Individual differences in behavior, neurochemistry and pharmacology associated with voluntary alcohol intake

SHIMA MOMENI
Abstract

Alcohol use disorder is a worldwide public health problem and is a disorder with substantial individual variation. There are suggested links between various behavioral traits, comorbid psychiatric diseases and excessive alcohol consumption. Moreover, the endogenous opioid system is involved in alcohol reward and reinforcement, and implicated in the action of alcohol. However, less is known about the complex associations between individual differences in behavior, alcohol consumption, pharmacotherapy response and related neurochemical mechanisms. Experimental animal models are critical for understanding the neurobiological underpinnings of alcohol use disorder.

The overall aims of this thesis were: i) to study the association between behavior and voluntary alcohol intake in outbred rats; ii) to study the association of voluntary alcohol intake, behavior, opioid receptor density and response to naltrexone; and iii) to obtain detailed behavioral characterizations of the animals on the basis of their voluntary alcohol intake.

The results revealed that the multivariate concentric square field™ (MCSF) test was a complementary method for understanding mechanisms underlying various mental states. The MCSF broadened the perspective on risk-related behaviors, including aspects of risk assessment. Individual differences in alcohol intake using the modified intermittent access paradigm enabled analyses of drinking patterns in high and low alcohol-drinking rats. There was an alcohol deprivation effect in high-drinking animals only. The behavior profiling of high alcohol drinking-rats before and after alcohol access suggested that this subgroup was consuming alcohol for its anxiolytic properties. Long-lasting changes were found in the mu and the delta opioid receptors after long-term, intermittent voluntary alcohol intake; some of these changes are in line with findings in humans. The voluntary alcohol consumption and the concomitant response to naltrexone were different for Wistar rats from different suppliers. Moreover, the Rcc Wistar rats may be more suitable for studies of alcohol use disorders due to increasing alcohol intake and the presence of a high-drinking subpopulation with increasing alcohol intake over time. The high-drinking subpopulation showed pronounced effects of naltrexone on alcohol intake.

In conclusion, studies of individual differences increase understanding of variability in behavior, pharmacotherapy response and factors involved in vulnerability of alcohol use disorders.

Keywords: intermittent access, multivariate concentric square field, open field, risk assessment, risk taking, individual difference, behavior, strain variation, endogenous opioid system, naltrexone

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This time, I will be
Louder than my words
Walk with lessons that
Oh, that I have learnt

Hard time,
Seinabo Sey
List of Papers

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


III  Momeni S., Bergström L. and Roman E. Intermittent voluntary alcohol intake and long-term modulation of brain $^3$H-DAMGO and $^3$H-DPDPE binding in outbred Wistar rats. *In manuscript*


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<tr>
<td>ACB</td>
<td>Nucleus accumbens</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AMPA</td>
<td>Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<td>AUD</td>
<td>Alcohol use disorders</td>
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<tr>
<td>BNST</td>
<td>Bed nucleus stria terminalis</td>
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<tr>
<td>C</td>
<td>Center</td>
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<tr>
<td>CG</td>
<td>Cingulate cortex</td>
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<tr>
<td>CPU</td>
<td>Caudate putamen</td>
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<tr>
<td>CRL</td>
<td>Crl:WI</td>
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<tr>
<td>CTRCI</td>
<td>Central circle</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala², N-MePhe⁴, Gly-ol]-enkephalin</td>
</tr>
<tr>
<td>DCR</td>
<td>Dark corner room</td>
</tr>
<tr>
<td>DOP</td>
<td>Delta opioid receptor</td>
</tr>
<tr>
<td>DPDPE</td>
<td>D-Penicillamine(2,5)-enkephalin</td>
</tr>
<tr>
<td>DSM</td>
<td>Diagnostic and statistical manual of mental disorder</td>
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<td>GABA</td>
<td>Gamma aminobutyric acid</td>
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<td>HCR</td>
<td>High cocaine responder</td>
</tr>
<tr>
<td>HD</td>
<td>High drinking</td>
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<tr>
<td>HR</td>
<td>High responder</td>
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<tr>
<td>HRA</td>
<td>High risk assessing</td>
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<tr>
<td>HRT</td>
<td>High risk taking</td>
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<tr>
<td>IC</td>
<td>Inner circle</td>
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<td>ICD</td>
<td>International classification of diseases</td>
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<td>ID</td>
<td>Intermediate drinking</td>
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<tr>
<td>KOP</td>
<td>Kappa opioid receptor</td>
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<tr>
<td>LD</td>
<td>Low drinking</td>
</tr>
<tr>
<td>LCR</td>
<td>Low cocaine responder</td>
</tr>
<tr>
<td>LR</td>
<td>Low responder</td>
</tr>
<tr>
<td>LRA</td>
<td>Low risk assessing</td>
</tr>
<tr>
<td>LRT</td>
<td>Low risk taking</td>
</tr>
<tr>
<td>M1/M2</td>
<td>Motor cortex (primary and secondary)</td>
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<tr>
<td>MCSF</td>
<td>Multivariate concentric square field™</td>
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<tr>
<td>MEPD</td>
<td>Medial amygdaloid nucleus, posterodorsal</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OC</td>
<td>Outer circle</td>
</tr>
<tr>
<td>OF</td>
<td>Open field</td>
</tr>
<tr>
<td>RCC</td>
<td>RccHan™:WI</td>
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<tr>
<td>RSG</td>
<td>Retrosplenial granular cortex</td>
</tr>
<tr>
<td>S1BF</td>
<td>Primary somatosensory cortex</td>
</tr>
<tr>
<td>SAP</td>
<td>Stretched attend posture</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TAC</td>
<td>HanTac:WH</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction

Alcohol use disorders
The condition alcohol use disorders (AUD) comprise a person's abuse and addiction to alcohol. Alcohol use occurs globally and is rooted in many cultures, even if variations exist among different countries and societies. According to the World Health Organization (WHO), 3.3 million people—5.9% of the world population—die every year as a result of alcohol use (WHO 2014). Europe has the highest alcohol consumption in the world which, after tobacco-use and high blood pressure, is the third largest risk factor for morbidity and mortality in Europe (WHO 2014). The estimated number of alcohol abusers varies in surveys since the concept and definition of abuse differs. Sweden is estimated to have about 285,000 men and 161,000 women alcohol abusers, which is considered to be 5.9% of the Swedish population (CAN 2014). Besides the direct impact on the health of the patient suffering AUD, it entails social consequences and creates an economic burden for the society.

Diagnose criteria and classifications
Various manuals are used in diagnosing AUD. WHO uses the International Classification of Diseases (ICD) system, currently in its tenth edition (ICD-10), which is considered to be the most widely-used statistical classification system for diseases in the world (WHO 2010). The Diagnostic and Statistical Manual of Mental Disorders (DSM) is also frequently used, introduced by the American Psychiatric Association in 1952. Currently in its fifth edition, the DSM-5, provides standardized criteria for mental disorders (Roehr 2013). DSM-IV separated alcohol disorders into abuse and dependence, whereas DSM-5 combines the two into one, termed AUD (Casey et al. 2012). The DSM-5 criteria for AUD are shown in Figure 1.
Figure 1. Alcohol use disorders (AUD) according to DSM-5. A minimum of two symptoms are required to diagnose an AUD. AUD can be classified as mild (two to three symptoms), moderate (four to five) or severe (6 or more). Figure modified from (NIH 2013).

### Impulsivity in alcohol use disorders

Impulsivity is a multifactorial phenomenon and there are a number of definitions of the construct of impulsivity. An elegant summary is provided by Daruna and Barnes (1993): "The behavioral universe thought to reflect impulsivity encompasses actions that appear poorly conceived, prematurely expressed, unduly risky or inappropriate to the situation and that often result in undesirable consequences". Its complex construct includes inadequately sampled sensory evidence, failure of motor inhibition, intolerance to delay of gratification or delay aversion and risk-taking behavior in the context of decision-making (Dalley et al. 2011; Pattij and Vanderschuren 2008).

DSM-5 includes criteria for impulsivity or impulsive behavior (Roehr 2013), not as separate diagnoses but in different standards that diagnose a specific disorder. One disorder highly associated with impulsive behavior is substance use disorders, wherein AUD is included. Among the criteria for AUD that indicate poor impulsive control are the use of the substance longer than intended, the inability to control substance use, and continuing the use despite being aware of physical and/or psychological consequences (Figure 1) (Evenden 1999).

Impulsive behavior is associated with excessive alcohol use. This personality trait is more pronounced in people with AUD and populations at risk for excessive alcohol intake, such as children of parents with AUD. The association between impulsivity and alcohol has been studied using tasks
such as the 5-choice serial reaction time, delay discounting, and probability discounting instrumental response. The results support links between impulsive-like behavior, excessive alcohol intake and the propensity to develop AUD (Lejuez et al. 2010).

Risk-taking behavior and decision-making processes are considered to be aspects of impulsivity. Risk-taking and decision-making are involved in the development and maturation of many mental functions, but, when impaired, they are associated with negative consequences (Balogh et al. 2013). For instance, individuals scoring high on the personality trait novelty-seeking are characterized by exploration in response to novelty, impulsive decision-making, and extravagance in approach to reward cues (Leggio et al. 2009), all of which are associated with risk-taking behavior. Increased novelty seeking and/or risk taking are associated with an augmented predisposition to rewarding and addictive behaviors (Balogh et al. 2013; Blanchard et al. 2009; Laviola et al. 1999; Leggio et al. 2009).

Types of AUD are categorized by behavior traits underlying the structure of the disorder (Leggio et al. 2009). Impulsive behavior is mostly associated with the type of alcohol use that is defined by early onset, spontaneous alcohol seeking, and a highly aggressive behavior (Cloninger et al. 1988; Dom et al. 2006; Dougherty et al. 2004). When these features become more dominant, the risk for developing AUD increases and consequently, excessive alcohol use exacerbates them (Noel et al. 2007). This reciprocal process further complicates the assessment of whether pre-existing personality traits and/or behavioral changes are a result of excessive alcohol use or if they are the main determinant in initiating this form of AUD. This question, together with poorly understood mechanisms associating impulsive behavior to AUD, remains unanswered and needs research (Lejuez et al. 2010; Stautz and Cooper 2013).

Anxiety in alcohol use disorders

Anxiety, graded from mild to severe, is characterized by a feeling of unease, such as worry or fear, and is a normal reaction to stress. When the reaction escalates to severe and the worry and fear are excessive and difficult to control, anxiety can affect daily life. Anxiety disorders are among the most common mental disorders and a wide variety of subtypes are classified. Some categories of anxiety disorders have been revised in the DSM-5, by splitting them into distinct groups. The developmental approach of disorders is an additional revision, as it now includes children and older adults (Mohr and Schneider 2013).
Anxiety disorders are frequently associated with AUD, where either condition is sufficient to initiate and sustain the other in a reciprocal fashion; the anxiety can also contribute to the maintenance and relapse of AUD (Kushner et al. 2000). The link between anxiety and alcohol intake and whether anxiety increases the vulnerability for excessive alcohol intake are two debated questions. As alcohol consumption does have acute anxiolytic effects, anxiety is a motivation for drinking alcohol (Spanagel et al. 1995). However, alcohol simultaneously activates the body’s stress response systems (Becker 2012). A confounding problem in studies on anxiety and AUD is that diagnoses of anxiety are often based on subjective self-reporting. It is therefore difficult to deduce whether the AUD or the anxiety were initially present, or which of them is the cause and the consequence (Langen and Fink 2004).

Genes and environmental factors
Genetic and environmental factors, particularly in early life, play an important role in the risk of developing AUD (De Bellis 2002; Kaufman et al. 2007; Nylander and Roman 2013). The individual variation in vulnerability to AUD and level of heritability are approximately 50% or more. Because AUD is a polygenic disorder with complex underlying aspects, the search for specific genes responsible for vulnerability to the disorder is complicated (Ducci and Goldman 2012; Pautassi et al. 2010). The change from habitual alcohol intake to an addictive one is individually different, and strongly influenced by early life environment (De Bellis 2002). Negative early life experiences such as abuse or insecure environmental elements are major risk factors for developing psychiatric disorders later in life, e.g., depression and anxiety (Gibb et al. 2007), which show comorbidity with AUD (Cerda et al. 2010; De Bellis 2002; Kushner et al. 2000). The mechanisms behind the gene–environment interactions and the susceptibility of developing AUD are not fully understood, and more knowledge is necessary to understand the underlying features that contribute to AUD (Ducci and Goldman 2012; Kalsi et al. 2009; Strat et al. 2008).

Mechanisms and brain regions of importance
Many neural pathways and neurotransmitters, including dopamine (DA), γ-aminobutyric acid (GABA), glutamate, endogenous opioids (see the section Alcohol and the opioid system), serotonin and the endocannabinoids, have been implicated in the effects of alcohol; together these systems interact in a complex manner (Koob and Volkow 2010).

It is well established that alcohol facilitates the activity of GABA, and GABA’s role in the acute effect of alcohol has been extensively studied. The
GABA_A receptor has been implicated in the motor impairment and anxiolytic effects of alcohol (Santhakumar et al. 2007). Studies of the GABA_B receptor have demonstrated the important role of this receptor in decreased self-administration, alcohol deprivation effect and alcohol craving (Agabio et al. 2012). Glutamate receptors, the most abundant excitatory neurotransmitter system in the mammalian brain, are also strongly involved in the effects of alcohol. In general, alcohol inhibits the function of the glutamate receptors. The ionotropic N-methyl-D-aspartate (NMDA) receptors exhibit the highest sensitivity to alcohol but α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors are also involved. Despite the availability of knockout animal models and drugs targeting specific receptor subunits, experimental research has not been able to show the exact mechanisms exerted by alcohol at the receptor and receptor subunit level (Holmes et al. 2013).

The dopaminergic system is one of the most well-studied when it comes to alcohol effects. The dopaminergic circuitries in the central nervous system consist of four different dopaminergic pathways—the mesolimbic, the mesocortical, the nigrostriatal and the tuberoinfundibular. The mesolimbic and mesocortical pathways (referred to mesocorticolimbic pathway), known as the "reward pathway", has cell bodies in the ventral tegmental area and projections to the nucleus accumbens, septum, bed nucleus of stria terminalis (BNST), amygdala, hippocampus and medial prefrontal cortex (Di Chiara and Imperato 1988; Koob and Volkow 2010; Soderpalm and Ericson 2013). Almost all drugs of abuse, including alcohol, activate the mesolimbic DA system, resulting in an increased DA release in the nucleus accumbens (Di Chiara and Imperato 1988; Koob and Volkow 2010; Soderpalm and Ericson 2013). DA, together with the endogenous opioid system, activates the positive reward pathways and has been implicated in the reinforcing effects of alcohol (Trigo et al. 2010). The DA-alcohol connection was established when alcohol was shown to increase extracellular levels of DA; moreover DA receptor antagonists decrease alcohol consumption (Soderpalm and Ericson 2013).

The extended amygdala is another pathway of importance for reward mechanisms and is composed of the central amygdala, the BNST and a transition zone in the medial (shell) subregion of the nucleus accumbens (Koob and Volkow 2010). This basal forebrain circuitry is also involved in the reinforcing effect of alcohol as administration of GABA antagonists to this area, specifically the central nucleus of the amygdala, decreases alcohol intake in animal studies (Roberto et al. 2012).

The striatum is a major DA-containing brain area. The progression from controlled alcohol intake to addiction has been hypothesized to involve a
shift from the acute reinforcing effects in the nucleus accumbens to recruitment of the dorsal striatum in compulsive intake (Everitt and Robbins 2013; Koob and Volkow 2010).

The fronto-striatal circuitry—anterior-cingulate cortex, orbitofrontal cortex and subthalamic nucleus—plays an important role in inhibitory function, impulse control and decision-making. A lack of prefrontal regulation on mechanisms of attention and inhibition has been suggested to infer a risk for AUD (Tessner and Hill 2010; Winstanley et al. 2010).

**Pharmacotherapy in alcohol use disorders**

The present pharmacotherapy approved for AUD treatment is limited to only four substances: disulfiram, acamprosate, naltrexone and nalmefene (Nutt and Rehm 2014). They all reduce alcohol intake and/or increase time spent abstinence by various mechanisms. Disulfiram has an aversive mechanism, causing a severe physical reaction when mixed with alcohol, whereas acamprosate causes anti-craving effects and naltrexone and nalmefene block the rewarding effects of alcohol (Muller et al. 2014). These treatments show wide individual differences in efficacy and only moderate therapeutic benefits, thus highlighting the urgent demand for more effective and individualized treatments. It also emphasizes the need for a more complete knowledge of the neural mechanisms of alcohol to understand the heterogeneity among patients with AUD (Heilig et al. 2011; Kranzler and McKay 2012).

**Individual differences in alcohol use disorders**

The heterogeneity among AUD patients is extensive and to understand the complex etiology behind AUD is challenging. AUD patients diverge in many aspects such as age of onset, pattern of drinking and comorbidity with other psychiatric disorders.

Categorizations of AUD patients into subgroups have been made in order to understand the individual differences, to further identify prevention needs, and to benefit the treatment process by matching subtypes with individualized treatment (Leggio et al. 2009). Clinicians as early as the 1850s grouped AUD patients into a variety of subtypes, although no further empirical investigations were made. In the 1960s, Dr. Jellinek categorized AUD subtypes as alpha, beta, gamma, delta, and epsilon, with most focus on delta and gamma. Delta patients were more socially focused and could not abstain from alcohol, whereas gamma patients were able to abstain from alcohol, but demonstrated a higher psychological risk and showed loss of control when drinking (Jellinek 1960). In 1981 Cloninger and colleagues
introduced Type I and II AUD patients, on the basis of genetic data from a group of Swedish adopted sons of patients with AUD. Type I and II patients differ in the age of AUD onset, genetic predisposition, environmental factors and comorbidity of psychiatric disorders. Type I patients have late onset of AUD, self medicate with alcohol, have the ability to abstain and generally respond better to treatment. Type II patients have an early onset of AUD, show a strong genetic predisposition of the disease together with antisocial behaviors, are unable to abstain, and generally show poor response to treatment (Cloninger et al. 1988). In 1992, Babor and colleagues introduced Type A and Type B typologies, based on 17 different characteristics of AUD patients such as personality traits, comorbidity with other psychiatric disorders, level of alcohol consumption and family history of AUD. Type B patients have a more serious form of AUD, similar to Cloninger's Type II patients (Babor et al. 1992).

Over time, the investigations of typologies have expanded and a variety of individual different groupings have been suggested in order to find the most optimal categorization. Type III, a third subtype in addition to Cloninger's two, has been proposed (Hill 1992). Further development of Babor's typology led to the categories of mild, high risk/severity, internalizing, and externalizing AUD patients (Del Boca 1994). Lesch and colleagues suggested a four-group typology—Types I-IV (Lesch et al. 1988; Lesch and Walter 1996) and the more recent typology introduced by Windle and Scheidt (2004), suggests four groups of AUD patients: mild course, polydrug, negative affect, and chronic.

The need of finding reliable ways of categorizing AUD patients based on genetic, biological, physiological, and behavioral data is considerable. Many of the AUD subtypes overlap and no categorization to date has been clearly superior. The ideal typology classification would give greater understanding of AUD, the ability to support prevention, identify individuals at risk, reduce drinking and prolong periods of abstinence, and subsequently support clinical trials to find individualized pharmacotherapy for each subtype. However these categories and classifications must be complex enough to include all AUD patients and to be clinically useful (Kranzler and McKay 2012; Leggio et al. 2009; Pombo and Lesch 2009).

The endogenous opioid system

The opium poppy (*Papaver somniferum*) is one of the most important medical plants in the history of pharmacology. The plant has been cultivated and used for medical and non-medical purposes already by ancient civilizations. Opium is produced through scratching the pod of the plant and
collecting the sticky, white-colored latex exudation that is further dried into a brown resin. The first opioid to be extracted was morphine, which was isolated from opium in 1806 (van Ree et al. 1999). The pharmacological effect of morphine consequently led to hypothesized existence of endogenous receptors and in the early 1970s it was shown that endogenous, stereospecific binding sites for opioid substances exist in the brain (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). Further studies of the binding sites led to the identification of three heterogenous opioid receptors named mu, delta and kappa (Lord et al. 1977; Martin et al. 1976). During the same time, parallel work in two research groups with different scientific approaches, revealed opioid-like activity in brain extracts indicating the presence of endogenous opioid ligands. Two homologous opioid peptides, Leu-enkephalin and Met-enkephalin, were characterized as the first endogenous ligands for these receptors; more peptides have since been identified (Akil et al. 1998; Le Merrer et al. 2009).

The opioid peptides are classified in three main groups: enkephalins, endorphins, and dynorphins (Akil et al. 1998; Terenius 2000). They originate from the precursor proteins proopiomelanocortin, proenkephalin A and prodynorphin, respectively (Kakidani et al. 1982; Nakanishi et al. 1979). Through enzymatic processes, the precursor proteins are cleaved into smaller segments, which are further chemically modified to the final peptide. Thus, beta-endorphin is generated from proopiomelanocortin; enkephalins—including Leu-enkephalin, Met-enkephalin, Met-enkephalin-Arg\(^6\)Phe\(^7\) and Met-enkephalin-Arg\(^6\)Gly\(^5\)Leu\(^8\)—all originate in proenkephalin; and dynorphin A, dynorphin B, neoendorphins and Leu-enkephalin are generated from prodynorphin (Le Merrer et al. 2009). The ligands differ in amino acid sequences and have varied affinities to the opioid receptors, but they all share an NH\(_2\)-terminus with the sequence Tyr-Gly-Gly-Phe (van Ree et al. 1999).

Both the endogenous opioid peptides and the receptors are widely distributed throughout the brain (Akil et al. 1998; Kieffer and Evans 2009; Mansour et al. 1987). The system is important for many basal physiological functions (motivation, reproductive behavior, food and fluid intake), and it is also involved in analgesia, stress reactivity, learning and memory, endocrine regulation, motor function, and reward and reinforcement (Trigo et al. 2010; Van Ree et al. 2000). Additionally, the opioid peptides function as neuromodulators, where their involvement relates to mechanisms important for reward, reinforcement and addiction (Christensson-Nylander et al. 1986; Spanagel et al. 1992).
The opioid receptors

The three opioid receptors mu, delta and kappa are members of the G-protein coupled receptor family. They have seven transmembrane domains and exert their cellular effects via coupling with the GTP-binding proteins $G_\text{i}/G_\text{o}$ (Figure 2). Activation of the receptor consequently leads to inhibition of neuronal activity and a reduction in neurotransmitter release (Kieffer and Evans 2009).

![Figure 2. General structure of the opioid receptors. The filled circles demonstrate homology between the mu, delta and kappa receptor. Reproduced figure with permission from Hoffmann (2015).](image)

With the development of advanced molecular tools, i.e., the ability to isolate and genes, and produce genetically modified animals, the understanding of the receptors has expanded (Kieffer and Evans 2009; Minami and Satoh 1995). The endogenous opioid system has been implicated in the rewarding effects of alcohol. In response to alcohol, the endogenous opioid system activates the positive reinforcement pathways together with DA (Trigo et al. 2010). However, the opioid receptors mediate opposite effects on DA transmission as stimulation of the mu receptor increases DA levels (Shippenberg et al. 1992). Both the mu and delta opioid receptors mediate euphoric effects, whereas stimulation of kappa is associated with dysphoria and results in reduced DA levels (Akil et al. 1998).

The affinity of the three classes of endogenous opioid peptides for their receptors varies. Beta-endorphins have high affinity to the mu and delta
receptors, enkephalins to the delta receptor, and dynorphins to the kappa receptor. However, natural alkaloids and synthetic molecules are also capable of activating these receptors. In addition to the primary classification of the opioid receptors, pharmacological studies give evidence of multiple subtypes of these receptors: mu 1-3, delta 1-2 and kappa 1-3 (Dietis et al. 2011; Pasternak 2014; van Ree et al. 1999). In addition, there are proposals that the opioid receptors exist as receptor heteromers by assembling subtypes into various combinations, thereby displaying distinct pharmacological effects (Brissett et al. 2012; Dietis et al. 2011).

Alcohol and the opioid system

The endogenous opioid system is involved in mediating the reward effect of alcohol. Likewise, it is well established that opioids modulate alcohol intake (Fields and Margolis 2015). It is therefore reasonable to study the link between alcohol and the endogenous opioid system. Despite the wide recognition of the alcohol-opioid relationship, there are still questions and ambiguities, which explains the large number of research groups that focus on the alcohol-opioid link (Fields and Margolis 2015; Gianoulakis 2009; Oswald and Wand 2004; Palm and Nylander 2015). For example, there is a body of work trying to understand the relation between both acute and chronic effects of alcohol on the endogenous opioid system using animal models (Gianoulakis 2009; Seizinger et al. 1983; Trigo et al. 2010).

In general alcohol increases brain activity and thereby also increases opioid neurotransmission, which certainly mediates the effects of alcohol, such as reinforcement. Regarding acute effects of alcohol on the opioid peptides, alcohol induces an increased release of endorphin and enkephalin (Gianoulakis 1990) in a dose-dependent manner in rodent brain (de Waele and Gianoulakis 1993; Lam et al. 2008). Some studies show an acute alcohol-induced increase of met-enkephalin, whereas contrasting studies show no such effects on enkephalin (Oswald and Wand 2004; Seizinger et al. 1983).

In contrast, chronic alcohol exposure generally decreases the positive properties of the endogenous opioid system, but increases the negative properties; however, the results are far from conclusive (Gianoulakis 2009; Koob 2014). Prolonged voluntary alcohol intake increase levels of enkephalin, which is involved in the positive effects of alcohol (Chang et al. 2010; Nylander and Roman 2012), whereas chronic alcohol exposure increases prodynorphin (Nylander et al. 1994) and dynorphin B (Chang et al. 2010), which are both involved in the negative effects of alcohol.
The effects of alcohol on the opioid receptors, as assessed by autoradiography, are varied and the relation appears to be complex. Alcohol reportedly increases mu receptor density in the caudate putamen (Fadda et al. 1999) and nucleus accumbens (Cowen et al. 1999; Djouma and Lawrence 2002) but a decrease has also been shown (Mendez et al. 2001; Turchan et al. 1999). The same is true for the delta receptor—alcohol increases delta receptor density in the substantia nigra pars reticulata, nucleus accumbens and caudate putamen according to one study (Mendez et al. 2004), but another study says alcohol decreases the density in nucleus accumbens (Turchan et al. 1999).

In conclusion, comparing findings of alcohol-induced effects on the opioid system is complicated since the studies differ widely on the focus of the parameters studied, such as brain region, part of the opioid system, alcohol paradigm, and variation of the animal species, strains, and lines (Gianoulakis 2009; Oswald and Wand 2004; Palm and Nylander 2015; Trigo et al. 2010; Van Ree et al. 2000). Throughout the years, theories of inherent basal levels of opioid activity have been proposed to describe the role and importance of the endogenous opioid system in the risk of developing AUD. "The opioid deficit theory" suggests that alcohol has a compensatory function in individuals with inherent abnormally low levels of endogenous opioid activity and that alcohol intake increases opioid activity in their brains (Trachtenberg and Blum 1987). "The opioid surfeit theory" suggests that enhanced alcohol intake occurs in individuals with inherent or acquired excess of endogenous opioid activity, which causes craving of alcohol (Reid et al. 1991). The loss of control of alcohol intake occurs when alcohol intake increases to silence the craving, which in turn leads to alcohol-induced opioid activity. A deficit or a surfeit of the basal endogenous opioid system, as vulnerability factors to develop AUD, is engaging theories since the propositions for prevention and treatment of AUD are apparent. However, the prevention and treatment of AUD is more complex than these theories portray (Oswald and Wand 2004).

**Individual differences in the opioid system**

Individual differences in the opioid system and its association with the development of AUD have been studied in both preclinical and human settings, in order to better understand their relation. In addition to genetic and environmental factors (see the section Genes and environmental factors), other aspects have been studied to clarify this intertwined relationship.

Individuals who are at higher risk for AUD, in other words individuals with a family history of AUD, show differences in their basal levels of beta-endorphin and alcohol induced beta-endorphin release compared to
individuals without a family history (Gianoulakis 1993; 1996). Based on this, a theory of beta-endorphin response to alcohol as a biomarker for AUD has been proposed to identify individuals who are at higher risk (Froehlich et al. 2000). However, contradictory results have also been presented, where there was no alcohol induced beta-endorphin response (Dai et al. 2002). Further, in development of the opioid deficit/surfeit theories, a third theory suggests that basal levels of opioid activity do not differ, but that the sensitivity and reactivity of the opioid system does, which causes enhanced alcohol intake in some individuals (Gianoulakis 1996). In a comparison of outbred Wistar rats from different suppliers, there was a variation in voluntary alcohol intake (Goepfrich et al. 2013; Palm et al. 2011b) as well as basal and alcohol-induced differences in endogenous opioid peptide levels (Palm et al. 2012). This underscores that individual differences in the opioid system play an important role in regulating alcohol intake.

The opioid system and pharmacotherapy for alcohol use disorders

The endogenous opioid system is implicated in two of the four substances currently approved as pharmacotherapy for AUD. Naltrexone has been used as pharmacotherapy in patients with opioid use disorders for many years (Martin et al. 1976) and it was approved as pharmacotherapy for AUD patients in 1994. It acts as a long-lasting, opioid antagonist with a preferred binding to the mu receptor; however, antagonistic properties on kappa and delta receptors are also known (Nutt 2014). The most recent pharmacotherapy for AUD is nalmefene, which was approved as pharmacotherapy for AUD in 2013 and acts as an antagonist to the mu and delta receptors and as a partial agonist to the kappa receptors (Bart et al. 2005; Nutt 2014). Both naltrexone and nalmefene block opioid receptors and decrease alcohol consumption in a dose dependent way in both animal and human studies (Gianoulakis 2009; Oswald and Wand 2004).

Naltrexone decreases alcohol intake, reduces alcohol craving, and reduces relapse rate in AUD patients (Nutt 2014; O'Malley et al. 1992; Volpicelli et al. 1992). It also reduces alcohol-induced activity of the dopaminergic system (Benjamin et al. 1993). However, naltrexone shows wide individual difference in treatment efficacy and only moderate therapeutic benefits. To thoroughly understand these differences, studies have focused on the effectiveness of naltrexone in AUD patients characterized according to Cloninger typologies (Kiefer et al. 2008) and multiple studies analyzing genes of importance for therapy (Ashenhurst et al. 2012; Roche and Ray 2015; Thorsell 2013).
More research is needed because of the multifactorial mechanisms of alcohol. It involves various targets of the opioid system, which in turn regulate alcohol intake. Furthermore, there are individual differences in response to treatment. The development of individualized treatment of AUD is urgently needed as well as a better understanding of why there are differences in pharmacological response (Bilbao et al. 2015).

Experimental animal methods

Various animal methods have been developed to study physiological, behavioral and biochemical processes with the aim of replicating the dysfunction underlying human disorders. Animal models allow the study of relationships between behavior and brain mechanisms. The aim is to gain an understanding of the underlying neuronal processes of human behavior (van der Staay 2006).

To fully resemble a human disorder, an animal model should fulfill criteria of validity, i.e. face, predictive, and construct validity. To acquire face validity, the symptoms and treatment of the animal model should resemble those of the condition being modeled. A model exhibits predictive validity when its results can predict conditions for the process being modeled. Finally, construct validity refers to an underlying mechanistic relevance, i.e., the homologous quality of a model to condition (Belzung and Lemoine 2011; van der Staay 2006).

Behavior refers to reactions to internal and/or external stimuli, and is observed as movements and vocalizations and/or the lack thereof. Four descriptors are used to describe behavior. The first of these is occurrence—if a specific behavior is observed or not. The second is latency—time elapsed before the first observation of the behavior. The third and fourth are frequency (how often the behavior occurs) and duration (total time of an occurrence) (Hinde 1970; Martin and Bateson 2007). These descriptive parameters can be brought into functional contexts for interpreting mental states in humans.

Risk assessment and risk taking are fundamental components in decision-making processes. Risk assessment is the investigation of a threat source, including approaching the source or scanning it from a distance. Risk-taking behavior is the willingness to take a risk, with the possibilities of positive and negative consequences of the action (Blanchard and Blanchard 2005).

Today there are behavioral tests to gain a broader understanding of animal’s mental state. Most of these tests are designed to evaluate a specific, predetermined mental state in the animal. In order to increase the number of
dependent variables, the tests are combined into a battery. Disadvantages with this approach include possible carry-over effects that may influence the response from one test to another, inter-test intervals that may impact the outcome, and lack of standardization of the tests in the batteries (McIlwain et al. 2001; Paylor et al. 2006).

In addition to observation of the animal's location in a test arena, complementary ethological behavior parameters can be assessed. For rats, this includes stretched attended postures (SAPs), rearing, and grooming. The SAP is considered a risk-assessment behavior as the animal hesitates to move from its present position but shows inquisitiveness and caution at the same time (Blanchard and Blanchard 2005). Cleaning the body, i.e. grooming, is a natural behavior of the rat and one in which it spends much of its waking time. This behavior is also elicited by novelty (Jolles et al. 1979), indicating that it is a displacement activity in reaction to sudden stimuli and conflict situations. Rearing—when the animal stands on its hind legs, either against a wall in a test arena or freely without support—is a commonly used measure of activity and exploration. Measuring defecation and urination as an indications of stress or fear is also common; however, the validity of this has been questioned (Hall 1934; Lister 1990).

Animal studies in alcohol use disorders

Experimental animal studies have played an essential role in understanding risk factors for AUD, by identifying behavior patterns fundamental to it (Sanchis-Segura and Spanagel 2006; Stephens et al. 2013). However, AUD consists of complex processes and preclinical modeling of this complexity is difficult, if not impossible, to achieve completely. Factors that can be clearly modeled in laboratory animals are: the initiation and maintenance of alcohol consumption, alcohol seeking and relapse (Sanchis-Segura and Spanagel 2006).

The procedures used to study AUD in animals are classified as methods studying drug-induced reinforcement and models studying addictive behavior (Figure 3). The drug-induced reinforcement methods are further divided into 'reinforcing tests' and 'self-administration models' (Sanchis-Segura and Spanagel 2006). In the drug reinforcing tests, a specific dose of the drug is administered to the animal in a forced paradigm, whereas in the self-administration models the animals consume the drug on a voluntary basis. Although the forced paradigm allows a higher level of control, it differs from situations of drug use in humans and therefore lacks face validity. Another limitation of the forced paradigm is the impossibility of studying individual predisposition to excessive alcohol intake, whereas self-administration models allow the animals to voluntarily decide to consume
the drug or not (Samson and Czachowski 2003). Self-administration models are widely used in basic drug addiction research and preclinical evaluations of abuse potential. The self-administration models are split into 'operant models', where animals are required to produce a response in order to gain access to the drug, and 'home-cage drinking models' where the animals are presented the drug in the home cage and voluntarily choose to consume it or not (Figure 3) (Samson and Czachowski 2003; Sanchis-Segura and Spanagel 2006). Compared to human consumption, the operant models are considered to be valid and reliable and show good reproducibility. The 'home-cage drinking model' is mainly restricted to studies of voluntary alcohol intake and shows both a stable face and construct validity, thus mimicking human alcohol consumption (Meisch and Lemaire 1993). The 'home-cage drinking model' has further been developed into different drinking paradigms to implement various schemes for different study intents.

<table>
<thead>
<tr>
<th>Experimental animal models for Alcohol Use Disorders</th>
<th>I) Drug-induced reinforcement</th>
<th>A) Self-administration models - Operant models</th>
<th>B) Reinforcing tests</th>
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<tr>
<td>II) Addictive behavior models</td>
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*Figure 3.* Overview of the most frequently used animal models for studies of AUD.

**Animal studies in individual differences**

Increasingly, the alcohol research is shifted towards highlighting the importance of studying individual differences and the attention on variability within disorders is emphasized. Animal models that detect vulnerable profiles and characterize individual response are important for understanding individual differences and variability in disorders and animal models that consider individual differences or apply these as a strategy are highly valuable. While standardized animal models based on individual differences in AUD research are still not common, there is a trend towards them. Greater knowledge concerning individual differences in animal models may help predict individuals at risk and assist in clinical trials to find optimal therapy and higher selectivity for treatment for all individuals (Harro 2010).

One model for detection of individuals vulnerable for substance abuse uses rats that are high or low responders to novelty. The animals are exposed to a novel environment. Based on their locomotor activity in this environment, they are classified as high (HR) or low responders (LR). HR animals are also more sensitive to the rewarding effects of psychostimulants compared to LR
animals (Blanchard et al. 2009; Piazza et al. 1989). Based on the HR/LR model and its effect on psychostimulants, a new model has been developed based on the animal's direct behavioral response after cocaine administration. These rats are classified as High Cocaine Responders (HCR) or Low Cocaine Responders (LCR) (Sabeti et al. 2002).

Methodology
Voluntary alcohol intake

The most critical and dependent experimental variables for voluntary alcohol consumption in rodents are the alcohol concentration and the access paradigm. Rodents consume liquids with low and high concentrations of alcohol very differently. Low concentrations have a mild sweet taste and are preferred over water (Richter and Campbell 1940), whereas high concentrations have a bitter, aversive taste and are rejected by the animal (Samson and Czachowski 2003). This results in a difficult but very important challenge in study design, since too low concentrations may not lead to sufficient pharmacological levels in the brain, but a voluntary consumption of the higher concentrations is difficult to achieve. To experimentally modify the consumption pattern, different drinking paradigms have been developed. The most common free-choice home-cage drinking paradigms have continuous, limited and intermittent access (Becker 2013). In continuous access paradigms, the animals have continuous access to alcohol and water for 24 hours during the whole experimental period. In limited access paradigm, the animals have access to alcohol together with a bottle of water for a limited time during their most active period, e.g., for 2 hours per day. The rats are most active during the dark phase of the light/dark cycle and having alcohol present in the home cage during the active phase consequently generates a higher intake. The limited access paradigm is used to model binge-like drinking and suitable for measurement of blood alcohol concentrations (Becker 2013). In the intermittent access paradigm, the animals have scheduled access to alcohol with repeated deprivation periods in between (Wayner et al. 1972; Wise 1973). Scheduled intermittent alcohol access results in higher alcohol intake compared with continuous access (Simms et al. 2008; Wise 1973) and also results in an increased intake over time (Simms et al. 2008).

The neurobiological explanation as to why the different paradigms show varied results is uncertain. These paradigms have further been elaborated on where parameters, including availability of one or several alcohol concentrations, the length of the alcohol period, the time of the limiting
hours, and the intermittent schedule, are varied in order to optimize the models for specific AUD research questions.

In this thesis voluntary alcohol intake was studied using the intermittent access paradigms (Paper I) and a modified intermittent paradigm (Paper II-IV). Further, rats classified as high drinking (HD) or low drinking (LD) animals were studied to obtain a detailed description of drinking patterns in rats with a high and low voluntary alcohol intake, respectively.

The open field test
The open field (OF) test is conducted in an open arena divided into zones. It was first introduced by Hall (Hall 1934) who used this arena to test emotional responses in rats in a novel environment, as measured by defecation and locomotion. Today it is a very common behavior test for animals to measure locomotion, thigmotaxis and exploration in a novel environment. Rodents naturally tend to remain close to the walls in a novel environment, avoiding open, central areas—this is known as thigmotactic behavior. Size, shape and level of illumination can vary in the OF. Because light conditions influence exploration of an open area, it is important to adjust the level of illumination depending on the study intent (Roman and Arborelius 2009; Walsh and Cummins 1976). The OF test was used in the present thesis for the characterization of risk-taking behavior. Animals were classified as high- (HRT) or low-risk taking (LRT), on the basis of time spent (duration) in the inner part of the OF.

The multivariate concentric square field™ test
The multivariate concentric square field™ (MCSF) test is an ethologically based test. Unlike many other common tests, it is unprejudiced with regard to mental condition. The MCSF tests the animals’ native behavior as the animal can choose between qualitatively different zones (Meyerson et al. 2006). Its multivariate feature gives the animal a free choice to move in different environmental settings, such as open or sheltered areas, areas with different lightning conditions, etc., within the same test. This allows for a more diverse characterization of behaviors, i.e., a behavioral profile, which generates many possibilities for understanding mechanisms behind various mental states (Meyerson et al. 2006; Meyerson et al. 2013).

The test arena consists of a square field surrounded by an outer wall (Figure 4). The square field is further divided into a central square called the center, and peripheral corridors by inner walls. Within the square shaped center, there is a circular zone, the central circle. From the center the animal can access the surrounding peripheral corridors through openings in the middle
of the walls. The corner end of one of the corridors is closed by walls and covered with a lid, providing a dark corner room (DCR) with only one entrance. At the corner end of the next corridor is an elevated zone located above the floor surface, known as the hurdle, which contains a hole board with a photocell device recording nose pokes into the holes. In another corridor, a stainless steel wire mesh bridge construction is located. The bridge has a transparent outer wall and this zone is more brightly illuminated compared to the other zones. An ascending slope leads up to the bridge. At the start of the test, the animal is placed in the center facing the wall without an opening (Figure 4).

![Figure 4](image)

*Figure 4. The MCSF arena (100 × 100 cm) with the defined zones as follows: 1) center, the open central area; 2-4) corridors, transit areas; 5) dark corner room (DCR), a sheltered area; 6) hurdle, an elevated area with a hole board as an exploratory incentive; 7) slope, the slope leading up to the bridge where the animal has to assess the risk of visiting the bridge; 8) bridge, an elevated and brightly illuminated bridge construction considered as an area associated with risk; 9) central circle, the area most associated with risk.*

The MCSF has been validated for areas associated with risk and shelter. This was done in experiments with food-deprived males and lactating dams. If the researcher placed food pellets (for the males) or pups (for the females) on the bridge, the rats relocated the food, respectively pups, to the DCR. However, when food pellets or pups were placed by the researcher in the DCR, no repositions occurred (Meyerson et al. 2006).

An operational categorization of the various parameters with regard to function (i.e. general activity, exploratory activity, risk assessment, risk taking and shelter seeking) is used in the interpretation of results. In addition a rank-order procedure referred to as the trend analysis is used (Meyerson et
This analysis takes individual strategies within a functional context into consideration and correlates the parameters. Values for parameters for each functional category are ranked so, the animal with the highest score is given the highest rank, the animal with the lowest score is given the lowest rank, and the ranked values are summed into a sum rank for each functional category (Meyerson et al. 2013).

In the present thesis the MCSF test was used to assess behavioral profiles before and after access to alcohol. Further, the MCSF test was used for characterization of risk-assessment behavior as animals were divided into high risk assessing (HRA) and low risk assessing (LRA) based on the trend analysis in the MCSF test.

The Y-maze

![Y-maze schematic](image)

*Figure 5. Schematic illustration showing zones A, B and C of the Y-maze test and examples of a correct and an incorrect alternation.*

The Y-maze has been used for many years for pharmacological studies in rodents. The maze allows the animal to move and explore freely the novel arena without stressful handling of the animals. The arena consists of three arms (zones A, B and C) oriented at a 120° angle relative to each other with a central triangular area (mid-zone). The arena allows measurement of the variable spontaneous alternation, i.e., the exploratory rotation of the animal. A correct alternation is defined as the animal visiting all arms in a sequential fashion without interruption in arm choice. Thus an alternation of, for example, A to B to C is correct (Figure 5A) whereas A to B to A is incorrect (Figure 5B), and the percent correct alternations is this number divided by the total number of alternations (Lalonde 2002). Many studies have used the Y-maze for studying locomotor activity and spatial working memory (Lalonde 2002). However it has also been used to assess cognitive function (Lalonde 2002; Pickering et al. 2015; Pioli et al. 2008), which is known to be
highly affected by excessive drug use (Bazov et al. 2013; Goldstein and Volkow 2011). In the present thesis the Y-maze was used to study spontaneous alternations, as assay of cognitive function in association with voluntary alcohol intake.

Receptor autoradiography

The history of autoradiography dates back to as early as 1867 when an emulsion of AgCl and AgI accidently was blackened by uranium salt. This led to the discovery of radioactivity and to methods with detection by radioactivity, one of these being autoradiography (Barthe et al. 2012). An illustration of the theory behind the autoradiography technique is shown in Figure 6.

A certain amount of a radioactive ligand is added to a specimen, which is then placed in contact with a photographic emulsion. The radioactive ligand

Figure 6. Schematic illustration of the autoradiographic procedure.
emits particles and provides an image of the distribution of the radioactivity in the specimen. The photographic emulsion contains silver halide crystals (Ag+) which are reduced to Ag atoms as black silver grains when activated by the radioactive emission (Pelc and Welton 1967). This produces a permanent image, which is made visible through developmental and fixing processes. In the present thesis three specific ligands were used—$^3$H-[D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin (DAMGO) for mu receptors, $^3$H-D-Penicillamine(2,5)-enkephalin (DPDPE) for delta receptors and $^3$H-U96,593 for kappa receptors—to detect the opioid receptor density.
Aim

In alcohol research there are suggested links between various behavioral traits and excessive alcohol consumption. Chronic alcohol intake can result in a shift from alcohol use to alcohol addiction via mechanisms that are progressively being revealed and understood. However, less is known of the complex associations between individual differences in behavior, alcohol consumption, and related brain mechanisms.

The overall aims of the four studies in this thesis were:
1. To study the association between behavior and voluntary alcohol intake using two intermittent-access paradigms in outbred rats.
2. To study the association of voluntary alcohol intake and neurochemistry.
3. To study whether the level of voluntary alcohol intake differed with respect to the behavior before and after access to alcohol.
4. To study the association between behavior and voluntary alcohol intake, and the subsequent response to naltrexone.
5. To obtain detailed behavioral characterizations of the animals on the basis of their voluntary alcohol intake.

Specific aims:

**Paper I:** To investigate the association between individual differences in risk-related behaviors, voluntary alcohol intake, and preference using an intermittent access paradigm.

**Paper II:** To study voluntary alcohol intake using a modified intermittent access paradigm and to investigate behavioral profiles before and after alcohol access.

**Paper III:** To analyze the long-term effects of voluntary intermittent alcohol intake on the density of brain opioid receptors in Wistar rats.

**Paper IV:** To study individual differences in Wistar rats from three different suppliers in: OF activity, Y-maze performance, intermittent voluntary alcohol intake, and response to naltrexone treatment.
Materials and methods

Animals
In Paper I, 40 outbred male Sca:WI rats were ordered from Scanbur BK AB (Sollentuna, Sweden). Ten of the animals were used in the pilot experiment and the remaining 30 animals were used in the main experiment. In Papers II and III, outbred male RccHan™:WI rats were ordered from Harlan Laboratories B.V. (Horst, The Netherlands). In paper IV, 20 outbred male RccHan™:WI animals were ordered from Harlan Laboratories B.V., 20 outbred male HanTac:WH from Taconic Farms A/S (Ejby, Denmark), and 20 outbred male Crl:WI from Charles River GmbH (Sulzfeld, Germany). The animals were housed 3-4 per cage in transparent cages (59 × 38 × 20 cm) containing wood-chip bedding material and two paper sheets (40 × 60 cm; Cellstoff, Papyrus). All cages were placed in temperature-controlled (21 ± 1°C) and humidity-controlled (50 ± 10 %) housing cabinets with a reversed 12 h light/dark cycle. The rats were maintained on rat chow (R36; Lantmännen, Kimstad, Sweden) and water ad libitum. The test rooms were kept at similar conditions as the housing room and all rooms had a masking background noise to minimize unexpected sound disturbance to the animals. All animal experiments were approved by the Uppsala Animal Ethical Committee and followed the guidelines of the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998: 56) and the European Communities Council Directive (86/609/EEC).

Voluntary alcohol intake
In Paper I, voluntary alcohol intake was studied using the intermittent access paradigms, whereas Papers II, III and IV used a modified intermittent paradigm. The animals were individually housed (42 × 26 × 18 cm) and given access to alcohol in the home cage. Water and ethanol solutions at room temperature were available in 150 ml plastic bottles with ball-valve nipples (Scanbur). Ethanol solutions at 20% v/v were made from 96% ethanol (Solveco Etanol A 96%; Solveco AB, Rosersberg, Sweden) diluted with tap water. Water and ethanol intake was measured at the end of the 24-hour period by weighing the bottles. Bottle positions were changed for each session to avoid position preference. On days between alcohol sessions, the
animals had access to two bottles of water. In order to minimize disturbance during intake measures, cages were changed and animals were weighed outside of periods of access (Saturdays: Paper I, and Fridays: Paper II-IV).

**Intermittent access paradigm**

In Paper I, the rats were given 24 h access to one bottle of water and one bottle of 20% ethanol (v/v) on Mondays, Wednesdays and Fridays for five weeks (15 sessions in total).

**Modified intermittent access paradigm**

In Papers II, III and IV, the rats were given 24 h access to one bottle of water and one bottle of 20% ethanol (v/v) for three consecutive days (Tuesdays, Wednesdays and Thursdays). In Papers II and III, the alcohol period lasted for seven weeks (21 sessions in total) and in Paper IV for 6 weeks (18 sessions in total)

**Behavior tests**

**The open field test**

The OF test was used in Papers I and IV. The OF (Figure 7) was a circular black arena (90 cm in diameter) with a stainless steel wire-mesh floor (10 mm between bars) enclosed by black stainless steel walls (35 cm high). The level of illumination in the center was 100 lux. The test started by placing the rat facing the wall in the outer circle, and each rat was given 20 min to freely explore the arena.

*Figure 7. The open field arena (90 cm in diameter) with the defined zones center, inner circle and outer circle.*
For analysis, the arena area was divided into zones (Figure 7) — the center (C; 30 cm in diameter), was surrounded by an inner circle (IC; width 15 cm), which in turn was surrounded by an outer circle (OC; width 15 cm). In Papers I the percentage of time spent in the inner circle and center (%D IC+C) was used for classification of risk-taking behavior, thus dividing rats by central activity versus thigmotaxis. In Paper IV, the OF test was used to control for potential differences in activity.

The multivariate concentric square field™ test

The MCSF test was used in Papers I and II. The MCSF arena (Figure 4), the general testing procedure, and the behavioral recording have been described in detail in Methodology and elsewhere (Meyerson et al. 2006; Roman and Colombo 2009). In brief, the arena is divided into zones, which form the basis of the description and the variables of the animals’ performance in this test. The light conditions (lux) in the arena were as follows: dark corner room <1; central circle approximately 20; corridors and hurdle <5-20; slope approximately 30; bridge 600-650. The test started by placing the rat in the center, facing the wall between the center and bridge. The animal was given 20 min to freely explore the arena.

The Y-maze

The Y-maze was used in Paper IV. The Y-maze is constructed of grey, non-reflective plastic and consists of three arms (zone A, B and C) each 50 cm long, 10 cm wide and 20 cm high oriented at a 120° angle relative to each other with a central triangular area (mid zone) (Figure 5). The level of illumination in the arena was 100 lux. The animals were started in arm A facing the mid-zone and allowed to freely explore for 10 min and the percent correct alternations were calculated.

Behavioral recordings

All behavior observations were made during the dark period of the light/dark cycle, and the animals were monitored through camera recording from an adjacent room. The number of rearings and groomings were observed and recorded in the OF and MCSF. In the MCSF, the SAPs and number of head dips into the hurdle hole board were also recorded. The latency (s) of first visiting a zone, frequency of visits, and duration (s) of time spent in a certain zone, and also the number of animals visiting each zone (occurrence) were manually scored using the program Score 3.3 (Copyright Soldis, Uppsala, Sweden). Visits to the defined zones were only scored as such if both hind legs crossed over into that section. For tracking of total distance (cm) and the mean velocity (cm/s) in the arenas, Ethovision version 2.3 (Noldus
Information Technology, Wageningen, The Netherlands) was used (Papers I-II). In Paper IV, all recordings and analyses of behavior were made in the Ethovision version XT10 and the macro Sequence Analysis toolkit (Noldus Information Technology).

**Brain preparations**

In Paper III, brains were collected and prepared for analysis by autoradiography. The animals were decapitated, the brains removed and quickly frozen at -20°C isopentane and stored at -80°C. One hour before sectioning, the brains were transferred to -20°C. The CryoStar™ NX70 Cryostat (Thermo Scientific) was used to section the frozen brains in 12 µm coronal sections. The brains were sectioned at -20°C, at the bregma levels 2.28, -0.72 and -2.76 mm with aid of a brain atlas (Paxinos and Watson 2007). Sections were thaw-mounted on microscope glasses coated with polysine (Polysine™, Menzel-Gläser) and stored at -80°C until further use.

**Receptor autoradiography**

Paper III reports the receptor autoradiography of the opioid receptors. Frozen brain sections on slides were allowed to thaw for 1 hour at room temperature and then pre-incubated in room-temperature buffer containing 50 mM Tris-HCl, pH 7.4, 0.9% NaCl for 30 minutes. Receptor labeling, slightly modified from Kitchen et al. (1997), was started by adding specific ligands (American Radiolabeled Chemicals, Inc, St. Louis, MO, USA) for each receptor: 4 nM ³H-DAMGO (50 Ci/mmol) specific for the mu receptor, 8 nM ³H-DPDPE (57.4 Ci/mmol) specific for the delta receptor, or 4 nM ³H-U96,593 (40 Ci/mmol) specific for the kappa receptor. These were incubated at room temperature in 50 mM Tris-HCl buffer (pH 7.4) for 60 minutes. The unspecific binding was obtained using 1 µM of the opioid receptor antagonist naloxone for the mu receptor and 10 µM for the delta and kappa receptors. Following incubation, the slides were washed 3 times in ice-cold washing buffer (50 mM Tris-HCl, pH 7.4) for 2 minutes each time and thereafter quickly dipped in ice-cold MilliPore water. The slides were subsequently placed to dry overnight, and then exposed to Biomax MR films (Kodak, Sweden) for 8 weeks (mu receptor), 38 weeks (delta receptor) and 56 weeks (kappa receptor) in cassettes together with ³H-microscale standards (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA). The films were manually developed in Kodak D-19 developer (5 min), fixed in Kodak unifix (5 min), rinsed in tap water and scanned using an Epson Perfection 4870 PHOTO scanner.
Receptor densities were quantified by densiometric measurements using the image analysis system ImageJ (Abramoff 2004). Brain structures were identified according to a rat brain atlas (Paxinos and Watson 2007). The optical receptor density readings of the standards were computer-fitted by regression analyses to generate a standard curve and were used to normalize the values for tissue sections exposed to each autoradiographic film. The average of three density values per brain structure from each animal was calculated and corrected for non-specific binding and background film noise. Optical density values were converted to fmol of radioligand specifically bound per milligram of tissue, by reference to the $^3$H-standards.

**Naltrexone treatment**

Pharmacological treatment was given in a Latin square design so that all animals received all doses of naltrexone (0.03, 0.3 and 3 mg/kg) and control (saline) before the first alcohol session. Doses of naltrexone were followed by two wash out sessions each week for four consecutive weeks. Naltrexone (Sigma-Aldrich, Schenndorf, Germany) was dissolved in saline and administered subcutaneously at 1 ml/kg 30 min before alcohol access. Alcohol intake, assayed by bottle weighing, was measured at 30 minutes, 2 hours, and 24 hours after alcohol access; the data are presented as $\Delta$0-30, $\Delta$30-2, and $\Delta$0-24, respectively.

**Statistical analyses**

Classical statistical analyses were performed using Statistica 10 (StatSoft Inc., Tulsa, OK, USA). Differences were considered statistically significant at $p \leq 0.05$. As a first step, the Shapiro-Wilk’s W test was used to define the distribution of the data. For normally distributed data, parametric statistics were used, otherwise non-parametric statistics were used.

**Parametric statistics**

The MCSF trend analysis (Paper II) and the densiometric measurements (Paper III) were normally distributed; hence comparisons between the groups were done using one-way analysis of variance (ANOVA). In Paper II, over-time comparison trials I and II were made using repeated measurements ANOVA. Body weight data were normally distributed; hence the parametric repeated measurement ANOVA was used, followed by the Fisher's Least Significant Difference (LSD) post-hoc test.
Non-parametric statistics
The non-parametric Kruskal-Wallis test was used for group analysis, followed by the Mann-Whitney U-test for between-group comparison of descriptive behavior parameters (Papers I, II and IV) and fluid intake during the period of access to alcohol (Papers I-IV).

The Friedman test, followed by the Wilcoxon matched pair test, were used for within-group analysis of total activity and distance moved during the four 5-minute periods in the MCSF (Paper I). They were also used for within-group analysis of weekly fluid intake (Paper I-II) and for comparing fluid intake over time during the period of naltrexone and saline administrations (Paper IV). Possible correlations were assessed by the Spearman rank order correlations test.
Results and discussion

Four studies were performed to study the associations between behavior, voluntary alcohol intake, neurochemistry and pharmacology.

Paper I studied the association of risk-related behavior and voluntary alcohol intake. The animals were behavior tested before a five-week long period of intermittent alcohol access. In Paper II a different approach was used to study the effect of voluntary alcohol intake on behavior. Here a modified, intermittent drinking paradigm was implemented during a seven-week long period, and the behavior studied before and after the period of alcohol access. The behavior of subgroups of high (HD), intermediate (ID) and low drinking (LD) animals was also studied before and after. In Paper III the same modified intermittent drinking paradigm was used during a seven-week long period to analyze the long-term effects of voluntary alcohol intake on the density of brain mu, delta and kappa opioid receptors using autoradiography technique. In Paper IV, the modified intermittent drinking paradigm was used during six weeks to study individual differences in behavior and its association with voluntary alcohol intake and subsequent response to naltrexone. Wistar rats from three different suppliers were used to create a seamless heterogenic group, thereby mimicking heterogeneity within the human population. In all four studies, outbred Wistar rats were used to maximize heterogeneity for the study of individual differences.

Voluntary alcohol intake

The modified intermittent access paradigm

In Papers II-IV, a modified intermittent drinking paradigm was used with free access to 20% alcohol on three consecutive days per week. In Paper II, the median alcohol intake increased over time with a significant difference between the first and the last week of alcohol access (Figure 8A). The median alcohol preference reflects the alcohol intake and this also increased over time with a significant difference between the first and the last weeks of alcohol access (Figure 8B).
Figure 8. Ethanol intake (g/kg) (A) and ethanol preference (%) (B) during the period of intermittent access to 20% alcohol and water for three consecutive days per week. Data are shown as median and 25-75 percentiles. **p<0.01, ***p<0.001 comparing week seven to week one (Wilcoxon matched pair test).

In Paper III, the animals had access to 20% alcohol for seven weeks. The pattern of weekly alcohol intake and alcohol preference resembles that of the larger group of animals presented in Paper II.

In Paper IV, the animals had access to 20% alcohol for seven weeks using the modified intermittent paradigm. However, the data revealed supplier-dependent differences of such magnitude that the voluntary alcohol intake was analyzed supplier-wise. This is discussed in the section "Voluntary alcohol intake and supplier dependent differences".

Individual differences in voluntary alcohol intake

In Paper II, the animals were further grouped by a tertiary split into low (LD), intermediate drinking (ID) and high drinking (HD) based on the average alcohol intake during the last week of access to alcohol. As shown in Figure 9A, the alcohol intake was significantly higher in the HD group compared to the LD during all weeks of alcohol access except for week four. When comparing the alcohol intake week one to week seven, an increase over time was seen in the HD group only.

The alcohol preference followed a pattern similar to that of the alcohol intake and alcohol preference week one to week seven was higher in the HD group. Furthermore, the HD animals had a significantly higher alcohol intake on all weekdays where alcohol was in access. The modified intermittent drinking paradigm with alcohol access on three consecutive days per week also revealed that the alcohol intake in the HD group was 30% higher on the first weekday of alcohol access (Tuesdays) compared to the second day (Wednesdays) and 20% higher compared to the third day (Thursdays). The pattern looked somewhat different in the LD group with a
significantly lower intake on the second day relative to the first and the third, respectively (Figure 9B).

**Figure 9.** A) Alcohol intake (g/kg) during the period of intermittent access to 20% ethanol and water for three consecutive days per week in low drinking (LD), intermediate drinking (ID) and high drinking (HD) animals. B) Average alcohol intake during Tuesdays, Wednesdays and Thursdays. Data are shown as median and 25-75 percentiles. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 comparing LD and HD groups (Mann–Whitney U-test). #p<0.05, ##p<0.01 compared to the intake on Tuesdays within the respective group, @p<0.05 compared to the intake on Wednesdays within the respective group (Wilcoxon matched pair test).

**Discussion**

The intermittent alcohol access paradigm was used in Paper I. In this paradigm alcohol consumption up to 6 g/kg/24 hours has been reported (Hargreaves et al. 2009; Simms et al. 2008), compared to continuous access where intake varied from 1 to 4 g/kg/24 hours (Gustafsson and Nylander 2006; Hargreaves et al. 2009; Nylander and Roman 2013; Simms et al. 2008).

In Papers II-IV, the use of the modified intermittent access paradigm with three consecutive days of access to 20% alcohol for seven weeks resulted in a higher alcohol intake, a higher preference, and an increased intake and preference over time compared to Paper I. One possible explanation for the higher intake and preference is the choice of Wistar sub-strain used herein (Palm et al. 2011b). The choice of supplier in alcohol studies is further discussed in the section "Voluntary alcohol intake and supplier dependent differences". This new intermittent model was in agreement with previous studies showing a higher alcohol intake and preference compared to studies in which Wistar rats were given continuous alcohol access (Gustafsson and Nylander 2006; Nylander and Roman 2013).

The large individual differences within the alcohol-drinking animals in Paper II allowed the formation of subgroups with high, intermediate and low alcohol intake (Steensland et al. 2012). The HD group had significantly higher alcohol intake and alcohol preference compared to the LD group, and also significantly higher alcohol intake over time compared to them. Not
only did the alcohol intake differ between these groups, but also the drinking pattern. The HD animals had a significantly higher alcohol intake on Tuesdays, after the 4-day alcohol deprivation, compared to Wednesdays and Thursdays, which was not seen in the LD group. This increased intake demonstrates that the HD animals not only consumed and preferred more alcohol, but were also more sensitive to the 4 days of enforced deprivation. This modified intermittent paradigm gives rise to an alcohol deprivation effect in the HD animals (Sinclair and Senter 1968), which to our knowledge has only been reported once when using the intermittent access paradigm (Mill et al. 2013).

Behavior and voluntary alcohol intake

The open field

In Paper I, the rats were divided by a median split into high-risk taking (HRT) and low-risk taking (LRT) groups based on the proportion of the percent duration in the inner part of the OF. However, there were only minor differences in the HRT and LRT groups for weekly alcohol intake (g/kg) (Table 1A) and preference (%) (Table 1B).

Table 1. The weekly (A) median ethanol intake (g/kg) and (B) ethanol preference (%) in low risk-taking (LRT) and high risk-taking (HRT) rats during the weeks of intermittent access to 20% alcohol.

<table>
<thead>
<tr>
<th></th>
<th>LRT</th>
<th>HRT</th>
<th>Mann-Whitney U-test</th>
</tr>
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<tbody>
<tr>
<td><strong>A. Ethanol intake (g/kg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>1.0</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Week 2</td>
<td>0.8</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Week 3</td>
<td>1.0</td>
<td>0.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Week 4</td>
<td>1.0</td>
<td>0.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Week 5</td>
<td>0.9</td>
<td>0.5</td>
<td>5.0</td>
</tr>
</tbody>
</table>

| **B. Ethanol preference (%)** |     |     |                     |
| Week 1 | 10.8 | 9.2 | 17.8 | 10.0 | 8.1 | 29.8 | n.s. |
| Week 2 | 10.1 | 7.2 | 14.8 | 13.6 | 10.0 | 36.5 | U=37.0, p<0.01 |
| Week 3 | 11.2 | 6.9 | 33.8 | 14.8 | 9.3 | 52.3 | U=47.0, p<0.05 |
| Week 4 | 11.1 | 6.4 | 44.2 | 12.8 | 7.5 | 55.1 | n.s. |
| Week 5 | 12.7 | 4.9 | 62.1 | 14.2 | 7.0 | 55.7 | n.s. |

The MCSF

In Paper I, the same animals that had been divided into HRT and LRT groups, were regrouped into high risk assessing (HRA) and low risk assessing (LRA) by a median split based on their risk-assessment behavior in the MCSF trend analysis. The HRA group had higher alcohol intake (g/kg)
than the LRA during weeks four and five (Figure 10A). The alcohol preference (%) also was higher for HRA than LRA in weeks 4 and 5 (Figure 10B). Moreover, HRA rats had increased alcohol intake over time with higher intake and preference at week five relative to week one, while no such difference was found in LRA rats.

![Figure 10](image-url)

*Figure 10. (A) The weekly alcohol intake (g/kg), and (B) preference (%) in high risk-assessing (HRA) and low risk-assessing (LRA) rats. Values represent median and quartile range. **p<0.01, ****p<0.0001 comparing HRA and LRA rats (Mann-Whitney U-test); #p<0.05, ##p<0.01 compared to week one in the respective groups (Wilcoxon signed rank test).*

The Y-maze

The Y-maze was used in Paper IV to study spontaneous alternations as a metric for cognitive function and its possible association with voluntary alcohol intake and preference. However, no such association was found between Y-maze behavior and voluntary alcohol intake or preference.

Individual differences in behavior

The rats in Paper I were divided by a median split into HRT and LRT groups based on the proportion of the % duration in the inner part of the OF. The trend analysis of the functional categories general activity, exploration, risk assessment, risk taking and shelter seeking is shown in Figure 11A. This analysis revealed a significant difference in the category risk taking—HRT rats showed higher risk taking behavior than the LRT rats. The same animals were further regrouped based only on the risk-assessment behavior in the MCSF, i.e., a median split into HRA and LRA. The MCSF trend analysis in HRA and LRA rats is shown in Figure 11B. In addition to the difference in risk assessment, a tendency towards higher risk-taking behavior was found in HRA than LRA rats.
Figure 11. The MCSF trend analysis with sum rank values for parameters included in the functional categories general activity, exploratory activity, risk assessment, risk taking and shelter-seeking behavior in (A) high risk-taking (HRT) and low risk-taking (LRT) rats and (B) in high risk-assessing (HRA) and low risk-assessing (LRA) rats. Values represent median and quartile range. *p<0.05 comparing HRT and LRT rats (Mann–Whitney U-test).

In Paper II, the animals were tested in the MCSF before and after the seven-week period of alcohol access. MCSF trends for the functional categories of general activity, exploratory activity, risk assessment, risk taking and shelter seeking revealed no significant differences between animals in the alcohol and water (control) groups. However, a comparison of the MCSF trend analysis for trials I and II revealed, over time, an interaction between group and duration of exploratory activity. Further post-hoc analyses did not reveal other significant differences. The MCSF before and after the seven-week period of alcohol access was also analyzed for the LD and HD subgroups.

Figure 12. Sum rank values for parameters in the functional categories shelter-seeking (A) and risk taking in the central circle (CTRCI) (B) in low drinking (LD) and high drinking (HD) animals prior to (trial I) and after (trial II) access to alcohol. Values represent mean ± S.E.M. *p<0.05 comparing LD and HD animals (one-way ANOVA).
There were no differences for these two subgroups in the trend analysis of the functional categories general activity, exploratory activity, risk assessment, risk taking and shelter seeking prior to alcohol access. However, when the risk-taking category was split into bridge and central circle, HD rats had less risk-taking behavior in the central circle than LD rats (Figure 12A). Results from the repeated testing after the seven-week period of alcohol access revealed lower shelter-seeking behavior in HD relative to LD rats (Figure 12B).

Discussion

In Paper I, the classification of risk-taking behavior was based on the duration (%) in the inner part of the OF (HRT/LRT), which also correlated well with previously used classifications such as locomotion. This is in line with the classification of animals into high (HR) and low responders (LR) based on activity in a novel environment, which is commonly used to model traits of sensation- or novelty-seeking in humans (Blanchard et al. 2009; Dellu et al. 1996; Kabbaj 2006; Piazza et al. 1989). Thus, the present classification of animals based on risk taking behavior (HRT/LRT) seems to share some traits with the extensively used high/low responder (HR/LR) classification. The classification of HRT and LRT rats was also consistent with risk taking parameters in the more complex MCSF test. These parameters were similar to the OF parameters, i.e., duration in the central circle. In Paper I there was a positive correlation between time spent in the inner part of the OF and risk taking in the MCSF, with HRT rats displaying higher risk-taking behavior than the LRTs in the MCSF. In Paper II, the risk-taking category was further divided into central circle-related parameters and bridge-related ones. Risk-taking behavior is measured by exploration of open areas, and the elevated brightly illuminated bridge is different. Therefore high risk taking in an open area is not necessarily a predictor of high risk taking on an elevated and brightly illuminated surface (Meyerson et al. 2013), in agreement with recent findings (O'Leary et al. 2013). In the MCSF there are several other options to explore and this is most likely the reason for the poor predictability of open field behavior for categories other than risk taking (Meyerson et al. 2006). The discrepancies could also be supplier-dependent, which has shown to differences previously in risk-taking behavior in the MCSF (Palm et al. 2011a).

Despite a lack of differences in MCSF performance between water- and alcohol-drinking rats in Paper II, a difference did emerge when studying the HD and LD animals in more detail. Prior to alcohol access the HD animals displayed a lower risk-taking behavior in the central circle of the MCSF. When tested again after the period with intermittent access to alcohol, the HD animals spent a shorter time in the DCR and had a higher risk/shelter
index value. Together this is interpreted as lower anxiety-like behavior compared to the LD rats. Thus, the HD rats were characterized by a tendency towards lower risk-taking behavior prior to alcohol access and lower anxiety-like behavior after alcohol consumption, compared to the LD rats. This may indicate that the HD animals in this study consumed alcohol for its anxiolytic properties. This finding is in line with many previous studies showing an association of high anxiety-like behavior and high alcohol consumption e.g., (Hayton et al. 2012; Spanagel et al. 1995).

**HRT and LRT**

In Paper I it was hypothesized that HRT rats would have a higher voluntary alcohol intake and preference than LRT rats, but only minor differences were found. Given the strong association between risk-taking behavior and excessive drug intake in humans, the reasons for the outcome in the present study are unclear. It has been suggested that risk-taking behavior is more common in adolescence than in adulthood (Laviola et al. 2003) and therefore its association with alcohol intake may be more pronounced if adolescent animals are used. Moreover, the HRT rats showed a more pronounced reduction of DA uptake than the LRTs in the dorsal striatum after amphetamines, which indicates that differences in this area may contribute to the sensitivity of these animals to the effects of psychostimulants and, in turn, proneness to addiction (Palm et al. 2014). Additionally, it may be that voluntary consumption of alcohol is a too weak reinforcer (Samson and Czachowski 2003) compared to, for example, operant self-administration of psychostimulants, where there are strong associations with activity in a novel environment (Blanchard et al. 2009; Dellu et al. 1996; Kabbaj 2006; Piazza et al. 1989).

**HRA and LRA**

In Paper I, the animals were also divided into groups based on risk-assessment behavior in the MCSF. This resulted in a different outcome than for HRT/LRT regarding alcohol intake. In HRA rats, alcohol intake and preference increased over time and was significantly higher than in LRA animals during the fourth and fifth weeks of access. LRA rats had a similar, lower intake throughout the experiment.

One of the most common measures of risk assessment is the SAP, often interpreted in terms of anxiety-like behavior (Carobrez and Bertoglio 2005; O’Leary et al. 2013). Behaviors in the elevated plus maze, OF and light/dark box tests have been shown to load on different components in a principal component analysis suggesting that each test measures different dimensions. In contrast, SAPs correlate across tests (O’Leary et al. 2013). However it has also been shown that mice display obvious differences in behavioral strategies related to risk assessment and risk taking where risk assessment is
not interpreted in terms of anxiety-like behavior (Augustsson and Meyerson 2004). Previous studies on risk assessment and alcohol intake are sparse. Mice selectively bred for high and low handling-induced convulsions have baseline differences in SAPs but administration of ethanol reduces SAPs in both lines (Atkins et al. 2000). An etho-experimental approach found that administration of alcohol increases risk assessment from a freezing baseline with a threat present, and decreases stretched attend/stretched approach activities in a situation with a less intense threat stimulus (Blanchard et al. 1993).

Palm et al. (2014), studying the relationship between risk assessment and DA, have found correlations between dopamine responsiveness in parameters related to risk assessment and not to the risk-taking parameters used for classification. A fairly strong correlation exists between the classification parameter and parameters used to classify HR/LR rats in previous studies (Dellu et al., 1996; Kabbaj, 2006; Blanchard et al., 2009).

The effect of alcohol on the opioid receptor density

Opioid receptor density

The specific binding of mu and delta receptors was analyzed successfully using autoradiography, whereas the specific kappa receptor binding was not detectable. Representative brain sections of $^3$H-DAMGO- and $^3$H-DPDPE binding are shown in Figure 13.

![Figure 13. Representative autoradiograms from coronal brain sections at bregma 2.28 mm, -0.72 mm and -2.76 mm displaying $^3$H-DAMGO (A) and $^3$H-DPDPE (B) binding.](image)

In the water-drinking animals, quantitative receptor autoradiography using $^3$H-DAMGO and $^3$H-DPDPE resulted in mu and delta receptor densities similar to previous studies (Kitchen et al. 1997; Mansour et al. 1987). $^3$H-
DAMGO binding to the mu receptor and $^{3}$H-DPDPE binding to the delta receptor were compared for the water- and alcohol-drinking animals.

The major finding was a decrease in $^{3}$H-DAMGO binding in the nucleus accumbens shell in the alcohol-drinking animals (Figure 14A). Further, a significant increase in $^{3}$H-DPDPE binding was found in the cingulate cortex area 2, caudate putamen lower part, primary and secondary motor cortex, the retrosplenial dysgranular cortex and medial amygdaloid nucleus, posterodorsal. A decrease of $^{3}$H-DPDPE binding was found in the caudate putamen upper part and the outer layer of the primary somatosensory cortex (Figure 14B).

![Figure 14. $^{3}$H-DAMGO (A) and $^{3}$H-DPDPE (B) binding in alcohol-drinking animals as percent of the binding in water-drinking rats. Data are expressed as mean ± SEM. *p≤0.05 compared to water-drinking animals (one-way ANOVA). Abbreviations: Acb Shell, Nucleus accumbens shell; M1/M2, Motor cortex (primary and secondary); CG2, Cingulate cortex (area 2); Cpu upper, Caudate putamen upper part; Cpu lower, Caudate putamen lower part; RSG, Retrosplenial granular cortex; S1BF outer, Primary somatosensory cortex (outer layers); MePD, medial amygdaloid nucleus, posterodorsal.](image)

Discussion

Paper III aimed to analyze the long-term effects of intermittent voluntary alcohol intake on the density of opioid receptors in male Wistar rats. The endogenous opioid system plays an important role in modulating alcohol-induced effects (Gianoulakis 2009; Oswald and Wand 2004; Palm and Nylander 2015). However, much of the knowledge is derived from studies using forced administration paradigms and the literature on long-term alcohol-induced effects on the opioid system after voluntary drinking is limited. The novelty of the findings presented here is that voluntary intermittent alcohol intake over seven weeks induced changes in mu and delta receptor density that persisted after 10 days of alcohol intermission.
Specifically alcohol-induced effects following voluntary alcohol intake were more pronounced on delta than on mu receptor density, which is in line with previous studies (Pradhan et al. 2011; Saland et al. 2004). The delta receptor has been implicated in mood disorders (Filliol et al. 2000; Saitoh et al. 2004), and previous research has shown that genetic deletion of the delta receptor is associated with anxiety-like behavior (Filliol et al. 2000; Pradhan et al. 2011). Increased alcohol consumption (Hall et al. 2001; Roberts et al. 2001) is believed to be related to the anxiolytic properties of alcohol (Roberts et al. 2001; van Rijn et al. 2010). This is further supported by increased delta receptor density in the amygdaloid nucleus and the cingulate cortex area 2, areas that have been implicated in various emotional processes (Davis and Whalen 2001; Watanabe et al. 2015). Moreover, increased delta receptor density was found in the lower part of the caudate putamen, whereas decreased density was found in the caudate putamen upper part. The transition from initial drug use to compulsive use and addiction involves long-lasting changes in neural networks (Koob and Volkow 2010) and is hypothesized to involve a shift from the acute reinforcing effects in the nucleus accumbens to compulsive intake and recruitment of the dorsal striatum (Everitt and Robbins 2013). Thus, the altered delta receptor density in the caudate putamen may be involved in such processes along with the habit formation related to the seven weeks of voluntary alcohol intake. The increased $^{3}$H-DPDPE binding in the lower part of the caudate putamen is consistent with previous studies showing higher delta receptor mRNA levels in the striatum of mice 3 weeks after removal of alcohol (Winkler et al. 1998). Finally, the increased density found in the lower part of the caudate putamen is notable in light of the recent gene expression analyses that reveal a down regulation of Met-enkephalin-Arg$^{6}$-Phe$^{7}$—a marker of proenkephalin—in the dorsal striatum of human AUD patients (Sarkisyan et al. 2015) and a trend towards increased delta receptor density in this area in AUD patients (Wand et al. 2013).

Mu opioid receptors are central for reward processing and important for alcohol reward and reinforcement (Gianoulakis 2009; Oswald and Wand 2004). Genetic deletion of mu receptors results in lower voluntary alcohol intake (Hall et al. 2001; Roberts et al. 2001). The A118G polymorphism in the mu opioid receptor gene modulates voluntary alcohol intake as well as response to naltrexone (Bilbao et al. 2015; Thorsell 2013). Thus, the alcohol-induced effects on mu receptors appear to be complex.

Lower mu receptor density was found in the nucleus accumbens shell after voluntary alcohol intake, an area central in reward circuits and motivation. This could reflect an adaptive down-regulation to counteract increased levels of dopamine after alcohol drinking. These result are in line with the previous literature which has found a lower level of $^{3}$H-DAMGO binding in the
nucleus accumbens one hour after acute oral administration of 2.5 g/kg alcohol (Mendez et al. 2001), after increasing concentrations of alcohol up to 6% in drinking water for one month (Turchan et al. 1999) and after two weeks of increasing alcohol concentration up to 6.7% in a liquid diet (Saland et al. 2005). In contrast, higher mu receptor density was found in the nucleus accumbens after voluntary intake of 5% alcohol and water (Cowen et al. 1999) as well as in rats withdrawn from alcohol for 10 days after voluntary intake of 5% alcohol and water for six weeks (Djouma and Lawrence 2002).

To compare the results of this study to previous results side by side is difficult due to differences in animal strains used, choice in alcohol paradigm, duration of alcohol access, and time after last alcohol session/administration until decapitation. Some of these difficulties are discussed below.

Supplier dependent differences

Paper IV aimed to study individual differences in behavior, its association with voluntary alcohol intake and preference, and subsequent response to naltrexone. Wistar rats from three different suppliers (Rcc, Crl, Tac) were included to create a seamless heterogenic group of animals, representative of heterogeneity in the human population. However, the data revealed supplier-dependent differences of such magnitude that the aim was shifted to behaviorally characterize Wistar rats from three different suppliers in OF activity, Y-maze performance, and thereafter to study intermittent voluntary alcohol intake and preference, and response to naltrexone treatment. Below are the results from the supplier-dependent differences in Paper IV.

Behavior and supplier-dependent differences

When analyzing behavior in the Y-maze, no differences between the three supplier groups were found comparing the percentage correct alternations (Figure 15). Crl rats displayed increased mobility and longer total distance travelled than Rcc and Tac, while no differences between Rcc and Tac were found. Analysis of time spent in the arms (zone A, B, or C) revealed no differences and the pattern of alternation was similar for all groups, but Crl spent more time in the mid-zone relative to the others. An overall correlation was found between total distance moved in the OF and the Y-maze, but with no correlations within groups.
**Figure 15.** The level (%) of correct alternations when comparing Wistar rats from different suppliers, i.e. RccHan™:WI (Rcc), Crl:WI (Crl) and HanTac:WH (Tac). Data are presented as mean±SEM (ANOVA, Fisher's LSD post-hoc test).

Voluntary alcohol intake and supplier-dependent differences

In Paper IV, overall differences were found for week 1 and 4 with the highest intake in the Rcc group (Figure 16A). An increase in voluntary alcohol intake over the six weeks of access was observed for all animals, and Crl and Tac separately, but not Rcc. Overall differences in alcohol preference (%) between groups were found for all weeks except 2 and 3. Post-hoc analysis revealed a higher preference in Rcc than Tac weeks 1 and 4, and higher than Crl for all weeks except 2 and 3. At week 6, alcohol preference was higher in Tac than Crl (Figure 16C).

Further, the average intake for alcohol intake on days 1, 2, and 3 was compared and differences were assessed in intake upon regaining alcohol access (drinking day 1 each week) compared to when accustomed (drinking day 3) (Figure 16B). Group-wise comparisons revealed a higher alcohol intake in Rcc on drinking days 1 and 3, with a tendency towards significance on day 2, compared to Tac and drinking days 1 and 2 compared to Crl. The alcohol intake within groups showed an evident pattern in Rcc with the highest alcohol intake on day 1 relative to 2 and 3, whereas in Crl and Tac the pattern was less pronounced although both still had their highest alcohol intake on day 1 (Figure 16B).
Figure 16. (A) Weekly voluntary alcohol intake (g/kg), (B) average alcohol intake (g/kg) on the three days of access (drinking day 1, 2, and 3), and (C) alcohol preference (%) during the six weeks of intermittent access prior to naltrexone treatment in Wistar rats from different suppliers, i.e. RccHan\textsuperscript{TM}:WI (Rcc), Crl:WI (Crl) and HanTac:WH (Tac). Data are shown as median with shaded areas indicating min and max (A and C), and median and quartile range (B). \( ^{c}p \leq 0.05, \quad ^{cc}p < 0.01, \quad ^{ccc}p < 0.001 \) compared to Crl rats, \( ^{t}p \leq 0.05 \) and \( ^{tt}p < 0.001 \) compared to Tac rats (Mann-Whitney U-test), \( ^{1}p \leq 0.05, \quad ^{11}p < 0.01, \quad ^{111}p < 0.001 \) compared to the intake on drinking day 1 within the respective group, and \( ^{2}p \leq 0.05, \quad ^{22}p < 0.01, \quad ^{222}p < 0.001 \) compared to the intake on drinking day 2 within the respective group (Wilcoxon matched pairs test).

Response to naltrexone and supplier-dependent differences

An overall effect of naltrexone on alcohol intake was found in all rats 30 minutes and 2 hours after alcohol access. After 30 minutes dose-dependent differences were observed when comparing all doses of naltrexone to saline (Figure 17A). In contrast, naltrexone at 0.3 along with 3 but not 0.03 mg/kg resulted in decreased alcohol intake compared to saline in all rats 2 hours after alcohol access (Figure 17B). After 24 h a minor effect of naltrexone on alcohol intake was seen (Figure 17C).

Comparisons of rats from the three suppliers showed an overall effect within each group after 30 minutes. All three doses of naltrexone reduced alcohol intake in Rcc compared to the saline control, but no intra-dose differences were observed between the two highest doses of naltrexone. A dose-dependent effect of naltrexone on alcohol intake was seen in Crl, and a general, but not dose-dependent effect, in Tac (Figure 17A). After 2 hours, group-wise comparisons revealed an overall effect of naltrexone on alcohol intake in Rcc and Tac, while a different pattern was observed in Crl. In Rcc, a decrease in intake was found after naltrexone at 0.3 and 3 but not after 0.03 mg/kg. An effect of naltrexone on alcohol intake after all doses was found in Tac, but no dose-dependent effect. In Crl, there was no decrease in alcohol intake found for any dose. Moreover, naltrexone at 0.03 mg/kg resulted in a higher intake compared to 0.3 and 3 mg/kg (Figure 17B). After 24 h no supplier-dependent differences were found (Figure 17C).
Figure 17. Voluntary alcohol (A-C) and water (D-F) intake (g/kg) after treatment with saline or naltrexone (0.03 mg/kg, 0.3 mg/kg or 3 mg/kg) administered s.c. 30 minutes prior to alcohol access in Wistar rats from three different suppliers, i.e. RccHan"TM:WI (Rcc), Crl:WI (Crl) and HanTac:WH (Tac). Alcohol and water intake was measured at 30 minutes (A and D, respectively), 2 hours (B and E, respectively), and 24 hours (C and F, respectively) after access. Data are presented as median and quartile range. *p ≤ 0.05, **p<0.01, ***p<0.001 compared to saline; @p ≤ 0.05, @@p<0.01, @@@p<0.001 compared to naltrexone at 0.3 mg/kg; #p ≤ 0.05, ##p<0.01, ###p<0.001 compared to naltrexone at 3 mg/kg (Wilcoxon matched pairs test).

Individual differences and suppliers

Each supplier group was subgrouped by a tertiary split into HD, ID and LD based on alcohol intake during weeks 1-6. Rcc showed a large variation in alcohol intake before naltrexone treatment. A difference between HD and LD was found for all weeks of access, whereas differences between HD and LD in Crl were found weeks 4-6 and in Tac weeks 3-6.

The response to naltrexone on alcohol intake after 30 minutes in HD, ID and LD subgroups within each supplier group is shown in Figure 18. In Rcc-HD, all three doses of naltrexone reduced alcohol intake compared to the saline, but no intra-dose differences were observed between the two highest doses. In Rcc-LD, naltrexone at 0.03 and 0.3 mg/kg resulted in lower alcohol intake, while no effect of naltrexone was found in Rcc-ID. In Crl, all groups responded to naltrexone compared to saline, except at 0.03 mg/kg in Crl-HD, and 0.3 in Crl-LD. In Tac, naltrexone at 0.03 and 3 mg/kg resulted in lower alcohol intake in HD compared to saline, whereas no effect was seen for ID
or LD (Figure 18). No effect of naltrexone on alcohol intake was seen 2 or 24 h after alcohol access.

**Figure 18.** Voluntary alcohol intake after treatment with saline or naltrexone (0.03 mg/kg, 0.3 mg/kg or 3 mg/kg) administered s.c. 30 minutes prior to alcohol access in Wistar rats from three different suppliers, i.e. RccHanTM:WI (Rcc), Crl:WI (Crl) and HanTac:WH (Tac), divided by a tertiary split into high drinkers (HD), intermediate drinkers (ID) and low drinkers (LD) based on the alcohol intake during weeks 1-6. Data are presented as median and quartile range. *p ≤ 0.05 compared to saline; @p ≤ 0.05 compared to naltrexone at 0.3 mg/kg; #p ≤ 0.05 compared to naltrexone at 3 mg/kg (Wilcoxon matched pairs test).

**Discussion**

The outbred Wistar rats differed in voluntary alcohol intake in a supplier-dependent manner. The Rcc group displayed the highest alcohol intake and preference, which is supported by previous studies (Goepfrich et al. 2013; Palm et al. 2011b). The level of voluntary alcohol intake in the Rcc group, using the same intermittent two-bottle model, was also replicated from Paper II. Notably, the pattern of alcohol intake differed between the Wistar groups. Rcc rats exhibited a stable intake that peaked more or less upon initial access, which has been shown previously (Goepfrich et al. 2013; Palm et al. 2011b), while Crl and Tac rats showed an initial increase followed by stabilization (Palm et al. 2011b). The lack of a pronounced escalation in intake was similar to previous reports on intermittent access paradigms.
(Adermark et al. 2011; Palm et al. 2011b; Suchankova et al. 2013), but in contrast to others (Carnicella et al. 2014). In a comparison of intake on the days of alcohol access, i.e., drinking days, the Rcc group drank more alcohol on drinking day 1 relative to days 2 or 3, which implies an alcohol deprivation effect, again replicating the findings from Paper II; however, this was not as distinct in the Tac and Crl groups.

Examining the data as one heterogeneous group, i.e. in all rats, a dose-dependent response to naltrexone was revealed. The effect of naltrexone compared to saline was seen following all doses—as early as 30 minutes after alcohol access—and it was still evident in the highest doses (0.3 or 3 mg/kg) after 2 h. This is in agreement with others using similar doses of naltrexone at 30 min (Daoura and Nylander 2011; Simms et al. 2008) and 2 hours (Daoura and Nylander 2011) after alcohol access.

Supplier-dependent differences in the effect of naltrexone were observed, as the effect was discontinued in Crl rats and only observed for the two highest doses in Rcc rats at 2 hours. An overall effect was seen for all doses in Tac rats at 2 hours, and the effect had ceased in all groups 24 hours after access. Others report that naltrexone at 0.02 mg/kg decreased alcohol intake after 2 hours in a subgroup of Sprague-Dawley rats displaying high novelty-induced activity (Barson et al. 2013), which may resemble the Crl rats studied here with high OF activity and loss of naltrexone effect at 2 hours. In line with the present results, naltrexone at 1 and 2 mg/kg decreased alcohol intake 4 hours after alcohol access in Rcc rats (Fredriksson et al. 2015; Steensland et al. 2012).

An assessment of naltrexone effects at 30 minutes in the HD, ID and LD groups revealed that Rcc-HD animals stood out with a dose-dependent decrease in alcohol intake; this was not evident in the Rcc-ID or LD animals or any other supplier subgroup. The effect in the Rcc-HD group can be attributed to increased intake in this subgroup, as previous studies describe a more striking effect of naltrexone in individuals with a high level of alcohol intake (Mitchell et al. 2009). In addition, naltrexone, at 1 and 2 mg/kg, reduces alcohol intake in Rcc rats with a similar intermittent voluntary alcohol intake as shown herein (Fredriksson et al. 2015; Steensland et al. 2012). Furthermore, naltrexone is more effective in reducing voluntary alcohol intake in animals subjected to rearing conditions simulating an unsafe early life environment and concomitant higher adult voluntary alcohol intake relative to a simulated protective environment (Daoura and Nylander 2011; Nylander and Roman 2013).
General discussion

In this thesis, we were able to study the association between risk-related behaviors and voluntary alcohol intake using the OF and the MCSF and two different intermittent access paradigms in outbred rats. Parts of the opioid system, the mu and the delta receptors, were studied and the long-term alcohol induced effects on these receptors were analyzed. Further, Wistar rats from different suppliers were studied with the aim of creating a seamless group of heterogenous animals and to divide the animals into subgroups based on individual differences in behavior, voluntary alcohol intake, and subsequent response to naltrexone treatment.

The findings revealed associations between risk-related behavior and alcohol intake. Notably, individual differences in risk-assessment behavior were important for voluntary alcohol intake as the high risk-assessing individuals showed elevated alcohol intake and preference. These associations were only revealed with the use of the multivariate options in the MCSF, as the multivariate test situation allows for several measures taken simultaneously. This can provide a profile rather than focusing on any particular behavior. This approach broadened the perspective on risk-related behaviors, including aspects of risk assessment. An operational definition of risk assessment was used based on activity in zones where the animal has to assess the risk of venturing into areas associated with risk (Meyerson et al. 2006; Meyerson et al. 2013).

The subgroups of extreme high- and low-alcohol drinking animals exhibited not only differences in alcohol intake and preference, but also drinking patterns. The individuals with the highest alcohol consumption also showed a stronger alcohol deprivation effect compared to the individuals consuming less alcohol. These findings show that even if clear differences cannot be seen between the alcohol- and water-drinking groups regarding behavior, clear individual differences emerge when studying subgroups in more detail.

Studies of the mu and the delta opioid receptors revealed long-lasting changes, mainly on delta opioid receptors, and some of which are in line with findings in humans (Sarkisyan et al. 2015; Wand et al. 2013). Results presented herein also demonstrate the importance of taking supplier-dependent differences into account. These are in line with previous results of large differences in alcohol intake (Palm et al. 2011b) and differences of importance for behavioral interpretations (Palm et al. 2011a). From a population validity point of view, it has been argued that the use of heterogeneous outbred animals is advantageous for translatability into human disorders, especially upon division into subgroups for studies of individual differences, responders, and non-responders etc. (Stewart and
Kalueff 2015). Most researchers are aware that differences between rat strains and lines from different suppliers exist; however, there are few articles on this topic. This is surprising considering how important the awareness of such differences is when selecting the appropriate animals for testing experimental hypotheses as well as for comparison of results between studies.

The findings herein indicate that the heterogeneity of Wistar rats can offer advantages in translational research. Although AUD is diagnosed by diagnostic criteria that evaluate several aspects of the disorder, the patient group displays great heterogeneity (Hines et al. 2005; Leggio et al. 2009; Moss et al. 2007). The confirmation of a wide heterogeneity within the Wistar rat and the possibility for detailed studies of subgroups is therefore an advantage for understanding different pharmacological responders.
Conclusions

This thesis was based on four studies. They identified important behavioral profiles for individuals at risk for high alcohol intake. They also analyzed alcohol-induced effects on the endogenous opioid receptors and identified individual pharmacological responses to naltrexone in relation to behavior profiles and voluntary alcohol intake. The main findings of this thesis were:

- The MCSF made possible a complementary method for understanding mechanisms underlying various mental states, rather than focusing on any particular behavior. The MCSF broadened the perspective on risk-related behaviors, including aspects of risk assessment.

- Alcohol intake and preference increased over time in high risk-assessing rats and was significantly higher than in low risk-assessing animals.

- There were large individual differences in alcohol intake in Rcc rats using the modified intermittent access paradigm, which enabled detailed analyses of drinking patterns in the high- and low-alcohol drinking rats.

- There was an alcohol deprivation effect in high drinking Rcc animals only. The behavior profiling of high alcohol-drinking rats before and after alcohol access suggested that this subgroup of rats was consuming alcohol for its anxiolytic properties.

- There were long-lasting changes mainly on delta, but to some extent also on mu receptor density after long-term intermittent voluntary alcohol intake; these changes persisted after 10 days of alcohol intermission. Some of these long-lasting alcohol-induced changes are in line with findings in humans.

- The voluntary alcohol consumption and the concomitant response to naltrexone were different for Wistar rats from different suppliers. In addition, there were differences in the behavior in the OF—and to a lesser extent in the Y-maze—that adds to the complexity of individual differences versus supplier-dependent effects.
The RCC Wistar rats may be more suitable for studies of individual differences in voluntary alcohol intake and response to pharmacological treatment. This due to increasing alcohol intake and the presence of a high drinking subpopulation with increasing alcohol intake over time. The high drinking subpopulation showed pronounced effects of naltrexone on alcohol intake.
Populärvetenskaplig sammanfattning


Naltrexon är idag ett av de läkemedel som används för behandling av alkoholberoende och verkar genom att blockera de opioida receptorerna och leder därigenom till minskat alkoholintag hos patienter med alkoholberoende. Behandlingseffekten av naltrexon uppvisar stor individuell variation, och det är idag okänt exakt varför olika individer svarar olika på behandlingen och mer kunskap behövs för att kunna förstå den heterogeniteten som finns hos alkoholberoende patienter.

Djurexperimentella studier har en nyckelroll för att förstå de bakomliggande genetiska och neurobiologiska mekanismerna men även för att kunna identifiera riskmarkörer och beteendemönster som ligger till grund för alkoholberoende.

Det övergripande syftet med studierna i denna avhandling var att studera individuella skillnader i beteende, frivilligt alkoholintag, neurokemi och farmakologisk behandling av naltrexon i utavlade Wistar råttor.

För att kunna studera individuella skillnader i riskrelaterat beteende samt dess samband med frivilligt alkoholintag, beteendeprofilerades djuren och klassificerades baserat på djurens risktagande och riskbedömande beteende. Analys av djurens frivilliga dryckesmönster studerades genom att
klassificera djuren i subgrupper om hög- och lågdrickare, baserat på deras alkoholintag. Ytterligare analyser av alkoholens effekt på hjärnan gjordes genom att med hjälp av autoradiografi undersöka hjärnans endogena opioidea system och densiteten av dess receptorer efter en period av frivilligt alkoholintag. För att studera individuella skillnaderna i behandlingseffekten av naltrexon undersöks djur från tre olika leverantörer i beteendetester innan de fick tillgång till frivilligt alkoholintag och behandlades sedan med tre olika doser av naltrexon samtidigt som alkoholintaget mättes. I alla fyra studier användes utavlade Wistar-råttor, vilket ger en större variation och möjliggör studier av individuella skillnader och subgrupper.

De sammanfattande resultaten av studierna i denna avhandling är att en detaljerad beteendeprofilering av djuret möjliggör en bredare och samtidigt mer ingående analys av de riskrelaterade beteendena, såsom risktagande och riskbedömning, och visar att djur med högt riskbedömmande beteende hade ett högre alkoholintag samt ett ökat alkoholintag över tid i jämförelse med djur med en låg grad av riskbedömning. Vidare, genom att subgruppera djuren baserat på deras alkoholkonsumtion, kan subgrupper med en depriveringseffekt under perioden utan tillgång till alkohol och med lägre ångestlikt beteende efter perioden med frivilligt alkoholintag identifieras. Således kan subgrupper som konsumerar mer alkohol för att eventuellt dämpa ångestlika beteenden identifieras. Ytterligare analyser av alkoholens effekt på hjärnan visar mätbara skillnader i det endogena opioida systemet, vilket aldrig tidigare kunnat påvisas efter längre tids frivilligt alkoholdrickande. I analysen av djur från tre olika leverantörer återfanns skillnader i beteende, frivilligt alkoholintag samt tids- och dosberoende skillnader i svaret på naltrexonbehandling. Resultaten påvisar den utavlade Wistar-stammens heterogenitet och belyser behovet av en ökad kunskap om fenotypskillnader inom samma djurstam, vilket kan vara av betydelse för såväl den kliniska och prekliniska forskningen.

Sammantaget visar resulterna att ingående studier av djurens beteendeprofil är av stor betydelse för att kunna fylla kunskapsluckorna kring de individuella skillnaderna man ser i benägenhet för högt alkoholintag samt farmakologiskt behandlingsresultat av naltrexon. Behovet för ökad kunskap är fortfarande stort för att förstå mer av de inblandade mekanismerna.
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