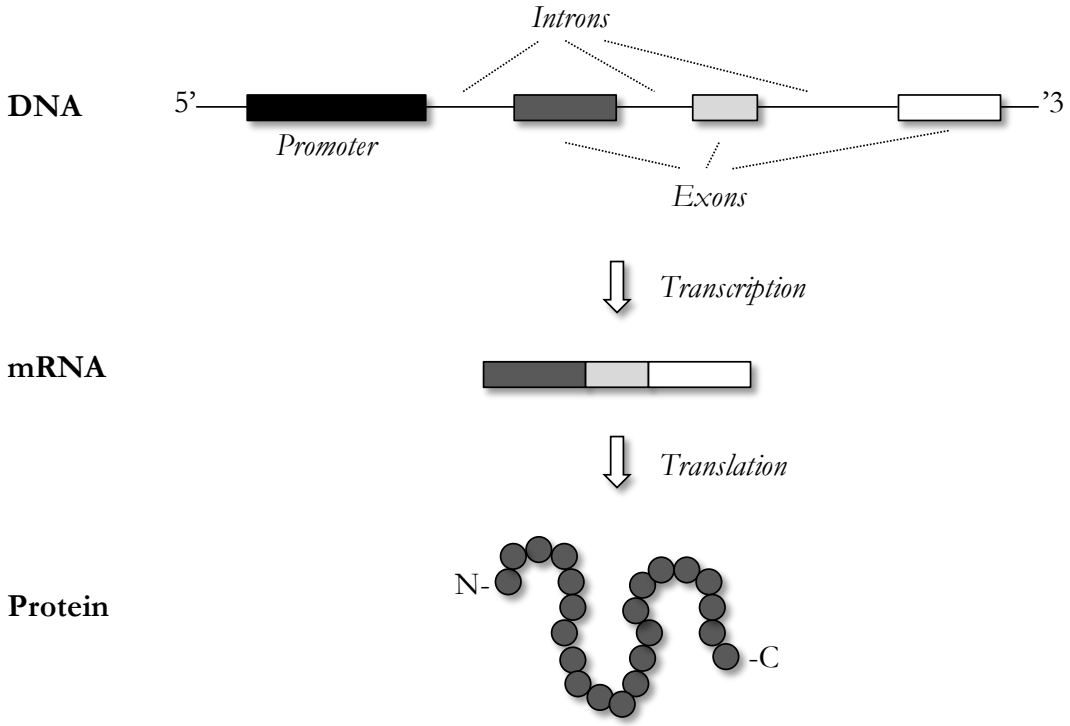

Figure 3. From DNA to protein: Overview of a gene and its structural components, as well as the transcription / translation process.

Genetic variation

The genetic code is very similar in all humans, even though variations in the genetic code are what make us unique as individuals. The most common form of genetic variation is single nucleotide polymorphisms (SNPs), *i.e.* at

one single position in the genome there are two (or in rare cases more) possible nucleotides present in different individuals, occurring in at least 1% of the population. The possible variants of a SNP are called alleles, and the two alleles an individual carries are called genotypes, *i.e.* for a SNP the alleles could be A and G and the possible genotypes would be AA, GG, and AG. Having the same allele on both chromosomes (AA or GG) means that you are homozygous, while having different alleles (AG) means that you are heterozygous for this SNP. SNPs are found once every 100-300 base in the genome. This nucleotide switch can either result in codon changes representing a new amino acid, or alterations in the binding capacity of DNA binding factors, such as transcription factors that regulate the activity of the gene. SNPs are the form of genetic variation studied in this thesis.

Other genetic variations in our genome that are also involved in susceptibility to diseases include; repeat polymorphisms where a segment of DNA is repeated in various numbers, insertions and deletions of smaller DNA regions, and copy number variations (CNVs) where large regions of DNA have been inserted or deleted.

Epigenetics

Epigenetics is defined as modifications of the DNA affecting gene expression without involving the actual DNA sequence itself. Epigenetic modifications can be inherited, but they are also affected by environmental stimuli such as stress, nutrition, *etc.* Hence, epigenetic mechanisms are sometimes referred to as the link between the genes and the environment. There are various forms of epigenetic modifications; both histones and DNA can be modified by, for example methylation or acetylation.

DNA methylation occurs at certain positions in the DNA called CpG sites, *i.e.* when a C is followed by a G in the genomic sequence, a methyl group can be attached to that C. When the number of CpGs in a region of DNA exceeds 50% of all Cs, this region is called a CpG island. CpG islands often occur in promoter regions of genes, and are yet another kind of gene regulation. When a gene is highly methylated, the gene expression of that gene is decreased. The exact mechanism for this reduction in gene

expression is not fully understood, but may, in all probability, depend on the methyl groups being a sterical hinder for transcription factor binding.

DNA methylation is thought to be site-specific, involving transcription factor binding of individual genes (Comb and Goodman, 1990), but it also involves global mechanisms that induce general promoter-methylation, silencing gene transcription depending on the number of CpG sites in a certain gene (Nan *et al.*, 1997). These mechanisms are involved in several psychiatric disorders including autism (Jones *et al.*, 2008), depression (McGowan and Kato, 2008) and schizophrenia (Gavin and Sharma, 2009), as well as in addictive behaviours, such as eating disorders (Frieling *et al.*, 2009) and alcohol dependence (Shukla *et al.*, 2008).

Genetic association studies

When it comes to genetic association studies of complex diseases, studying SNPs have many advantages over other sorts of genetic variations. Some of the advantages are the high frequency of the SNPs in the genome, how easily they are genotyped, the fact that groups of SNPs may have alleles that show distinctive inherited patterns, *etc.*, *vide infra* (Collins *et al.*, 1998; Schork *et al.*, 2000).

It may seem irrelevant that such small changes of the DNA as a single base exchange would have an impact on the individual, but actually rare inherited monogenetic diseases, such as cystic fibrosis, are caused by one SNP only. This is however not the case for common diseases with a more complex genetic as well as environmental origin such as diabetes, Alzheimer's disease or addiction. Rather a large number of polymorphisms in several different genes are thought to contribute to susceptibility of complex diseases that do not follow classic Mendelian inheritance. The same genotype may result in different phenotypes or different genotypes can result in the same phenotype. Thus the genotype at a given locus may affect the probability of disease, but not fully determine the outcome (Lander and Schork, 1994; Schork, 1997).

In a genetic association study, the allele and genotype frequencies of a genetic marker, such as a SNP, are compared between individuals who have

or do not have a certain disease or trait. If the frequency is significantly higher in one of these groups, this genetic marker is said to be associated with that disease or trait. Often, when one or more SNPs have been associated with a disease, the gene where these variants are located is called a risk gene or a susceptibility gene.

The genetic association studies of the 21st century have evolved considerably, not only due to the finishing of the HUGO project and the following Haplotype Mapping project (HapMap), but also due to the vast improvement of genotyping techniques, allowing massive parallel genotyping. These improvements lead to the development of SNP microarray chips covering the genetic variation of the entire genome (Gunderson *et al.*, 2005; Lockhart *et al.*, 1996). The use of SNP microarrays has made genome wide association studies (GWAS) possible.

Linkage disequilibrium and haplotypes

Previously, the effect of single SNPs on disease traits were studied in genetic association studies. However, today usually several SNPs in a gene are used. Such a sequence of SNP alleles, located on the same parental chromosome, is called a haplotype. When the whole HUGO project was finished and the sequence data were analysed for individual differences, it was found that some regions of DNA have been conserved during evolution *i.e.* have not been broken up by recombination. This means that, within such a region, SNPs are inherited together. This correlation between two SNPs is known as linkage disequilibrium (LD). An LD of 1 means 100% probability that two SNPs are inherited together (Lawrence *et al.*, 2005). High LD also means that the haplotype frequency does not equal the combined frequency of the individual alleles. This has also resulted in the fact that all possible haplotype combinations are not present in a population, but rather a few combinations exist in rather high frequency.

The International HapMap project is an extension of HUGO that aims at creating a catalogue of human common genetic variants. It describes these variants and how they are distributed in populations of different origin. Genetic data have been collected from four different populations with

African (Nigeria, YRI), Asian (Japan, JPT and China, CHB), and European (CEU) ancestry (The International HapMap Project, 2003; Thorisson *et al.*, 2005). These data are freely available and can be used in haplotype studies.

When designing a haplotype study, the LD pattern of SNPs is used. Instead of genotyping all SNPs of a gene in all individuals of a study, a small representative subset of so-called haplotype tagging SNPs (htSNPs) that are in perfect, or nearly perfect, LD with other SNPs in the haplotype block can be used. These htSNPs, covering the whole variation of a gene or region, are then used for genotyping and haplotype reconstruction in genetic association studies of complex diseases (The International HapMap Project, 2003; Gabriel *et al.*, 2002).

The strength of a haplotype study compared to a single SNP study is that the entire genetic variation of the gene is studied as a larger segment of the chromosome is covered. The probability of finding an association increases and, if complex genetic disorders are studied, it is more likely that a combination of SNPs is responsible for the phenotype than one single SNP alone. However, to perform haplotype analyses a larger study population than for single SNP analyses is needed (Johnson *et al.*, 2001).

Genetics of alcohol dependence

Genetic association studies can be useful when exploring and understanding the aetiology of drug abuse and dependence (Tyndale, 2003). In the future, such genetic markers could perhaps be used for identifying risk individuals, find new targets for pharmacological treatment and perhaps more individualized treatment strategies. As an example, the Asn40Asp polymorphism in the opioid receptor μ_1 gene (*OPRM1*) has been suggested to be predictive of naltrexone treatment outcome (Oroszi *et al.*, 2009). A family history of alcohol dependence greatly increases the risk of developing alcohol dependence compared with the offspring of non-alcoholics. When summarizing the results from several twin and adoption studies, the heritability of alcohol dependence is over 50%, and the concordance in monozygotic twins is significantly higher than for dizygotic twins. As there is

no classic pattern of inheritance in alcohol dependence, it is regarded as a complex disorder (Köhnke, 2008).

Despite the large genetic component of alcohol dependence, few major susceptibility genes have been identified. The two major focus areas have been on genes that affect DAergic transmission as well as on alcohol metabolizing enzymes. The *Taq*A1 polymorphism in the *DRD2* gene is probably the most studied genetic marker for alcohol dependence. However, results are inconsistent (reviewed in Dick and Foroud, 2003; and in Noble, 2003). Some of this discrepancy might be attributed to sample size, but also to the fact that this marker is situated in the gene neighbouring *DRD2*, *i.e.* in the ankyrin repeat and kinase domain containing 1 *(ANKK1)* gene. Other genetic studies on alcohol dependence have focused on known functional variants in the genes that encode the alcohol metabolizing enzymes, alcohol dehydrogenasee (*ADH*) and aldehyde dehydrogenase (*ALDH*). These variants alter the enzymatic activity of these enzymes resulting in alterations in alcohol consumption, specifically in Asian populations (Crabb *et al.*, 2004). Other association studies on alcohol dependence include studies of the known functional monoamine oxidase *(MAO_A* and *MAO_B)* gene variants; of the catechol-O-methyltransferase gene *(COMT)* Val158Met SNP; of the serotonin transporter *(5HTT)* long and short variants; and of genes in the GABAergic and noradrenergic systems (Dick and Foroud, 2003; Köhnke, 2008; Tyndale, 2003). These genes, as well as others found associated with alcohol dependence in association studies are summarized in Figure 4. From these studies, it is obvious that alcohol dependence is a complex multigenetic disorder.

In alcohol dependence there have not been as many successful GWAS as for other complex diseases such as diabetes type 1 and 2 (Cooper *et al.*, 2008; Zeggini *et al.*, 2008). However, three studies on alcohol dependence have been conducted including: One study using pooled genotype data (Johnson *et al.*, 2006); an Australian/Dutch GWAS of alcohol and nicotine dependence (Lind *et al.*, 2010); and a German study of alcohol dependence (Treutlein *et al.*, 2009). Interestingly, the findings from these studies show disease associated loci in genes primary related to cell adhesion, intracellular signalling and alcohol metabolism (*ALDH*) and not with the DAergic or

GABAergic systems, which raises more questions regarding the genetic basis of alcohol dependence than it answers.

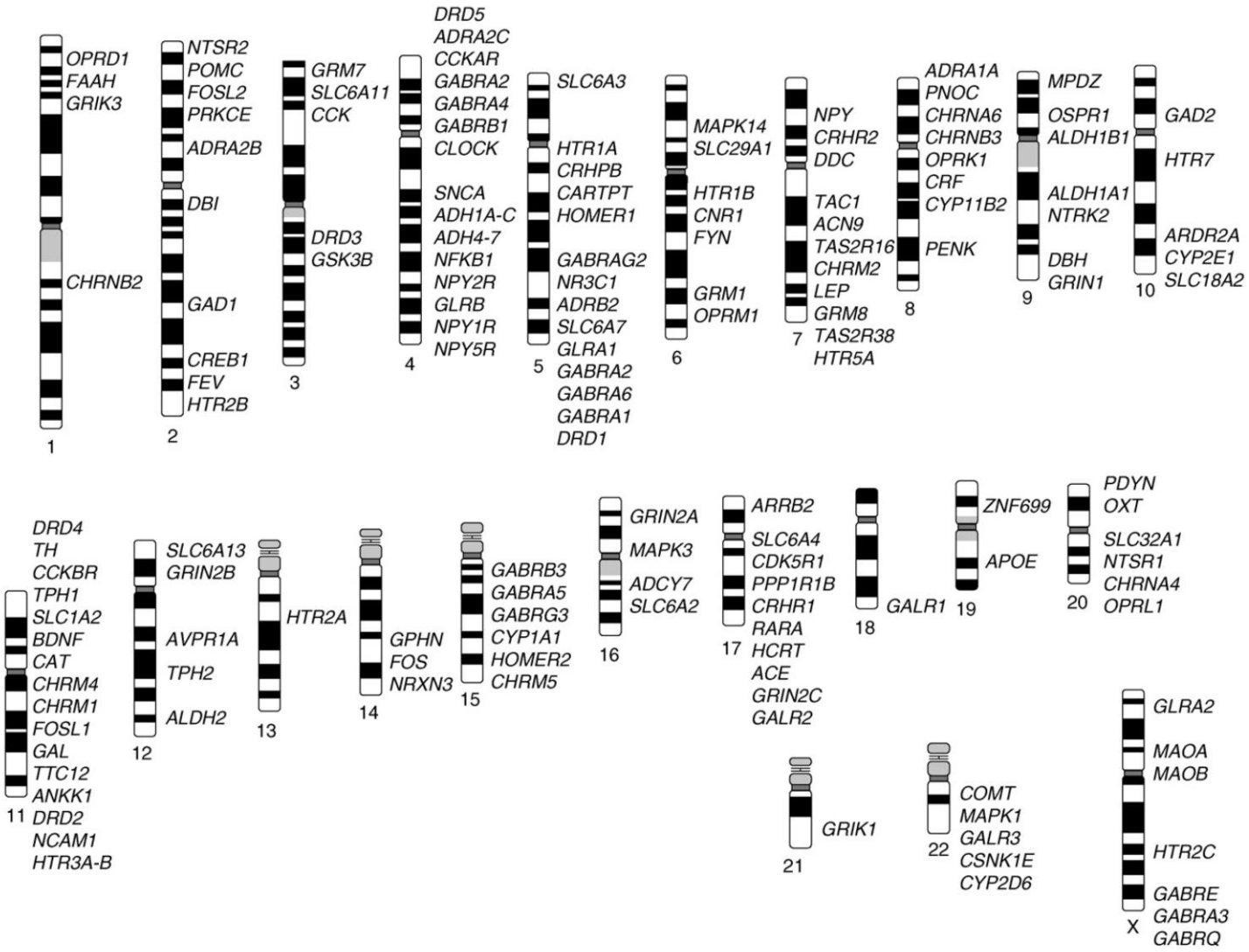

Figure 4. Map of the human chromosomes and of gene loci associated with alcohol dependence in association studies. (Kalsi *et al.*, 2009)

Genetic tools in animal studies

Today, there are a vast number of molecular genetic techniques used to alter the genes of an organism. When studying the function of a certain gene or protein, it is possible to eliminate this gene totally or partly in knock-out

models or to block the transcription of a gene for a certain amount of time using small interfering RNA (siRNA). Further, it is also possible to over-express a gene by inserting a second copy of a gene into the DNA, then identifying the phenotypes produced. However, this has not always been the case. Before the dawn of modern molecular genetic techniques, researchers had other ways of studying the genetics of a certain trait. By selectively breeding animals for a certain phenotype or for lack of a certain phenotype, *i.e.* bidirectional selection, specific lines of animals were obtained. The utility of using such animal lines is based on the theory of genetic selection pressure, which has gradually enriched alleles in genetic loci that promotes, for example high or low alcohol drinking. Further, it is hypothesized that these animals would also enrich other phenotypic traits associated with the selected trait, by pleiotrophic interactions, *i.e.* one gene influences multiple phenotypes. In the field of addiction, several such animal lines with different alcohol preferences have been constructed using this method.

The AA (Alko, Alcohol) and ANA (Alko, Non-Alcohol) rats: The AA and ANA rats were among the first lines to be developed for the purpose of studying alcohol intake and preference, by subjecting them to the two-bottle free choice drinking of 10% alcohol solution and water. These lines are of Wistar and Sprague Dawley origin, later also mixed by Lewis and Brown Norwegian rats. Despite the fact that AA rats drink up to 10 times more alcohol than ANA rats, AA rats show signs of behavioural disinhibition while ANA rats are more anxiety prone (Kiianmaa *et al.*, 1991). ANA rats are also more sensitive to stress than AA rats (Sandbak *et al.*, 1998). Interestingly, differences in AA and ANA rats in the behavioural model of the concentric field test have been observed in AA and ANA rats. ANA rats show more shelter seeking and anxiety-like behaviour than AA rats, which may be a result of the stress produced in this model (Roman *et al.*, 2007).

Several neurotransmitter systems have also been investigated in these rats, including the DAergic and noradrenergic systems. However, differences between AA and ANA rats have only clearly been observed in the endogenous opioid as well as in the endocannabinoid systems showing an up-regulation in AA compared to ANA rats (reviewed in Kiianmaa *et al.*, 1991; Sinclair *et al.*, 1989; Sommer *et al.*, 2006).

Other similar rat lines include the alcohol-preferring P and alcohol-non-preferring NP lines of rats developed by mass selection from a Wistar foundation stock (Bell *et al.*, 2006); the Sardinian alcohol preferring sP and non-preferring sNP rats (Colombo *et al.*, 2000); the alcohol preferring UChA and non-preferring UChB strains exhibiting differences in alcohol metabolism (Mardones and Segovia-Riquelme, 1983); as well as the high alcohol drinking (HAD) and low alcohol drinking (LAD) rat lines (Murphy *et al.*, 2002).

Even though these rat lines, having been bred for the same purpose, all display high and low alcohol drinking, they differ with regards to several other neurochemical and behavioural aspects. This is beneficial in the sense that they all model different aspects of alcohol dependence. Especially since alcohol dependence is a heterogeneous disorder caused by multiple pathogenic mechanisms.

AIMS

The overall aim of the present thesis is:

to investigate the signalling systems involved in the brain reward systems in relation to alcohol dependence, by using molecular genetic techniques.

The specific aim of each paper is:

- to investigate the role of the genetic variation of five different nAChR subunit genes in a human association study of alcohol dependence (Paper I).

- to investigate the genetic variation of the ghrelin signalling system in two human association studies of alcohol dependence (Papers II and III), also addressing the gender aspect (Paper III).

- to investigate the role of the ghrelin signalling system for alcohol consumption using GHS-R1A antagonist, plasma ghrelin measurements and gene expression analyses in combination with various alcohol drinking paradigms in rats with different alcohol preference (Paper IV).

MATERIAL AND METHODS

Ethics

All studies included in this thesis were approved by Ethics Committees in the respective cities, *i.e.* at the University of Santiago de Compostela (Papers I and II), at Uppsala University (Paper III) and at the University of Gothenburg (Papers III and IV). Regarding the human studies, all patients and controls provided written informed consent to participate, and the studies were conducted following the tenets of the Helsinki Declaration.

Study subjects

Patients with heavy, moderate or no alcohol use

In Papers I and II, a Spanish population (n=418) including (a) a sample of heavy drinkers admitted to the hospital in the Northwest of Spain (n=138) and (b) a sample of the general adult population (n=280) from the same area was used. Thus, the final study population consisted of 418 participants. As a whole, 142 individuals were alcohol abstainers, 112 were moderate drinkers (1–280 g EtOH/week), and 164 were heavy drinkers (>280 g EtOH/week). The median age of the participants was 50 years (range, 18–92 years). A total of 214 individuals (51.6%) were male.

The sample of heavy drinkers admitted to the hospital: The hospital group consisted of a series of 138 heavy drinkers consecutively admitted to the Internal Medicine Department. The main reasons for admission were alcohol withdrawal syndrome (67 patients), complications of advanced alcoholic liver disease (35 cases), general symptoms plus liver biochemical abnormalities (20 patients), infection (7 cases), and pancreatitis (6 cases). A detailed description has been reported elsewhere (Campos *et al.*, 2005). *The sample from the general adult population:* The general adult population group consisted of individuals previously included in the A-Estrada Allergy Study (Gonzàlez-Quintela *et al.*, 2003). A total of 280 individuals from this study agreed to further participate in an investigation of genetic causes of disease.

Alcohol intake was registered in each subject by adding up the total number of standard drinking units (one bottle of beer, one glass of wine, or one unit of spirit, all of them approximately equivalent to 10 g EtOH) habitually consumed/week (Gual *et al.*, 1999). Subjects who had stopped alcohol consumption during the last 12 months after years of alcohol use were still considered drinkers. Alcohol consumption was significantly linked to male gender. Regular consumers of at least 1 cigarette/day were considered smokers.

Female severe alcohol-dependent individuals

In Paper III, a population of female alcohol-dependent individuals is studied. These are severely dependent individuals with heavy alcohol problems and they are in treatment as a result of court order. They are well-characterized on the basis of alcohol consumption, other drugs, personality profile, *etc.*

110 Caucasian inpatients, all fulfilling the criteria for alcohol and/or substance dependence according to ICD-10 diagnostic criteria, were included in the study. The patients were recruited between July 2001 to July 2006 from a long-term inpatient treatment facility for females suffering from alcoholism and drug abuse. Patients are sent to the facility by social authorities from all over Sweden. Although there are possibilities for patients to initiate contact with the institution themselves, most patients treated are sent there by court order (107 out of the 110 were treated in accordance to a court order). Usually the social authorities have been alerted by a physician that there was a substantial risk for the patient in question to endanger her own mental or physical health or to endanger the wellbeing of other persons. This system allows the physician to immediately contact social authorities and the social authorities to send the patient to a long term drug abuse treatment facility in accordance with the law (the Swedish substance abuse treatment act). The age of the patients ranged between 18 and 75 years. All patients were examined by a physician and a specialist in psychiatry for mental and somatic parameters. Recruited patients were asked to fill in a questionnaire on past and present somatic and mental illness, past and present substance use, social factors such as employment and marital status,

family history concerning alcohol and drug use, criminality and mental illness. Nursing staff at the treatment facility completed the questionnaire with information about vital parameters, withdrawal symptoms and medication at day 1. The lifetime diagnoses of co-morbid disorders such as anxiety disorders and depression were made by a trained specialist in psychiatry, also responsible for the treatment of the patients. Antisocial behaviour and the presence of aggressiveness were diagnosed based upon overt, documented behaviour such as arrests, sentences, and times in prison and overt aggressiveness, usually documented in the criminal reports. Blood samples for genetic analyses were collected from patients that had agreed to participate in the study.

The population cohort INTERGENE

In Paper III, the controls were selected from a cohort from the normal population studied regarding alcohol consumption, drinking and eating habits in relation to genetic factors.

INTERGENE is a population based research programme that assesses the INTERplay between GENEtic susceptibility and environmental factors lifestyle, gender and psychosocial background for the risk of chronic diseases in western Sweden. The survey started in April 2001 and continued until August 2004. The study population consists of randomly selected women and men, aged 25–74 (at the time for sampling), living in the Västra Götaland region. The selected men and women (n=3610) received a postal questionnaire together with information about the study. For the purpose of this study pregnant women (n=16) were excluded. The response rate increased with age. The study procedure is described in detail in a previous article (Berg *et al.*, 2005) and at www.intergene.gu.se. The main questionnaire has been developed and tested for the INTERGENE programme. It includes information on environmental and lifestyle-related exposure variables including physical activity, alcohol and dietary habits, smoking, psychosocial stress, social network, education, occupation and financial status. In addition to measures of current weight status, a series of questions related to body weight history (including birth weight) history of weight losses and weight gains and reasons for weight losses are included in the

questionnaire. The participants were asked about the frequency of intake of different types of alcoholic beverages (low alcohol beer, medium-strong beer, strong beer, wine, dessert wine and spirits). For each alcoholic item, there were 8 possible response categories, ranging from never to three or more times per day. The data on frequencies and standardized portions of alcoholic beverage consumed per occasion were used to calculate the total consumption of pure alcohol in g/day.

Molecular genetics

The polymerase chain reaction

The polymerase chain reaction (PCR) is a technique widely used in molecular biology to amplify small regions of DNA from a few copies to thousands. This technique was developed during the early 80's (Mullis, 1990) and awarded Kary Mullis the Nobel Prize for chemistry in 1993. It has been the cornerstone for the HUGO project and is now fundamental for all molecular biology laboratories (Bartlett and Stirling, 2003). The basis of PCR relies on the ability of the enzyme DNA polymerase to copy a small piece of single stranded DNA, and in this thesis the thermostable *Taq* DNA polymerase has been used. The PCR reaction comprises several cycles, normally 35-40 cycles in total, of heating and cooling steps as follows:

1. Denaturation, 95ºC: The reaction mixture containing DNA, salt buffer, primers, deoxynuclotide triphosphates (dNTPs) and DNA polymerase is heated causing separation of the double stranded DNA, giving single stranded DNA.
2. Annealing, 55-65ºC: Primers, *i.e.* small synthetic oligonucleotide pieces complementary to the DNA sequence of interest, bind to the single stranded DNA template.
3. Elongation, 72ºC: The DNA polymerase copies the DNA sequence of interest, using the primer as the starting point and the DNA as a template, adding dNTPs to the newly synthesized DNA strand.

The amount of DNA is doubled in each cycle, resulting in an exponential increase of PCR product.

All of the molecular genetic techniques used in this thesis, described below, are based on the PCR principle in different applications.

DNA Sequencing

In DNA sequencing, the exact order of nucleotides in a piece of DNA is determined. The automated DNA sequencing method used today is developed from the original method proposed by Sanger *et al.* (1977). First, the DNA region of interest is amplified using PCR. In a second PCR run, both normal dNTPs as well as fluorescently labelled terminating dideoxynucleotide triphosphates (ddNTPs) are used. As the ddNTPs randomly terminate DNA synthesis, this second PCR reaction generates DNA fragments of all lengths possible, all ending with a fluorescent ddNTP. These fragments are then separated by size using capillary gel electrophoresis, and the fluorescence is detected using laser at the end of the capillary, giving the sequence of the amplified DNA.

This method was used in Papers I and II to determine the genetic variation of a gene when comparing the sequence between individuals, and also to genotype certain genetic variants in patients when other techniques were not applicable.

TaqMan Allelic Discrimination

TaqMan Allelic Discrimination (TAD) is used for genotyping of SNPs. A PCR that amplifies the region around the SNP is used. This PCR reaction also includes two oligonucleotide probes, specific for one SNP allele each. These probes are labelled with one fluorescent reporter dye each (VIC and FAM) at one end, and a quencher that absorbs fluorescence at the other end. This method takes advantage of the 5' nuclease activity of the DNA polymerase. This means that when the DNA polymerase extends the newly synthesized DNA strand, it cleaves any probe that is tightly bound to the DNA. This results in an increase in fluorescence, as the quencher no longer absorbs the light emitted by the reporter. A single mismatch between the probe and the target sequence leads to displacement instead of cleavage of

the probe, not increasing the fluorescence signal. After PCR amplification, the signal strength of each reporter molecule is measured and displayed in a scatter plot with the fluorescence of the probes plotted on the X and Y axis, respectively, where each point represents one sample, thus making it possible to determine the genotype of each sample. A signal from only one probe means that this individual is homozygous for the allele represented for that probe, while signals from both probes mean that this individual is heterozygous (Holland *et al.*, 1991; Lee *et al.*, 1993; Livak, 1999; Ranade *et al.*, 2001).

This method was used in Papers I-III for genotyping of SNPs in all patients and controls.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) is used to measure the expression of a gene by determining the amount of mRNA for that gene. To enable PCR amplification, the mRNA is converted to the more stable complementary DNA (cDNA) by reversed transcriptase PCR (RT-PCR). The mRNA (in this case of the cDNA produced) is quantified using sequence-specific fluorescently labelled probes. The principle of this method is basically the same as for the TAD technique, even though only one single probe (reporter molecule FAM) is used. The difference from TAD is that the fluorescence emitted is measured in every amplification cycle instead of just at the end of the PCR. As the fluorescence intensity increases proportionally to the amount of PCR product, this information can be used to calculate the amount of mRNA in each sample. When plotting the fluorescence intensity against cycle number, the resulting curve can be divided into four phases: A linear ground phase, followed by an exponential phase, a linear phase and finally a plateau phase. The greater the starting amount of mRNA the sooner the plateau phase is reached (Ishmael and Stellato, 2008).

There are two methods for analyzing data from qRT-PCR: Absolute and relative quantification. This thesis uses relative quantification. Relative quantification is done by using the C_T value, which is the cycle number at which the threshold set at the exponential phase, is crossed (Livak and

Schmittgen, 2001). The larger amount of mRNA present in the sample, the faster the threshold will be reached and the lower the C_T value will become. To account for differences in starting material, the expression of the target gene is normalized to the expression of an endogenous reference gene for every individual (ΔC_T). It is important that the endogenous reference gene is equally expressed in all tissues studied. To account for differences between individual runs, all samples are normalized against a calibrator sample included in all runs ($\Delta\Delta C_T$). The $2^{-\Delta\Delta CT}$ value (*i.e.* fold expression) can now be used to compare gene expression between different tissues or between different individuals *etc.* A $2^{-\Delta\Delta CT}$ value of 2 means that the sample of interest has a 2-fold expression of the gene compared to the control sample (Ishmael and Stellato, 2008; Livak and Schmittgen, 2001).

$C_{T\ target} - C_{T\ ref} = \Delta C_T$

$\Delta C_{T\ sample} - \Delta C_{T\ control} = \Delta\Delta C_T$

$2^{-\Delta\Delta CT}$ = fold expression value

This method was used to measure the GHS-R1A gene expression in Paper IV. The endogenous control gene used was RNA polymerase II and the calibrator sample was a whole brain sample from an untreated rat.

Statistical concepts in genetic association studies

Statistical significance

Basically, all statistical analyses aim at testing if a null hypothesis can be rejected at a specific significance level (normally set to 0.05 or 0.01). This limit means that the probability (p-value) for rejecting a true null hypothesis, *i.e.* making a type I error, is less than 5% or 1%. The other case, when a null hypothesis is not rejected despite being false, is called a type II error. When the risk of making a type II error is low, the power of the study is high. Normally a power above 80% is requested, and depends on sample size, the quality of the data, the significance level, and effect size.

Odds ratio

Odds ratio (OR) is a measure representing the risk of an outcome (*i.e.* disease) if exposed or if not exposed to a certain risk factor (*i.e.* whether or not an individual is a carrier of a genetic marker such as a SNP allele).

OR is calculated as: $\frac{\text{(risk/1-risk)exposed}}{\text{(risk/1-risk)un-exposed}}$

The OR is interpreted by the outcome in relation to the number 1 since OR=1 means that the exposure has no effect on the outcome, *i.e.* the SNP allele does not affect the disease risk. An OR<1 indicates decreased risk and an OR>1 indicates increased risk. When the OR is low, OR=risk ratio. However, it is not possible to use risk ration in case-control studies using regression models, and therefore OR has been used throughout this thesis.

Hardy Weinberg equilibrium

Hardy Weinberg equilibrium (HWE) is a measure of the stability of allele and genotype frequencies in a population that is randomly mating without selection pressure. This balance can be measured using χ^2 statistics, and has been assessed for all genetic markers in the statistical analyses in this thesis, in patients and controls separately. When a genetic marker significantly differs from HWE, this is normally a sign of genotyping errors or poor data quality, but can also be a result of genetic drift caused by a true disease association.

Correction for multiple testing

In the haplotype analyses, several genetic markers are analyzed in patients and controls in relation to several disease associated parameters. As the significance level is set to 0.05, meaning that 5% of the associations found could possibly be false, the risk of making a type I error grows with the number of statistical analyses. Therefore, the use of methods correcting for such multiple testing are essential. In this thesis two different methods have been used; the simpler Bonferroni correction where the corrected p-value is

obtained by multiplying all p-values with the number of tests performed; and the more advanced permutation test where multiple random shuffling of phenotype and genotype data results in a corrected p-value estimated as the proportion of the simulated p-values that are smaller than the original one (Rice *et al.*, 2008).

Animal studies

Rat Strains (Wistar, AA/ANA)

In Paper IV three different rat strains were used. The AA (Alko, Alcohol) and ANA (Alko, non-Alcohol) rats are two strains selectively bred for their high and low alcohol preference (Eriksson, 1968, 1971). These rats were obtained from the National Public Health Institute in Helsinki, Finland. To also study an un-bred strain, having a non-genetic control for any innate differences in the AA and ANA rats, normal Wistar rats (B&K Universal AB, Sollentuna, Sweden) were also used.

Drinking models (continuous, intermittent, limited access)

AA/ANA rats were used for the continuous and limited access two-bottle choice drinking paradigms, since these strains are known to consume high (AA) and low (AA) amounts of alcohol when exposed to such paradigms. Wistar rats were exposed to the intermittent access 10% alcohol two-bottle choice drinking paradigm, since it has been demonstrated that distinct high, medium and low consuming rats are obtained by this paradigm in a similar setup (Simms *et al.*, 2008).

Continuous and limited access two-bottle choice drinking paradigms: Initially, all AA and ANA rats were group housed and had continuous access to both tap water and increasing concentration of alcohol (2, 4, 6, 8, 10%) over a two-week period (approximately 3 days at each percentage alcohol). Thereafter, they were housed individually for nine weeks with continuous access to tap water and alcohol solution (10%). During this period of time it was confirmed that AA rats consumed high amounts of alcohol whereas ANA

rats consumed low amounts of alcohol. The fluid (alcohol and water) consumption was measured twice a week and the body weight of the animals was measured once a week. Subsequently, the access to alcohol solution was limited to the first 90 min of the dark period (*i.e.* a limited access paradigm) (Rhodes *et al.*, 2005) for half of the AA and half of the ANA animals. The two-bottle (alcohol/water) free choice limited access paradigms were maintained for two weeks prior to GHS-R1A antagonist treatment of the AA rats.

Intermittent access two-bottle choice drinking paradigm: The Wistar rats were introduced to a two-bottle free choice paradigm with access to tap water and increasing concentrations of alcohol (2, 4, 6, 8, 10% vol/vol) over a 2-week period to gradually familiarise them with the alcohol solution. The rats were then housed individually, and at altering days given continuous access to two bottles each containing either EtOH 10% and water, or to two bottles of water. The intermittent access 10% alcohol two-bottle choice drinking paradigm was adapted from Simms *et al.* (2008) and does not require sucrose fading. On the Monday after the end of the housing acclimatization period, rats were given access to one bottle of 10% (vol/vol) alcohol and one bottle of water. After 24 h, the alcohol bottle was replaced with a second water bottle that was available for the next 24 h. This pattern was repeated on Wednesdays and Fridays. All other days the rats had unlimited access to water. Low alcohol consumption individuals were characterised as <2 g EtOH/kg/day, medium alcohol consumption individuals were characterised as 2-3 g EtOH/kg/day and high alcohol consumption individuals were characterised as >3 g EtOH/kg/day.

Radioimmunoassay

The radioimmunoassay (RIA) is used to measure the levels of a certain antigen in a sample (*i.e.* levels of hormones or peptides in blood). This method was first developed in the 50's by Yalow and Bernson (1960) to measure insulin in blood. An antibody specific for the antigen of interest is added to the sample at a concentration sufficient to bind all of the antigen present. Then, an antigen labelled with radioactivity (normally ^{125}I) is added to the sample, binding all left-over antibodies. The more antigen that was

present in the sample from the beginning, the less antibody is left to bind the tracer-labelled antigen. Then a secondary antibody is added, precipitating the whole complex when bound to the primary antibody. The precipitate is isolated and the radioactivity of the sample is measured. A high signal represents a low concentration of antigen. A standard curve is generated to calculate the exact amount of antigen in a sample.

This method was used in Paper IV to measure plasma ghrelin concentration.

RESULTS AND DISCUSSION

Genetics of 5 different nicotinergic acetylcholine receptor subunit genes

Genetic studies of nAChRs

Since addictive behaviours such as smoking, alcohol dependence and eating disorders seem to overlap both biologically and genetically, genetic variants of the nAChR genes are strong candidates for addiction susceptibility factors (see Schlaepfer *et al.*, 2008; and Steinlein and Bertrand, 2008). The nAChR subunits are coded for by one gene each. Several of these nicotinic receptor subunit genes (*CHRN*s*) are genomicly mapped one after the other in so called gene clusters. In this thesis, the subunits previously implicated in alcohol and nicotine reward, *i.e.* the α_3, α_4, α_6, β_2, β_3 subunit genes (*CHRNA3,4,6* and *CHRNB2,3*), were investigated (Paper I). Previous publications on nAChR genetics have been studied nicotine dependence, where risk loci as have been identified in the *CHRNA5-A3-B4* as well as in the *CHRNB3-CHRNA6* and *CHRND-G* gene clusters (Bierut *et al.*, 2007; Hoft *et al.*, 2009b; Li *et al.*, 2005; Saccone *et al.*, 2007; Stevens *et al.*, 2008). *CHRNA5-A3-B4* has also been associated with the quantity of cigarettes smoked (Caporaso *et al.*, 2009) as well as with serum cotinine levels (Keskitalo *et al.*, 2009), indicating a role for these genetic variants in nicotine consumption. As regards alcohol dependence, the *CHRNB2* has been previously associated with the subjective feelings of both alcohol and nicotine (Ehringer *et al.*, 2007), while the *CHRNA6* and *B3* have been associated with alcohol consumption (Hoft *et al.*, 2009a).

Even though numerous genetic association studies have been performed on these genes, few functional studies on any of the associated genetic variants have been performed. It has been postulated that rare missense SNPs in the intracellular loop of the receptor can alter receptor function, as this intracellular region influences the properties of the ion channel, and is also important for receptor assembly (Kracun *et al.*, 2008; Sabatelli *et al.*, 2009; Williams *et al.*, 1998).

nAChRs and alcohol dependence (Paper I)

Several nAChR subunits are important for mediating the stimulating and rewarding effects of alcohol (*vide supra*). In Paper I, the genetic variation in 5 subunit genes, *i.e. CHRNA3*, *CHRNA4*, *CHRNA6*, *CHRNB2* and *CHRNB3*, was investigated in a population of heavy and moderate alcohol consumers as well as in abstainers, to determine if these genes influenced the risk of developing alcohol dependence. Using HapMap data, htSNPs covering >80% of the genetic variation of each gene were selected, and this resulted in the genotyping of a total of 20 SNPs.

No significant differences in allele or genotype frequencies were observed for any of the investigated SNPs, even though a trend for an association with the AC genotype of SNP rs16891604 in heavy alcohol consumption could be seen compared with moderate alcohol consumption and abstainers.

When collapsing all of the genotype data, investigating the haplotypes of each gene, one haplotype block consisting of 4 consecutive SNPs in the *CHRNA6* was associated with heavy alcohol consumption as well as with the actual amount of alcohol consumed measured in g EtOH/week. Within this block, 2 individual haplotypes (CCCC and TCGA) were responsible for these associations, *i.e.* the frequencies of the haplotypes were higher in the heavy consumers both compared to the moderate consumers and the abstainers and the carriers of these haplotypes drank higher amounts of alcohol than non-carriers. The OR/allele for these associations were 1.63 and 1.64 respectively, meaning that carriers of these haplotypes have 1.63 or 1.65 times higher risk of being heavy alcohol consumers than non-carriers of these haplotypes. In addition, the weekly alcohol intake in carriers of these haplotypes was 126 g and 117 g higher/allele, than in non-carriers.

In human studies, verifying our results, SNPs in the *CHRNA6* have been associated with alcohol consumption (Hoft *et al.*, 2009a), as well as with nicotine dependence (Hoft *et al.*, 2009b), and the subjective response to nicotine (Zeiger *et al.*, 2008). In animal studies there is some evidence indicating that the α_6 subunit of the nAChRs is involved in mediating the rewarding properties of alcohol (Larsson *et al.*, 2004).

Even though there are no functional studies on these genetic variants, these results still strengthen the involvement preferably of α_6-containing nAChRs in alcohol consumption and dependence. The SNPs associated with alcohol consumption in this study are in high LD with the SNPs (rs1072003, rs892413 and rs2304297 in *CHRNA6*) also associated with alcohol consumption in another study (Hoft *et al.*, 2009a), suggesting that genetic variants of the *CHRNA6* may be involved in the vulnerability for developing alcohol dependence. Furthermore, the nAChR antagonist varenicline, having some affinity for α_6-containing receptors (Mihalak *et al.*, 2006), affects alcohol self-administration and reward both in rodents (Ericson *et al.*, 2009; Steensland *et al.*, 2007) and in humans (McKee *et al.*, 2009). Taken together, nAChR antagonists might thus have beneficial effects in the treatment of alcohol dependence (reviewed in Crunelle *et al.*, 2010).

nAChRs and body mass (Paper I)

Given that nAChRs have been implied in overeating and since co-morbidity between eating disorders and both alcohol and nicotine dependence is common (Field *et al.*, 2002; Wolfe and Maisto, 2000), variables related to body weight was also studied in Paper I.

No significant differences in allele or genotype frequencies were observed for any of the investigated SNPs when looking at the mean weight and BMI for all genotypes and alleles. However, a haplotype block in the *CHRNA4*, consisting of 4 consecutive SNPs, was associated with increased weight and BMI in heavy alcohol consumers. The haplotype GGTG responsible for this association resulted in a 9.1 kg weight increase/allele, *i.e.* heavy alcohol consuming individuals heterozygous for this haplotype weigh 18.2 kg more than non-carriers of this haplotype, which is quite a large effect. As there are more female individuals in the control group than in the patient group, this analysis is corrected for gender, and can hence not be explained by the lower body weight in females than in males.

The nAChR subunit gene *CHRNA2* has previously been associated with overweight (Kim, 2008), and one of the SNPs included in the associated *CHRNA4* haplotype in Paper I (rs1044396) has previously been associated

with nicotine dependence (Feng *et al.*, 2004; Li *et al.*, 2005). Neither the mechanism by which the associated haplotype exerts its effects on eating and body weight, nor the functionality of these genetic variants is known. However, both the rewarding properties of alcohol intake and food ingestion are, at least in part, regulated by the cholinergic input in the reward link. Both alcohol- and food intake increase tegmental ACh levels and accumbal DA levels (Larsson *et al.*, 2005; Rada *et al.*, 2000). These similarities between different kinds of addictive behaviours indicate a common neurobiological origin of these conditions. Hence, in addition to alcohol dependence, pharmacological treatments targeting nAChRs could also have beneficial effects on overeating.

Genetics of the ghrelin signalling system

Both the gene encoding ghrelin, *i.e.* the preproghrelin gene *(GHRL),* and the ghrelin receptor gene (*GHSR*) are situated on chromosome 3, even though not in close proximity. As the known function of ghrelin long was limited to food intake and appetite, genetic studies of the ghrelin signalling system has to a large extent been restricted to this area. Genetic variants of *GHRL* and *GHSR* have been associated with obesity/body mass (Ando *et al.*, 2007; Baessler *et al.*, 2005; Kuzuya *et al.*, 2006; Mager *et al.*, 2008; Miraglia del Giudice *et al.*, 2004; Ukkola *et al.*, 2002; Vartiainen *et al.*, 2006), diabetes/insulin sensitivity (Berthold *et al.*, 2009; Korbonits *et al.*, 2002; Ukkola and Kesaniemi, 2003; Zavarella *et al.*, 2008), anorexia (Dardennes *et al.*, 2007), binge eating (Monteleone *et al.*, 2007), and bulimia (Ando *et al.*, 2006; Miyasaka *et al.*, 2006). It should, however, be noted, that others have not been able to repeat these results (Garcia *et al.*, 2008; Garcia *et al.*, 2009; Gueorguiev *et al.*, 2009; Kim *et al.*, 2006; Larsen *et al.*, 2005; Martin *et al.*, 2008; Monteleone *et al.*, 2006; Ukkola and Kesaniemi, 2003; Wang *et al.*, 2004). Further, *GHRL* variants have been associated with emotional problems in methamphetamine dependence (Yoon *et al.*, 2005).

The *GHRL* gene consists of five exons. Initially, two mRNA transcripts (A and B) were identified, where the A form is the main form of ghrelin mRNA in humans. This mRNA encodes preproghrelin, a 117 amino acid peptide that is processed to pro-ghrelin that, in turn, is cleaved to yield the 28 amino

acid peptide ghrelin. To be biologically active, *i.e.* to be able to bind to its receptor, ghrelin also needs to be octanylated on the Ser^3 position of the peptide, yielding acyl-ghrelin. When ghrelin has been cleaved off from pro-ghrelin, the left-over part, called C-ghrelin, can be further processed yielding obestatin (Chen *et al.*, 2009). Obestatin is a peptide hormone suggested to have the opposite actions of ghrelin regarding food intake, but this has been debated (Tang *et al.*, 2008; Zhang *et al.*, 2005). Additionally, several more *GHRL* transcripts and products have been identified in mouse and humans with unknown or peripheral biological functions.

The *GHSR* gene consists of two exons. The gene is transcribed into the functional receptor GHS-R1A as well as the alternatively spliced, inactive GHS-R1B form lacking transmembrane domains 6 and 7 (Kojima and Kangawa, 2005). The GHS-R1A is a G-protein coupled 7 transmembrane spanning receptor (Howard *et al.*, 1996). It signals via a Gq/11 α-subunit that results in the release of inositol triphosphate and Ca^{2+}. This receptor is, as described above, the receptor for ghrelin. However, it has also been shown that it has high constitutive activity, *i.e.* a basal activity that is more than 50% of the ghrelin-induced maximum (Holst *et al.*, 2003; Holst and Schwartz, 2006), which might have some physiological role in energy expenditure and meal initiation.

In the field of ghrelin signalling system genetics, some efforts have recently been made to functionally characterize the associated genetic variants. One study has investigated different *GHRL* and *GHSR* gene haplotypes consisting of promoter SNPs, showing significant differences in promoter activity (Garcia *et al.*, 2009), while others have shown that *GHSR* promoter SNPs affect gene expression, probably by altering the binding sites of transcription factors (Mager *et al.*, 2008). Non-synonymous SNPs, *i.e.* SNPs that alter amino acid coding, in the *GHSR*, have been shown to alter the basal receptor activity, receptor binding of both agonists and antagonists, as well as receptor expression (Liu *et al.*, 2007), even though these SNPs are very rare and have not been studied in this thesis.

The ghrelin signalling system in alcohol dependence (Papers II and III)

The ghrelin signalling system has been shown to be involved in the rewarding properties of alcohol in numerous studies (*vide supra*). In Papers II and III genetic variation in the *GHRL* and *GHSR* genes were investigated to determine their impact on alcohol dependence and related traits.

In Paper II, the same study population as in Paper I was used, consisting of heavy and moderate alcohol consumers as well as abstainers. When selecting the studied htSNPs, three previously studied non-synonymous *GHRL* SNPs were included (Arg51Gln rs4684677, Leu72Met rs696217 and Gln90Leu rs34911341), 3 additional htSNPs in *GHRL*, as well as 4 htSNPs in the *GHSR* were chosen based on HapMap data to cover >80% of the genetic variation of these two genes. The GA genotype of the *GHRL* SNP, rs2232165 was associated with heavy alcohol consumption in this study. However, none of the other SNPs or any of the constructed haplotypes had any effect on the risk for alcohol dependence or on actual alcohol consumption.

Paper II was one of the first complete haplotype analyses conducted in the lab. Since then the statistical methods used have improved, as can be noted in Papers I and III. When recalculating the data from Paper II using these improved statistical models, including regression models, covariates and permutation analysis, the A allele of SNP rs2232165 was still found to be associated with heavy alcohol consumption (p_c=0.019; OR/allele=1.7; 95% CI=1.2-2.5). Again, no additional associations with any other SNP or haplotype were found regarding risk for alcohol dependence or actual alcohol consumption.

As there are known sex differences in the ghrelin system (Makovey *et al.*, 2007), a population of female severely alcohol dependent women were investigated in Paper III. All individuals were genotyped for the same *GHRL* and *GHSR* variants as in Paper II. Similar to Paper II, no associations with genetic variants of the ghrelin signalling systems and risk of alcohol dependence were found. However, associations with a haplotype in the *GHRL* (TAACGT) and reports of paternal alcoholism were found in the group of alcohol-dependent women, as well as associations with a *GHSR* haplotype (CCGG) and being characterized as a type 2 alcohol dependent-

individual. Both these features, paternal alcoholism and type 2 alcohol dependence, are parameters related to the genetic risk of developing alcohol dependence, since the heredity of alcohol dependence is mainly transferred from the father (Knop *et al.*, 2007), and since type 2 alcohol dependence is thought to be more heredity driven (Cloninger *et al.*, 1988). These results thus give indirect evidence for the involvement of genetic variation in the ghrelin signalling system in the addiction process. Comparing Paper II and III, there were no evident sex differences in the genetics of the ghrelin signalling system.

The weakness of both Paper II and III was the number of patients and controls investigated. This could be one of the reasons for the inconclusive results regarding whether these genetic variants affect the risk of developing alcohol dependence or not. In an effort to summarize all these data and the role of genetic variants in the risk for alcohol dependence, data from all patients and controls from the genetic studies in this thesis, as well as some additional alcohol-dependent patients and controls also genotyped for the same *GHRL* and *GHSR* variants, were analysed. Hence, the patients included heavy alcohol consumers from the Spanish study, n=164; female alcohol-dependent patients, n=113; and alcohol-dependent patients from southwest Sweden, n=46, while the controls included Spanish abstainers, n=138; female controls from a cohort in southwest Sweden, n=602, and controls from southwest Sweden, n=32.

Interestingly, when expanding the number of study subjects, a haplotype in the *GHRL* (TGGCGT) was associated with increased risk of alcohol dependence, while a haplotype in the *GHSR* (TTGA) was associated with a decreased risk of alcohol dependence, *i.e.* had a protective effect (Table 2). Only results for the 6 and 4 marker windows, respectively, are displayed. However, similar haplotypes were significantly associated with alcohol dependence in all of the other windows. These data supports the findings showing that the central ghrelin signalling system is required for alcohol reward and alcohol consumption in mice (Jerlhag *et al.*, 2009).

It should be noted that the haplotype TGGCGT associated with alcohol dependence in this extended analysis is quite similar to the haplotype TAACGT that was associated with reports of paternal alcohol dependence

in Paper III. This extended analysis, even though the patients and controls are not perfectly matched, show that *GHRL* and *GHSR* gene variants are in fact associated with alcohol dependence, even though the effect size is small, and a large sample size is needed to significantly show these associations.

Table 2. *GHRL* and *GHSR* gene haplotypes associated with alcohol dependence

Gene	Haplotype	Patients (n=323)	Controls (n=772)	p_c-value	OR (95%CI)/ allele
GHRL	TGGCGT	0.425	0.367	0.013	1.11 (1.05-1.46)
	TAACGG	0.180	0.211	0.096	-
	TGACGT	0.089	0.098	0.092	-
	TAACGT	0.055	0.073	0.489	-
	TGACTT	0.062	0.070	0.882	-
	AGACGT	0.070	0.068	0.756	-
	TGGCGG	0.071	0.054	0.070	-
GHSR	TCGA	0.427	0.414	0.694	-
	TTGA	0.287	0.328	0.021	0.91 (0.84-0.93)
	TCGG	0.151	0.136	0.060	-
	CCGG	0.096	0.098	0.432	-

GHRL=the pro-ghrelin gene; *GHSR*=the growth hormone secretagogue receptor gene; OR=odds ratio; CI=confidence interval; the p-values displayed was calculated using a stepwise logistic regression model, including age as a covariate, and are corrected for multiple testing by means of permutation test (p_c).

Genetics of the ghrelin signalling system, body mass and smoking (Papers I and III)

There is co-morbidity between different kinds of addictive behaviours such as alcohol and nicotine dependence as well as with eating disorders and obesity (*vide supra*). Since ghrelin is involved both in appetite regulation and food intake, as well as in mediating the rewarding properties of alcohol, it was interesting to investigate if *GHRL* and *GHSR* gene variants could affect body mass and smoking.

In Paper II, the C allele of SNP rs2948694 in the *GHSR* was associated with increased weight, and a trend for an association with body mass index (BMI), specifically in heavy alcohol consuming individuals. Further, 2 haplotypes of

the *GHRL* (TAAC/GT) were associated with both higher weight and BMI in non-smoking heavy alcohol consuming individuals, and 2 haplotypes of the *GHSR* (CCGG and TCGA) were associated with higher weight and BMI, respectively. In Paper III, 2 haplotypes in the *GHRL* gene (-CGG and TCGG) were associated with smoking and one haplotype in the *GHSR* (TCGG) was associated with reduced BMI in controls only.

Again, when recalculating the data from Paper II, using the improved statistical methods, haplotypes TGACTT and TAACGT of the *GHRL* were associated with increased BMI in all individuals, using alcohol consumption and smoking as co-variables ($p=0.002$, 2.3 units/allele and $p=0.004$, 2.5 units/allele respectively). Furthermore, a trend for associations of haplotypes CCGG and TCGA of the *GHSR* with increased body weight was seen.

Summarizing these data, both *GHSR* and *GHRL* gene variants may have an effect on increased body weight. While the *GHSR* haplotype CCGG was associated with higher body weight in Paper II, the haplotype TCGG was associated with lower body weight as well as with smoking in Paper III. The specific effect of these haplotypes seems to depend on the allele present in the first position (C or T), where C is related to higher and T is related to lower body weight. Accordingly, the C allele of this SNP was associated with increased weight in Paper II, and a trend for the same association was seen in Paper III. Since smoking is known to reduce body weight, the effect on body weight in smoking individuals could rather be a result of the smoking than the genotype. Even though these results further imply the role of the ghrelin signalling system in the regulation of body weight and in the co-morbidity of alcohol dependence and compulsive overeating, the effect size of these associations was quite small and their clinical relevance limited. No evident sex differences were observed in the associations with genetic variants of the ghrelin signalling system and body weight.

Genetics of the ghrelin signalling system and sucrose intake

When performing the study presented in Paper III a larger part of the INTERGENE population was genotyped for the *GHRL* and *GHSR* gene variants. Except for the female alcohol low-consumers used in Paper III it

also included both female and male alcohol high-consumers as well as male low-consumers of alcohol. For all these individuals, the total sucrose intake (g sucrose/day) was calculated from a questionnaire regarding eating habits, including consumption of sweets. When analyzing sucrose intake and the above mentioned genetic markers in *GHRL* and *GHSR*, an association with increased sucrose intake and one haplotype (AGACGT) in the *GHRL* was found (Table 3; preliminary data).

Table 3. Associations between *GHRL* haplotypes and higher sucrose consumption in heavy/moderate alcohol consumers (n=283).

Gene	Haplotype	Sucrose Intake (g/day)[1]	
		g/allele (95% CI)	p_c-value[2]
GHRL	AGACGT	30 (19-41)	0.008
	TGGCGT	-	0.308
	TAACGG	-	0.901
	TAACGT	-	0.756
	TGACGT	-	0.917
	TGACTT	-	0.218
	TGGCGG	-	0.103

GHRL=pro-ghrelin gene; CI=confidence interval; Individuals inlude heavy and moderate alcohol consumers from the INTERGENE study; [1]sucrose intake is calculated from reports of intake of sweets; [2]p-values are calculated with linear regression including relevant covariates, and are corrected for with permutation analysis

This finding is in line with published studies where a positive correlation with the response to sweet taste and excessive alcohol intake has been shown, an effect that may have a genetic origin (Kampov-Polevoy *et al.*, 2004). It is known, from animal studies, that ghrelin is involved in both food and alcohol intake (Blum *et al.*, 2009; Jerlhag *et al.*, 2009; Salomé *et al.*, 2009a; Salomé *et al.*, 2009b). Specifically, ghrelin has been known to increase the intake of rewarding food (Egecioglu *et al.*, 2010), which leads to the hypothesis that the associated *GHRL* haplotype might explain some of the

genetic basis for the relationship between high sucrose as well as high alcohol intake.

In genetic association studies where a case-control design is used, it is important to have a well-characterized study sample. The frequency or distribution of variables such as age, sex, body composition, *etc.* should be equal in both groups to reduce the risk of them influencing the results. However, in reality this is not easy to accomplish. In the samples used in this thesis, such differences have been accounted for by the use of co-variables in all of the statistical analyses. Another important issue is the selection of patients and controls regarding the actual studied disease. If, as a result of inadequate sampling method, there are individuals in the control group that actually belong to the patient group, it is more difficult to find significant associations. In this thesis, patients have been carefully selected using international diagnostic criteria. Though, it should be noted that the heavy consumers of the Spanish population included both alcohol-dependent individuals as well as un-diagnosed heavy alcohol consumers. In the same population, the alcohol abstaining controls should be regarded with caution, as the reason for them not drinking is not fully elucidated.

The ghrelin signalling system in high and low alcohol consuming rats

In Paper IV high (AA) and low (ANA) consuming rats as well as normal Wistar rats were used. These rats were all subjected to different alcohol consumption paradigms. Since the ghrelin signalling system is required for alcohol consumption, as well as for alcohol reward (Jerlhag *et al.*, 2009), different aspects of the ghrelin signalling system were investigated in a genetic model of high and low alcohol consumption using AA and ANA rats. The Wistar rats can be regarded as a non-genetic control in the expression analyses.

Effects of GHS-R1A antagonist (Paper IV)

The GHS-R1A antagonist JMV2959 significantly reduced the alcohol intake in the high consuming AA rats compared to vehicle treated controls, similar

to other studies with mice (Jerlhag *et al.*, 2009). These results show that GHS-R1A antagonists are effective in reducing alcohol intake both in normal animals, as well as in animals selectively bred for high alcohol consumption (*i.e.* animals with a genetic predisposition for high alcohol intake). The results from the AA rats strengthen the likelihood of GHS-R1A antagonist having beneficial effects in reducing the alcohol intake also in humans, since alcohol dependence is known to have a high genetic predisposition. It is also important to note that, despite the lower alcohol intake in the AA rats in this study compared to others (Kemppainen *et al.*, 2009; Roman *et al.*, 2005) the intake was still significantly reduced by the GHS-R1A antagonist.

It is known that both alcohol and ghrelin can activate the brain reward systems by increasing locomotor activity and accumbal DA overflow (Jerlhag *et al.*, 2007; Larsson *et al.*, 2004). Furthermore, ghrelin into the VTA or LDTg increases alcohol consumption (Jerlhag *et al.*, 2009), and ghrelin signalling is required for the rewarding effects of alcohol, since GHS-R1A antagonism blocks alcohol intake, alcohol-induced accumbal DA overflow, as well as CPP for alcohol (Jerlhag *et al.*, 2009). Thus, the effects of the GHS-R1A antagonist are probably mediated via the mesolimbic DA system, even though it is administered peripherally.

Plasma levels of ghrelin (Paper IV)

In alcohol naïve animals, there were no differences in plasma ghrelin between AA and ANA rats. After 14 weeks of alcohol consumption the plasma ghrelin levels were lower in both strains, however, the reduction was significantly smaller in the AA rats than in the ANA rats. It is known from previous studies that plasma ghrelin levels decrease with age in both humans and rodents (Rigamonti *et al.*, 2002; Sandström *et al.*, 1999). Based on the results from Paper IV, showing a smaller reduction in plasma ghrelin in AA and ANA rats, alcohol seems to presumably counteract this reduction in plasma ghrelin over time, since the AA rats have a significantly higher alcohol intake than the ANA rats. Even though this corresponds quite well with human studies showing higher plasma ghrelin levels in alcohol-dependent individuals than in controls (Kim *et al.*, 2005; Kraus *et al.*, 2005),

the mechanism for this effect is yet unknown. Alternative explanations for the differences in plasma ghrelin could be the amount of stress produced with the different sampling methods, *i.e.* tail incision and decapitation. Ghrelin is known to increase stress response (Kristensson *et al.*, 2006; Lutter *et al.*, 2008), and it has been shown that there are differences in the stress response of AA and ANA rats (Sandbak *et al.*, 1998).

Gene expression of the GHS-R1A in the brain reward systems (Paper IV)

The gene expression of GHS-R1A was studied in 5 different reward-related brain areas, *i.e.* NAc, VTA, amygdala, PFC and hippocampus, in AA, ANA and Wistar rats.

The GHS-R1A gene expression was significantly higher in the AA rats, especially in NAc and VTA, and a trend for the same effect could be observed in VTA of high compared to moderate and low alcohol consuming Wistar rats. These two brain regions are the two main constituents of the brain reward link, and mediate the rewarding properties of alcohol, food and ghrelin (*vide supra*). In this study it cannot be concluded if this is an alcohol-related effect or an innate difference between AA and ANA rats. However, since a trend for the same effects can be found in the high, moderate and low alcohol consuming Wistar rats, that do not have the same genetic predisposition, an alcohol-mediated effect is perhaps more likely. Conversely, the high alcohol consuming Wistar rats actually drink more alcohol than the AA rats, and still the differences in gene expression are quite small compared to the effects seen in the AA and ANA rats, favouring the hypothesis regarding innate genetic differences. A combination of the two, such as an epigenetic effect may be possible (*vide infra*). Of course the perfect scenario would have been to include alcohol naïve AA and ANA rats in the study, but the number of animals in this study was too low to be able to conduct such an experiment.

Since half of the animals were subjected to continuous access only while the other half was subjected to both continuous and limited access to alcohol, gene expression levels were investigated in these two groups of animals separately. The GHS-R1A gene expression was higher in the AA than in the

ANA rats in the amygdala of rats subjected to both the continuous and limited access paradigms. Amygdala is a brain nucleus involved in emotion and stress, and more importantly, with stress in addiction related to drug-seeking (Koob, 2009). The difference in gene expression might thus be a result of the increased stress produced with the change of drinking paradigm. Accordingly it has been shown that ANA rats have a lower stress threshold than AA rats (Sandbak *et al.*, 1998).

When comparing the results from the two drinking paradigms with each other for each strain of rats separately, there were significant differences in GHS-R1A gene expression between both AA and ANA rats in hippocampus and between AA rats only in PFC. The effects in hippocampus are probably related to the actual change of paradigm since this effect is seen in both strains of animals and since ghrelin in hippocampus is involved in learning (Diano *et al.*, 2006). The magnitude of the difference in gene expression in PFC is very subtle, but might reflect the increase in craving/withdrawal in the limited access paradigm.

Epigenetics

Epigenetic modifications, such as DNA methylation and histone acetylation, are known to affect gene expression (*vide supra*). It has also been shown that alcohol may influence both of these epigenetic mechanisms (Shukla *et al.*, 2008). Therefore, an attempt to measure GHS-R1A DNA methylation in brain tissue from AA, ANA and Wistar rats has been performed.

DNA was extracted from all brain tissue samples in the animal experiments above, *i.e.* from NAc, VTA, amygdala, hippocampus and PFC in AA, ANA and Wistar rats. An effort was made to measure the DNA methylation of the GHS-R1A gene in all of these samples using methylation-specific PCR (MSP). A trend towards a lower degree of DNA methylation was observed in VTA of the AA compared to the ANA rats, which corresponds very well to the higher expression of the GHS-R1A gene, while no significant differences were observed in the NAc and in the hippocampus, even though there is a trend for the opposite effect (calculated using χ^2-test) (preliminary data) (Table 4). No GHS-R1A DNA methylation could be detected in the

PFC or in the amygdala, and no differences in GHS-R1A DNA methylation were found in the high, moderate and low alcohol consuming Wistar rats (data not shown). The lack of methylation in these regions could, however, be a result of methodological problems.

Table 4. GHS-R1A DNA methylation in rat brain tissue. Percent methylated DNA samples in AA and ANA rats in three reward-related brain regions, i.e. ventral tegmental area (VTA), nucleus accumbens (NAc) and hippocampus.

Brain Region	AA	ANA	p-value[1]
VTA	33%	57%	0.069
NAc	63%	60%	0.791
Hippocampus	33%	23%	0.294

VTA=ventral tegmental area, NAc=Nucleus Accumbens; AA=high alcohol consuming rats; ANA=low alcohol consuming rats; [1]p-values are calculated using χ^2-test.

These results indicate that the higher GHS-R1A gene expression observed in the AA compared to the ANA rats could be mediated by an epigenetic mechanism, as a result of their higher alcohol intake. Alcohol has been suggested to affect both DNA methylation and histone acetylation, although the exact mechanism is not known (Shukla *et al.*, 2008). Conversely to the results presented here, some studies show a global hypermethylation, *i.e.* increased activity of the DNA methylating enzymes, in alcohol dependent patients (Bönsch *et al.*, 2004), that might be a result in altered gene expression of these enzymes (Bönsch *et al.*, 2006). Additionally, alcohol exposure during early embryonic development significantly increases DNA methylation of several genes in rats (Liu *et al.*, 2009).

Such an epigenetic mechanism could, hypothetically, also explain the smaller reduction in plasma ghrelin levels seen in the AA compared to the ANA rats, as it seems like alcohol has a protective effect on the reduction in plasma ghrelin over time. Even though hypomethylation of the GHS-R1A gene, and increased GHS-R1A expression could have an effect on plasma ghrelin levels the observed effects could also be a result of an altered DNA methylation of

the *GHRL* gene, which of course would be interesting to study in these animals.

CONCLUDING REMARKS

The data presented in this thesis suggest that genetic variations of reward-related genes may be involved in the pathogenesis of alcohol dependence, even though they cannot be regarded as major susceptibility genes. Rather, they contribute to an increased vulnerability in the reward systems that, in combination with environmental factors, may lead to the development of dependence.

The main findings of the genetic association studies in this thesis are that; (1) nAChR gene variants influence alcohol consumption and body weight in alcohol-dependent individuals (2) genetic variants of the ghrelin signalling system influence the risk of developing alcohol dependence, even though the effect size is small, and in addition, it is possible that these variants might have an effect on body weight. These effects seem independent of sex.

The findings regarding the nAChR system further strengthen the role of the cholinergic projection of the cholinergic-DAergic reward link in alcohol dependence, as well as in other addictive behaviours such as overeating and overweight. The functional significance of these genetic variants needs further evaluation, since they potentially could alter the capacity of alcohol and/or receptor ligands to interact with the receptor. Additionally, pharmacological agents affecting these nAChR subunits should be further evaluated as treatments for such addictive behaviours.

From the studies on the ghrelin signalling system in this thesis, it cannot be concluded whether innate genetic differences, or alcohol-induced brain plasticity involving the ghrelin signalling system, is the key mechanism that eventually leads to addiction. This is something that could be generalized to almost all systems in the brain involved in the effects of alcohol dependence and addiction, which also is in line with the individual centred and drug centred theories of dependence (*vide supra*). Though, a combination of both is most likely. In the case of the ghrelin signalling system, inherited genetic modifications of this system could increase the vulnerability for the effects of alcohol, which in turn, tentatively by epigenetic mechanisms, could alter the expression and function of the ghrelin signalling system. Ultimately this would cause enhanced alcohol-reward as well as alcohol-seeking behaviour, and the development of dependence. This is in accordance with the

hypothesis about a general reward mechanism, where ghrelin might play a part, that could be triggered by different environmental stressors such as drugs or addictive behaviours, and could also be the explanation for the high degree of co-morbidity for different addictive behaviours. Thus, we found associations between haplotypes of the *GHRL* and *GHSR* and increased sugar intake in individuals with a moderate/high alcohol intake as well as with increased body weight in heavy alcohol consuming individuals.

No evident sex differences could be found regarding the genetics of the ghrelin signalling system. Conversely, it is known that there are sex differences in plasma ghrelin levels (Makovey *et al.*, 2007; Wurst *et al.*, 2007). As women are known to be more sensitive to the addictive properties of alcohol (Mann *et al.*, 2005), perhaps according to the hypothesis above, women are more sensitive than men to the alcohol-induced brain plasticity that leads to dependence, and thus their ghrelin levels are affected differently.

It is known that genetics constitute at least half of the risk of developing dependence. However, it has proven to be difficult to identify these genes. So far, genetic association studies using the candidate gene approach has been the most common approach in the addiction field, as well as in this thesis. With the advancement of GWAS, and the success of using this approach in other complex disorders such as obesity, perhaps this is the way to go also in the addiction field. However, the few attempts that have been made for alcohol dependence, have so far been quite disappointing. The drawbacks of a GWAS are that a really large number of subjects need to be included. Further, as thousands of genetic markers are analysed, the problem of multiple testing increases and needs thorough correction, which in turn increases the likelihood of missing true associations. This can be a problem in complex disorders, such as alcohol dependence, where a large number of markers each exert a small effect on the outcome.

Another problem, with the association studies conducted today, as well as with the studies of this thesis, is the lack of functional verifications of the associated markers, making it hard to draw conclusions about what these associations mean. Perhaps the way to go in the future is to move towards what is sometimes called "functional genomics", where genotype data from

large genomic studies are functionally characterized. It is suggested that these studies should be hypothesis-driven regarding which genetic markers to include, and involve a large number of well-characterized alcohol-dependent patients where the genes should also be investigated regarding mRNA and protein levels as well as epigenetic modifications.

The main findings from the animal studies in this thesis are that; (3) GHS-R1A antagonism reduces alcohol intake in a genetic rat model of high alcohol consumption (4) the GHS-R1A gene expression is higher in high alcohol consuming rats than it is in low alcohol consuming ones in several reward-related brain areas (5) suggestively, alcohol counteracts the reduction of plasma ghrelin levels over time.

The fact that the GHS-R1A antagonist effectively reduces alcohol intake in several rat and mouse models makes it a promising candidate for treatment of alcohol dependence. As ghrelin was thought to be the cause of obesity when discovered, several GHS-R1A antagonists have been developed, and it would be of interest to investigate their effects on alcohol intake in humans, preferably in subtypes of alcohol-dependent patients with co-morbid eating disorder. Since the treatments of alcohol dependence available today have beneficial effects for some patients but not for others, such individualization of the treatment strategy either based on co-morbidity, patient history or, in the future, based on genetic background might perhaps improve treatment efficiency.

SUMMARY IN SWEDISH/SVENSK SAMMANFATTNING

De flesta personer med alkoholproblem är överens om att dessa har funnits i deras släkt i generationer. Det är förstås svårt att avgöra vad som är arv och miljö, men det verkar som att det finns en relativt stor ärftlig komponent i sjukdomsutvecklingen. Vad denna ärftliga komponent består i behöver klargöras. Det övergripande syftet med detta projekt har därför varit att studera gener involverade i hjärnans belöningssystem, olika varianter av dessa gener samt hur aktiva dessa gener är liksom deras eventuella koppling till alkoholberoende.

En gen är en enhet i DNA-koden som fungerar som ett recept för tillverkningen av ett visst protein. Det mänskliga DNA:t innehåller ca 20 000-30 000 gener. Alla individer har dock inte exakt likadana gener och det är bl.a. detta som gör att vi inte ser likadana ut eller har samma risk för att utveckla sjukdomar. De genvarianter som studeras i denna avhandling kallas för polymorfismer. Förenklat sett innebär en polymorfism att det finns två versioner av en och samma punkt i DNA-koden. Denna enkla förändring i DNA-koden kan t.ex. leda till att proteinet som tillverkas får andra egenskaper, eller tillverkas i för hög eller för låg mängd.

Man vet att alkohol verkar i system i hjärnan som kallas för belöningssystemen. Dessa system är till för att förmedla en belönande känsla av mat, vatten och sex, d.v.s. beteenden som förstärker individens och artens fortlevnad. Dock har det visats att alla beroendeframkallande droger också verkar på dessa system. Vidare tror man att dessa system är av vikt både för motivation och för sökandet efter naturliga belöningar och beroendeframkallande droger.

Då alkoholberoende anses vara en delvis ärftlig sjukdom är det av intresse att utreda vilka gener och vilka polymorfismer som är involverande i den ökade känsligheten för beroendeutveckling hos dessa individer. Alkohol är en substans som inte verkar enligt en enskild mekanism, utan påverkar de flesta typerna av nervbanor i hjärnan (exv. noradrenalin, serotonin och dopamin). Det är därför av intresse att försöka utreda vilka av dessa som är viktiga för alkoholens beroendeframkallande potential. Hypotesen är att några gener är viktigare än andra även om nervbanorna tillsammans medverkar i alkoholens belöningsprofil. På grundval av detta kan det på sikt vara möjligt att

klassificera alkoholberoende individer i olika grupper beroende på hur deras genetiska profil ser ut och därefter skräddarsy behandlingen utifrån detta.

Ett sätt att undersöka genetikens betydelse vid alkoholism är att studera alkoholberoende patienter och jämföra deras gener med friska kontrollpersoner. Detta har gjorts i Arbete I-III i avhandlingen. Ett annat sätt att undersöka genetikens betydelse för alkoholism är att studera råttor som får dricka alkohol och se hur detta påverkar deras genuttryck (dvs. hur aktiv en gen är). I Arbete IV användes dels vanliga laboratorieråttor som fått ett fritt val mellan alkohol och vatten och dels råttor som är specifikt avlade för att dricka mycket alkohol (AA-råttor) eller nästan ingen alkohol alls (ANA råttor). Genuttrycket mättes i olika områden i hjärnan som ingår i belöningssystemet och som är viktiga för att mediera alkoholens effekter. Dessutom mätte vi halten av ett ämne som vi tror är involverat i detta system i blod, liksom effekten av behandling med droger som påverka detta ämne.

I det första arbetet studerades det nikotinerga acetylkolinsystemet. I åtskilliga studier har en koppling mellan rökning och alkoholmissbruk/beroende påvisats. Man har funnit att 80-90% av alkoholisterna är storrökare och att alkoholism är 10-14 gånger vanligare hos rökare än icke-rökare. Det har även visats att nikotin kan förstärka alkoholens belönande effekter. Nikotinreceptorer finns i områden i hjärnan som ingår belöningssystemet. När vi undersökte polymorfismer i generna som kodar för nikotinreceptorerna hittade vi genetiska variationer som kunde kopplas till alkoholberoende, till hög alkoholkonsumtion men även till ökad vikt hos alkoholberoende individer i ett Spanskt patientmaterial. Det finns läkemedel som kan påverka de receptorer som dessa gener kodar för, vilket skulle kunna vara mål för behandling av alkoholberoende.

Flera studier har visat på en koppling mellan belöningssystemet, och de mekanismer som styr energibalansen. Detta faller sig ganska naturligt då båda systemen verkar för individens överlevnad. Ett ämne som verkar vara av betydelse för denna koppling är det aptitstimulerande ämnet ghrelin. Det hittades först i magen och utsöndras till största del därifrån, men finns även i mindre mängd i hjärnan, bl.a. finns ghrelinreceptorer i belöningssystemet. Det finns även en samsjuklighet mellan drogberoende och ätstörningar.

Alkoholister har förändrade ghrelin-nivåer i blodet jämfört med friska kontroller. Kliniskt, har symtom kallade ”matnoja” observerats vid abstinens hos alkoholberoende patienter då de läggs in för behandling, vilket eventuellt skulle kunna förklaras av den ökade halten ghrelin. Eftersom det även verkar finnas likheter mellan alkohol och ghrelin då de båda aktiverar belöningssystemet skulle ghrelin kunna vara involverat i alkoholens belöningsprofil och därigenom påverka alkoholintaget. När vi undersökte polymorfismer i ghrelin och ghrelinreceptor generna fann vi kopplingar mellan dessa genetiska varianter och alkoholkonsumtion samt med ökad vikt hos alkoholberoende individer i det spanska patientmaterialet. Vi undersökte även dessa genetiska varianter i ett svenskt material med alkoholberoende kvinnor. Vi fann inte identiska, men dock liknande effekter i dessa patienter.

Ghrelinsystemet undersöktes också i råttor som vi låtit dricka alkohol under en längre period. Uttrycket av ghrelinreceptorn var högre i alla de undersökta belöningsrelaterade hjärnområdena i de högdrickande AA råttorna jämfört med ANA råttorna. Vidare minskade alkoholkonsumtionen kraftigt i de AA råttor som behandlats med ett läkemedel som blockerar ghrelinreceptorn.

Sammantaget visar denna avhandling att genetisk variation i vissa av receptorerna i det nikotinerga acetylkolinsystemet skulle kunna påverka utvecklingen av alkoholberoende samt påverka kroppsvikten hos alkoholberoende individer. Dessa receptorer kan anses vara potentiella behandlingsmål för beroendetillstånd såsom alkoholberoende och ätstörningar. Dessutom har det visats att generna som kodar för ghrelin och ghrelinreceptorn inte kan anses vara de huvudsakliga riskgenerna för utveckling av beroende, men de skulle kunna leda till en ökad känslighet i hjärnans belöningssystem som, tillsammans med miljöfaktorer kan leda till alkoholberoende. Ifrån djurstudierna kan man konstatera att ghrelinsystemet, om än inte på en genetisk nivå, är involverat i alkoholberoende och kanske främst i reglering av alkoholintag i alkoholberoende individer. Ghrelinsystemet är också involverat i regleringen av kroppsvikt. Därför skulle läkemedel som påverkar ghrelinsystemet kunna vara en ny effektiv behandling för alkoholberoende. Främst skulle denna behandling kunna vara effektiv för alkoholberoende individer med samtidiga ätstörningsproblem.

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