The heterogeneity of alcoholics complicates the selection of suitable treatment for alcoholism. The composition and function of neurotransmitter systems affects the course of alcoholism. This thesis conducted a post-mortem investigation of brain specimens of anxious Cloninger type 1 and impulsive Cloninger type 2 alcoholics and controls. GABA receptor binding was decreased in all alcoholics. mGluR2/3 binding was increased in type 2 alcoholics; μ-opioid receptor binding was reduced in type 1 alcoholics.
Alterations in the Neurotransmitter Receptor Binding Densities in Cloninger Type 1 and 2 Alcoholics
VIRPI LAUKKANEN

Alterations in the Neurotransmitter Receptor Binding Densities in Cloninger Type 1 and 2 Alcoholics

Postmortem Human Whole Hemisphere Autoradiography Studies

To be presented by permission of the Faculty of Health Sciences, University of Eastern Finland for public examination in auditorium SN 200, Snellmania building, Kuopio, on Friday, June 10th 2016, at 12 noon

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ABSTRACT

Alcoholism is a disease that often goes undiagnosed and untreated. The current treatment results of alcoholism are far from satisfactory. One reason for this situation is that there is extensive heterogeneity among alcoholics. Cloninger’s typology divides alcoholics into two subgroups: late-onset, anxiety-prone type 1 alcoholics and early-onset, impulsive type 2 alcoholics. The composition of the neurotransmitter systems of a subject plays a central role in the development and maintenance of alcoholism. The γ-aminobutyric acid (GABA), glutamate and opioids are important neurotransmitters involved in alcoholism. The main aim of this thesis was to investigate possible differences between Cloninger type 1 (n=10) and 2 alcoholics (n=8) and control subjects (n=10) in the binding density of GABA_A receptor, metabotropic glutamate receptor 2 and 3 (mGluR2/3) and µ-opioid receptor (MOR), in the brain areas relevant for decision making, memory, habituation and reward.

The research method involved post-mortem human whole hemisphere autoradiography with [³H]flunitrazepam as a binding ligand to study GABA_A receptor binding density (study 1), [³H]LY341495 to reveal the binding density of mGluR2 and mGluR3 (study 2) and [³H]naloxone and [³H]DAMGO to study MOR binding density (study 3).

In study 1, GABA_A receptor binding density was statistically significantly decreased in the internal globus pallidus and hippocampus of both alcoholic subtypes, and in the dentate gyrus of Cloninger type 2 alcoholics, in comparison to the controls. These findings point to altered GABA_A receptor function in both alcoholic subgroups. In study 2, the mGluR2/3 binding density of Cloninger type 2 alcoholics was significantly increased in the perigenual anterior cingulate cortex, when compared with the controls. This alteration may be linked to the impulsive tendencies of this alcoholic subgroup. Study 3 detected a statistically significantly decreased [³H]naloxone binding density in the dentate gyrus of Cloninger type 1 alcoholics. There was a trend towards decreased [³H]naloxone and [³H]DAMGO binding in type 1 alcoholics in all brain areas examined in this work, perhaps indicative of impaired MOR function in Cloninger type 1 anxiety-prone alcoholics.

These findings reveal alterations in the GABA_A receptor, mGluR2/3 and MOR–mediated neurotransmitter function in alcoholism. Altered mGluR2/3 and MOR binding characteristics were specific to a certain subgroup of alcoholics, whereas reduced GABA_A receptor binding was observed in all alcoholics. These findings deepen our understanding of the neurotransmitter systems involved in different types of alcoholism. Hopefully, they will provide new prospects for the treatment of patients as well as a launching pad for future research.

Hermovälittäjäainejärjestelmien yksilöllinen rakenne ja toiminta vaikuttavat merkittävällä tavalla alkoholismin kehitykseen ja kulkuun. Keskeisessä roolissa ovat γ-aminobutyraatti-(GABA-), glutamaatti- ja opioidi-välittäjäainejärjestelmät. Tässä väitöskirjatyössä tutkimme Cloningerin tyypin 1 (n= 10) ja tyypin 2 (n= 8) alkoholistien ja verrokkihenkilöiden (n= 10) välillä havaittavia eroja GABA\(_\alpha\)-reseptorien, metabotrooppisten glutamaattiareseptoreiden 2 ja 3 (mGluR2/3) sekä µ-opioidireseptorien (MOR) sitoutumis- ja tuesta käytännössä keskeisillä aivoalueilla. 

Tutkimusmenetelmämme oli post-mortem -aineendosäilytteiden autoradiografia. Tutkimme GABA\(_\alpha\)-reseptorit sitoutumisesta käytävän ligandina \([{}^3H]\)flunitratsepaamia (koe 1). Toisessa työssä tutkimme \([{}^3H]\)LY341495:n mGluR2/3-reseptoreihin (koe 2). Kolmannessa työssä käytimme \([{}^3H]\)naloksonia ja \([{}^3H]\)DAMGOa tutkia kemmeen µ-opioidireseptorin sitoutumisesta (koe 3). 

Kokeessa 1 GABA\(_\alpha\)-reseptorisin sitoutuminen oli vähentynyt tilastollisesti merkittävästi sisemmän globus palliduksen ja hippokampuksen alueella molemmissa alkoholistaryhmissä, ja gyrus dentatukseen alueella Cloningerin tyyppi 2 alkoholistelle. Löydös viittaa GABA\(_\alpha\)-reseptoritoiminnan muutokseen molemmissa alkoholistaryhmissä. Toisessa kokeessa mGluR2/3-sitoutuminen oli tyyppi 2 alkoholistelle merkittävästi kohonnut verrokkihenkilöihin nähden etumaisen, pihtipainon alueella, mikä voi liittyä tämän alkoholistiryhmän impulsiivisuuteen. Kolmannessa kokeessa havaittiin tyyppi 1 alkoholistilla verrokkihenkilöihin nähden matalampi \([{}^3H]\)naloksonin sitoutuminen gyrus dentatukseen. Tyyppi 1 alkoholistilla \([{}^3H]\)naloksoni- ja \([{}^3H]\)DAMGO-sitoutuminen oli verrokkeja matalampaa kaikilla tutkituilla alueilla. Löydös viittaa muuttuneeseen MOR-toimintaan Cloningerin tyyppi 1 ahdistusherkillä alkoholistille.

Löydöksemme viittaavat poikkeavaan GABA\(_\alpha\)-reseptori-, mGluR2/3- ja MOR-toimintaan alkoholismissa. mGluR2/3- ja MOR-sitoutumismuutokset nähtiin vain toisessa alkoholistaryhmässä, kun taas GABA\(_\alpha\) reseptoritiheys oli muuttunut molemmissa alaryhmissä. Löydökset antavat lisätietoa välittäjäainejärjestelmien muutosista alkoholismissa ja tarjoavat lisäväliteitä alkoholisin hoitoon ja tutkimukseen.
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Kuopio, May 2016

Virpi Laukkanen
List of the original publications

This dissertation is based on the following original publications:


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<th>DSM-V</th>
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<td>ACC</td>
<td>anterior cingulate cortex</td>
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<td>Diagnostic and Statistical Manual of Mental Disorders 4th Edition</td>
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<tr>
<td>AEA</td>
<td>anandamide</td>
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<td>aINS</td>
<td>anterior insula</td>
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<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<tr>
<td>Amy</td>
<td>amygdala</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>aPFC</td>
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<td>BAC</td>
<td>blood alcohol content</td>
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<td>European Medications Agency</td>
</tr>
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<td>BOLD</td>
<td>blood oxygenated level dependent</td>
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<td>calcium</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>Cau</td>
<td>caudate</td>
<td></td>
<td>Gamma-hydroxybutyrate</td>
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<td>CBT</td>
<td>cognitive behavioral therapy</td>
<td></td>
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</tr>
<tr>
<td>Cl$^-$</td>
<td>chloride</td>
<td></td>
<td>G-protein coupled receptor globus pallidus</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>CPP</td>
<td>conditioned place preference</td>
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<td>external globus pallidus</td>
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<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>DAMGO</td>
<td>2-Ala-4-mephe-5-gly-enkephalin</td>
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<td>Classification of Diseases</td>
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<td>dopamine transporter</td>
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<td>DHEA</td>
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<tr>
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<td>$\kappa$-opioid receptor</td>
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<td>LTD</td>
<td>long-term depression</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
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<tr>
<td>mGluR2</td>
<td>metabotropic glutamate receptor 2</td>
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<tr>
<td>mGluR3</td>
<td>metabotropic glutamate receptor 3</td>
<td></td>
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</tr>
<tr>
<td>mGluR2/3</td>
<td>metabotropic glutamate receptor 2 and 3</td>
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<td>MOR</td>
<td>µ-opioid receptor</td>
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<tr>
<td>mPFC</td>
<td>medial prefrontal cortex</td>
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</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
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<tr>
<td>NAM</td>
<td>negative allosteric modulator</td>
<td></td>
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<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartate</td>
<td></td>
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</tr>
<tr>
<td>NMDA NR2B</td>
<td>N-Methyl-D-aspartate receptor with NR2B unit</td>
<td></td>
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</tr>
<tr>
<td>NICE</td>
<td>The National Institute for Health and Care Excellence</td>
<td></td>
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</tr>
<tr>
<td>OCD</td>
<td>obsessive-compulsive disorder</td>
<td></td>
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<tr>
<td>OFC</td>
<td>orbitofrontal cortex</td>
<td></td>
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<tr>
<td>OLS</td>
<td>ordinary least squares</td>
<td></td>
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<tr>
<td>pACC</td>
<td>perigenual anterior cingulate cortex</td>
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<tr>
<td>PAM</td>
<td>positive allosteric modulator</td>
<td></td>
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<tr>
<td>PET</td>
<td>positron-emission tomography</td>
<td></td>
<td></td>
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<tr>
<td>PCC</td>
<td>posterior cingulate cortex</td>
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</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
<td></td>
<td></td>
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<tr>
<td>PHG</td>
<td>parahippocampal gyrus</td>
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<tr>
<td>pINS</td>
<td>posterior insula</td>
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<tr>
<td>PMI</td>
<td>post-mortem interval</td>
<td></td>
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<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
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<td>PREG</td>
<td>Pregnenolone</td>
<td></td>
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<tr>
<td>Pu</td>
<td>putamen</td>
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<tr>
<td>Rec</td>
<td>receptor</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SERT</td>
<td>serotonin transporter</td>
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<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
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<tr>
<td>TC</td>
<td>temporal cortex</td>
<td></td>
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</tr>
<tr>
<td>SSR1</td>
<td>selective serotonin reuptake inhibitor</td>
<td></td>
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<tr>
<td>THC</td>
<td>tetrahydrocannabinol</td>
<td></td>
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<tr>
<td>US</td>
<td>The United States of America</td>
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<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>5HT1A</td>
<td>serotonin receptor type 1A</td>
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1 Introduction

The history of alcohol use as an integral part of many human cultures extends over thousands of years (alcohol refers to ethanol throughout this thesis). Typically, alcohol drinking has been an inherent part of communal activities, like festivals. Due to its mild psychoactive properties, alcohol is a legitimate psychoactive substance available for all adults in most cultures in which it is legal. Similar to coffee and nicotine, alcohol has become interwoven into many cultural traditions promoting social interactions. However, alcohol use has a darker side, the earliest descriptions about pathological aspects of alcohol use can be found in texts written in classical antiquity (Crocq 2007).

Nowadays, alcohol consumption is a worldwide phenomenon. Not only the highest rates of consumption but also that of alcohol use disorder, referred to as alcoholism in this thesis, have been recorded in the high-income countries. In 2010, the global alcohol consumption per person aged 15 years or older was equal to 6.2 liters of pure alcohol. Alcohol consumption per capita is increasing in most parts of the world (WHO 2014). In Finland, the consumption was constantly increasing since the time when statistics of alcohol consumption started to be compiled in the 1960’s till 2011, when the consumption reached 12.1 liters of pure alcohol per person aged 15 years or older. Since 2011, the alcohol consumption has been decreasing year by year, being 10.8 liters of pure alcohol in 2015 (THL 2013, THL 2016).

Although there are large regional and national differences in alcohol consumption, in most countries, the burden of disease and death attributable to alcohol is significant (WHO 2014). Alcohol ranks third in the list of risk factors for the global disease burden, surpassed only by high blood pressure and tobacco smoking. Alcohol consumption accounts for 5.1 percent of the total worldwide burden of disease, disability and death (Lim et al. 2012).

The costs of alcohol to the society and economy have also been estimated. Direct economic costs to the society, such as hospitalizations and ambulatory care, represent only from 9 to 24 percent of all alcohol-attributable social costs (van Gils et al. 2010). In addition to direct costs, there are huge indirect and intangible costs of alcohol, like lost productivity or pain and suffering of the alcoholic him/herself and his/her loved ones. Altogether, these costs have been estimated to amount to 125 billion euros in the European Union for the year 2003 (WHO 2014). Typically, the costs represent 1.3-3.3 percent of the gross domestic output (Rehm et al. 2009).

Alcoholism, the dark side of alcohol use, is a multifactorial disease. The hereditary component has an acknowledged impact on the liability to alcoholism (Ducci and Goldman 2008), as well as on the cascade of neurochemical adaptations in the brain to alcohol (Nutt 2013), which affect the chronicity of alcoholism. In addition to the hereditary component, the causes of alcoholism include social and psychological factors, which often extend over generations. A review of a large psychiatric epidemiologic survey identified a wide variety of risk factors for alcoholism, i.e. male gender, ethnicity, marital status, psychiatric comorbidity and externalizing as well as internalizing psychopathology. In this survey, having young children, involvement with religious activities and treatment participation predicted recovery during follow-up, whereas predictors of non-recovery included childhood maltreatment, personality disorders and low social support (Hasin and Grant 2015).

As a disease, alcoholism often goes undiagnosed and untreated. The poor efficacy of the currently available treatment options may be one cause for this situation, but also cultural attitudes towards alcohol and alcoholism play a role (Oroszi and Goldman 2004, Spanagel and Kiefer 2008). If one looks at the situation, one year after abstinence, one out of every three patients has fully relapsed, which is as many (i.e. one in three) who have managed to
remain abstinent (Oroszi and Goldman 2004). However, a longitudinal survey of three years’ duration suggested that up to 35 percent of untreated patients with substance-use disorder (including alcoholism) had a remission without any treatment (Henriksen et al. 2015). This study revealed several different risk factors in different categories for poor outcome of substance-use disorders, like male gender, youth, comorbid mental disorders and childhood maltreatment.

One reason for the unsatisfactory treatment result is the heterogeneity of alcoholism. It has been suggested that a patient’s response to different treatment options depends on the type of his/her alcoholism and this can be traced to the underlying neurochemical characteristics (Leggio and Addolorato 2008). In addition to neurochemical characteristics responsible for different types and causes of alcoholism, alcohol itself is a psychoactive substance and thus it is able to alter neurotransmitter function throughout the central nervous system (CNS). Not only will these changes evoke many of the typical symptoms of alcoholism but they may also increase an individual’s vulnerability to addiction. These altered neurotransmitter functions have been studied in this thesis in order to provide more information about this devastating disease.
2 Review of the Literature

2.1 ALCOHOL AND ALCOHOLISM

2.1.1 Definitions of alcoholism
Good definitions of alcohol dependence, referred to as alcoholism in this thesis, can be found in the diagnostic manuals. ICD-10, the International Classification of Diseases, which is used for diagnostic coding of diseases in the European countries (WHO 2011), defines the shared characteristics of the addiction syndrome that apply to all psychoactive substances, including alcohol. A core finding of dependence is the strong desire, the urge, to take the addictive substance, in this case - alcohol. A dependent person has difficulties in controlling the onset and length of substance use and the amounts of substance being used. Cessation of the use causes physiological and mental symptoms. A dependent person displays tolerance, in which a greater amount of the substance than before is required to achieve certain effects. Other daily activities suffer while more time is spent either using the substance or recovering from the period of usage. A dependent person is unable to stop or limit his/her use despite the obvious negative consequences for example the physical, psychological or social harm as well as the financial impact. According to ICD-10, at least three of the aforementioned symptoms should be present during a period of one year for a diagnosis. All these characteristics apply to alcoholism (WHO 2011).

The fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-V), the current diagnostic manual in the United States (US), subdivides substance-related and addictive disorders into two categories, substance use disorders and substance-induced disorders, the latter including intoxication, withdrawal and mental disorders induced by the use of the substance (APA 2013). Substance use disorder is a cluster of cognitive, behavioral and physiological symptoms, where a person continues to use the substance despite the presence of significant problems that are substance-related. DSM-V lists four clusters of symptoms characteristic to substance use disorder: impaired control, social impairment, risky use and pharmacological criteria. These clusters consist of eleven more specific criteria typically seen in the substance use disorder. Since the range of severity of substance use disorder is wide, its severity is rated as mild, moderate or severe depending on the number of criteria being fulfilled (APA 2013).

In the experimental part of this thesis, a sample of post-mortem brains of alcoholic and non-alcoholic study subjects was utilized. The diagnoses for alcohol dependence were set by two physicians according to Diagnostic and Statistical Manual IV (DSM-IV) (APA 1994), the precursor of DSM-V, which was the diagnostic manual in use in the US previously. According to a field trial of Cottler and colleagues, the implementation of the diagnostic criteria of either DSM-IV or ICD-10 produced similar results, demonstrating the equivalence of these two classifications (Cottler et al. 1995). In DSM-V, substance use disorders are not divided into the subgroups of dependence and abuse as previously in DSM-IV, but instead form one continuum of disorder severity from mild to severe (APA 2013). These changes reflect the temporal nature of the diagnostic criteria of substance use disorder, including alcoholism. However, the basis for current diagnostic criteria of both ICD-10 and DSM-V, as well as for DSM-IV, can be traced back to the description of “alcohol dependence syndrome” devised by Edwards and Gross in 1976 (Edwards and Gross 1976), which suggests an existence of some core characteristics of substance dependence, including alcoholism.
2.1.2 Vulnerability to alcoholism stems from individual neurotransmitter composition

Certain people are more vulnerable to alcoholism than others. More than 50% of the variance in alcoholism liability can be traced to genetic factors (Ducci and Goldman 2008). However, the number the genes influencing the alcoholism liability is huge, ranging from genes regulating the pharmacokinetics and pharmacodynamics of alcohol to genes implicated in several psychiatric disorders, for example those modulating behavioral control, stress resiliency and reward (Ducci and Goldman 2008). The different characteristic traits that predispose to addiction, such as impulsivity, low deliberation, anxiety etc., reflect individual differences in the composition and interactions of the neurotransmitter systems in the CNS. These characteristics and the underlying neurotransmitter systems modulate also the experience of alcohol and the adjustment to the pharmacological effects of alcohol (Nutt 2013). It is the composition of the neurotransmitter systems of the CNS, not only regulated by the genetics but also influenced by environmental factors (both past and present), which play a central role in the development and maintenance of alcoholism.

2.1.3 Classifications of alcoholism try to answer the need for better patient selection

The heterogeneity of alcoholism may be one of the reasons for the limited efficacy of current pharmacotherapies for this disease (Holmes et al. 2013), since different subgroups of alcoholics respond differently to therapeutic approaches (Wackernah et al. 2014). Therefore, there is a clear demand for better tools for patient selection. The classification of alcoholics into subgroups according to certain criteria represents an attempt to disentangle the heterogenic group of alcoholics into separate categories, which could lead to better targeted treatments and more detailed prognosis (Leggio et al. 2009, Spanagel and Kiefer 2008). Over the past decades, several models for the classification of alcoholism have been introduced. These include binary models, such as Cloninger’s typology, Babor’s classification and a simple dualistic division of early- and late-onset alcoholics, but also more detailed typologies categorizing alcoholics into several subclasses, e.g. Lesch’s typology (Leggio et al. 2009).

In the experimental part of this thesis, the typology proposed by Cloninger was used to classify the alcoholics into two separate groups. This classification is based on a sample of Swedish children adopted to the US (Cloninger et al. 1981, Cloninger et al. 1988), and it is applicable to all alcoholics, irrespective of which diagnostic manual is being used. The Cloninger typology is derived from personality traits, which, according to the underlying theory, are thought to predispose to alcoholism and associate with certain elements of the patient’s neurochemistry (Cloninger 1995, Cloninger et al. 1981, Cloninger et al. 1988). Cloninger type 1 alcoholism is more common. This subgroup of alcoholics consists of both sexes. The onset age of alcoholism is typically at the age of 25 years, or later. Type 1 alcoholics are cautious personalities; they are harm-avoidant, deliberative and prone to anxiety. Cloninger type 2 alcoholics are typically males; this type of alcoholism is highly hereditary from father to son. The onset age is under 25 years of age. A history of teenage-onset of antisocial behavior is common in type 2 alcoholics and they often have criminal record. Type 2 alcoholics score high in novelty-seeking aspects and their personality is low in terms of harm-avoidance and dependence of social reward. Typically, they use alcohol and other substances for their euphoric properties (Cloninger et al. 1981, Cloninger et al. 1988).

Cloninger suggested that Cloninger type 1 alcoholics would have a dopaminergic deficit, whereas type 2 alcoholics would have altered function of the serotonergic system (Cloninger 1994, Cloninger et al. 1988). In the previous human autoradiography studies of our research group, dopamine 2 receptor (D2 receptor) binding density was decreased in Cloninger type 1 alcoholics in the nucleus accumbens (NAc) and medial globus pallidus, whereas in other striatal areas, like caudate, putamen and lateral globus pallidus, D2 binding density was decreased in both alcoholic subgroups. This finding was accompanied by a decreased dopamine transporter (DAT) binding in type 1 alcoholics in the caudate,
putamen and NAc (Tupala et al. 2001b, Tupala et al. 2003a, Tupala et al. 2003b). These findings may suggest that Cloninger type 1 alcoholics have more deficits in the dopaminergic neurotransmission of the striatal areas than type 2 alcoholics. In addition, the previous autoradiography evaluations of our research group found some evidence of decreased serotonin transporter (SERT) binding density in the perigenual anterior cingulate cortex (pACC), caudate and amygdala in all alcoholics, and no difference between Cloninger subgroups was detected (Mantere et al. 2002, Storvik et al. 2006, Storvik et al. 2007). There are both pharmacological and genetic studies supporting the theory of serotonergic alterations in Cloninger type 2 alcoholics. The study of Johnson et al. indicated better treatment outcomes with the selective serotonin receptor 3 antagonist, ondansetron, in early-onset alcoholics, whose characteristics resemble Cloninger type 2 alcoholics (Johnson et al. 2000a, Johnson et al. 2000b, Leggio et al. 2009). Bordukalo-Niksic and colleagues found an increased frequency of certain single-nucleotide polymorphisms (SNPs) of three genes (5-HTTLPR-LL, STin2-1010 and MAO-A 3-repeat allele) involved in the regulation of serotonergic neurotransmission in Cloninger type 2 alcoholics (Bordukalo-Niksic et al. 2012). It has been suggested that Cloninger’s typology could be applied to select patients for serotonergic medications (Leggio et al. 2009).

2.1.4 The spiraling model of addiction proposed by Koob and Le Moal

Among the fundamental models describing different states of addiction, is the model postulated by George F. Koob and Michel Le Moal (Koob and Le Moal 1997, Koob and Le Moal 2006). They introduced a concept of spiraling distress, where preoccupation with and anticipation of a drug, for example alcohol, leads to binge-type use and intoxication followed by withdrawal and a negative affective state. Each of these three phases functions as a precursor for the following state (Figure 1). During the repetitive spiraling of these phases, the subject’s homeostasis is lost and replaced with allostasis, the balance of which becomes more and more fragile during the progress of the spiraling phases (Koob and Le Moal 1997). In the initial phases of addiction, preoccupation and anticipation, followed by binge-type intake are predominant in the life of the subject, but during the many repetitions of the cycle, as addiction takes over via altering allostasis, the states of withdrawal and negative affect become ever more present (Koob and Le Moal 1997, Koob and Le Moal 2006).
Figure 1. Three stages of addiction according to Koob and Le Moal (1997). These stages (Preoccupation/Anticipation, Binge/Intoxication and Withdrawal/Negative affect) are repeated. Each of the stages functions as a precursor of the following one. The repetition leads to the development of addiction.

2.1.5 Gradual alteration of neurotransmitter function
In alcoholism, as the various phases of the spiral described in the previous paragraph are repeated, the CNS is repeatedly exposed to alcohol. Gradually, all aspects of neurotransmission adapt to the chronic presence of alcohol. The consumption of alcohol initially increases GABAergic function, but when alcohol’s presence becomes chronic, either the composition or the number or both of GABA\(_A\) receptors change, leading to reduced GABAergic function (Harris and Allan 1989, Liang et al. 2009, Sauguet et al. 2013, Tabakoff and Hoffman 2013). Similarly, acute alcohol inhibits glutamatergic neurotransmission, but when intake is chronic, then glutamatergic tone becomes enhanced (Nevo and Hamon 1995). These major changes in the two main neurotransmitter systems regulating the rate of excitation are accompanied by alterations in other neurotransmitter systems, which are involved in the regulation of alcohol-induced effects in the CNS. These include the dopaminergic, opiodergic and cannabinoid systems (Nutt 2013). Overall, the disrupted balance of neurotransmitters during chronic alcohol use forms the basis for the symptoms of addiction. Below, I will shortly describe some core concepts of addiction, such as reward, craving and compulsion, since the neurotransmitter changes underlying these phenomena were the subjects of the experimental part of the thesis. Reward is emphasized especially in the early stages of alcoholism. Craving is present from the initial phase of
alcoholism, but its importance is highlighted as alcoholism proceeds. Compulsion prevails in the late stages of alcoholism.

2.1.6 Components of the brain reward system

In 1954, Olds and Milner reported that rodents would voluntarily press a lever for electrical stimulation, if the electrodes were placed in the septal area of the brain. This observation led to a conclusion that an activation of a specific area of brain produced feelings interpreted as reward (Olds and Milner 1954). A decade later, amphetamines were shown to potentiate this rewarding lever pressing of rodents (Stein 1964). Subsequently, it has been postulated that the experience of reward in the ventral tegmentum is dopamine-driven, since this area expresses dopaminergic neurons (Crow 1972, Nutt et al. 2015). Microdialysis studies in rodents have revealed that other addictive substances not only stimulants, but compounds like opioids, alcohol and nicotine, were able to enhance dopamine release in the NAc (Di Chiara and Imperato 1988), and thus the theory was developed of a dopaminergic pathway, including the ventral tegmental area (VTA), NAc and the cortical and limbic inputs from the NAc, as a “common reward pathway” (Robinson and Berridge 1993, White and Milner 1992).

The regulation of this dopaminergic pathway is extremely complex. For example, glutamate exerts extensive control on the activity of the mesolimbic dopaminergic pathway. In addition, the function of this pathway is strongly influenced by several neurotransmitter systems including GABAergic and opioidergic projections from the NAc and ventral pallidum, a cholinergic input from the pedunculopontine nucleus, and a glutaminergic input from the prefrontal cortex (PFC) to the VTA. In addition, the serotonergic system originating from the raphe nucleus regulates the dopaminergic activity in both the VTA and NAc. Furthermore, endorphins and endocannabinoids stimulate dopamine release within the NAc by disinhibiting the dopaminergic neurons in the VTA. The sum effect of the altered neurotransmission of the above neurotransmitters during acute alcohol intake, is that the dopaminergic activity becomes enhanced, indicative of reward-driven motivation (Nutt 2013).

However, nowadays it is acknowledged, that the mesolimbic dopaminergic pathway is not the only neuronal circuit of reward. The increase in dopamine levels in the striatum does not seem directly correlate to the experienced reward of drugs in general, since studies with tetrahydrocannabinol (THC) and opioids have exerted either a minor or insignificant change in the NAc (Nutt et al. 2015). In opioid addicts, the experienced euphoria from heroin or the reward while expecting heroin does not affect dopamine levels in the striatum (Daglish et al. 2008, Watson et al. 2014). Rather than providing feelings of reward, it is now hypothesized that the ultimate role of the NAc and its dopamine secretion is to direct the action of a subject towards adaptive and motivated behavior (Sesack and Grace 2010). Impaired dopaminergic function of the NAc interferes with the adaptation process during which the subject should change or tune his/her actions in a goal-directed manner. This is the case in addiction, in which the habituated response of drug taking prevails, although it is not a reasonable or optimal choice. This has been exemplified in cocaine addicts, who will choose the offer of cocaine with its instantaneous gratification property rather than a more valuable monetary reward (Martinez et al. 2007, Martinez et al. 2011). The addicted subjects are either experiencing an error in setting a ranking of competitive rewards, or their reward system is unable to respond in a sensible goal-directed manner.

2.1.7 Both positive and negative components trigger craving

Craving is the intense desire to re-experience the same kinds of effects as experienced when previously using the psychoactive substance. Craving is often present immediately after the use of the substance, but may reappear even after years of abstinence. Several triggers like stress, substance-associated stimuli and withdrawal symptoms can escalate craving (Nutt
In alcoholism, the reinforcement system has been pathologically sensitized to the stimuli that indicate alcohol use. In the positive reinforcement of alcoholism, rewarding stimuli are paired with alcohol consumption and thereby, they induce reward craving (Heinz et al. 2003). It has been suggested that positive craving is attributable to the alterations occurring in the dopaminergic and MOR systems (Di Chiara 1995, Volpicelli et al. 1995). In negative reinforcement, alcohol use is paired with negative emotional effects like withdrawal and this triggers relief craving (Heinz et al. 2003). Relief craving is thought to be a result of the altered GABA- and glutamatergic systems, accompanied by the input of other altered neurotransmitter systems like κ-opioid receptor (KOR) signaling and function (Verheul et al. 1999, Wee and Koob 2010). In addition to reward- and relief-craving, the concept of obsessive craving has been postulated by some researchers, describing the compulsive nature of addiction and also evidence of the impairment of an even wider variety of neurotransmitter systems, including the serotonergic system (Verheul et al. 1999). The entity of craving most likely consists of all the above-mentioned components, not only differently emphasized between the individual subjects but also differently expressed within the same subject at different time points.

2.1.8 Habituated response and compulsion in addiction

One of the important characteristics of alcoholism and other addictions is the compulsive use of the substance, where goal-directed behavior is suppressed by habitual function. Both goal-directed and habit-driven actions are required for the optimal function of the subject. In the normal situation, these two forms of action are used in a flexible and balanced manner in order to promote the homeostasis of the subject. This balance is lost when an individual becomes addicted to something.

The shift from goal-directed function, during which the subject is sensitive to the predicted value of his/her action, to the habit-driven function, where the reinforcing qualities of the predicted outcome of subject’s action are no longer able to modify his/her behavior, has been defined as a core phenomenon of addiction (Barker and Taylor 2014). In general, habit formation is a highly beneficial skill, allowing the subject to shift attention from a familiar action to novel external or internal stimuli.

Drug addiction has been likened to stereotypies, which are encountered in neurological and psychiatric disorders like Tourette’s syndrome or unmedicated schizophrenia. In stereotypies, flexibility is minimal, whereas repetitiveness is maximal (Graybiel 2008). These repetitive actions, like all habituated responses, once fully acquired, can go to completion without any conscious awareness. They are triggered by an internal or external cue, for example in the case of drug addiction, a stress, drug-priming or drug-related cue (Graybiel 2008). It has been suggested that the neural circuit of goal-directed actions might be actively suppressed during these kinds of stimulus-response actions (Graybiel 2008). This would, at least in part, explain why these actions, for example alcohol-intake, are so hard to extinguish, once triggered.

Animal studies of alcoholism have indicated that alcohol reinforcement causes a more readily formation of habit than natural reinforcers, but the reason why alcohol possesses this ability is still not clear (Barker and Taylor 2014). In the study of Oslund and colleagues, the habituated alcohol-related cues were able to disturb the goal-directed, (also known as motivated) behavior of rodents trying to obtain a natural reward (Oslund et al. 2010). This increased propensity towards habit formation has been detected also in human alcoholics (Sjoerds et al. 2013). It is postulated that the alterations in the neurotransmitter systems caused by chronic alcohol, like altered dopaminergic, glutamatergic, GABAergic and opioid signaling underlie the enhanced habitual learning encountered in alcoholism (Barker and Taylor 2014).
2.1.9 Involvement of memory and decision-making in addiction
The previous chapters 2.1.6 – 2.1.8 introduced three core characteristics of alcoholism, reward, craving and compulsion, and speculated that there are several alterations in neurotransmitter function involved in these phenomena. The formation and maintenance of these characteristics of addiction require cognitive processes, like decision-making and memory. For example, the different triggers of craving and habituated response require memory functions in order to evoke a response. Alcoholism impairs episodic memory and causes learning difficulties, which in turn participate in the maintenance of alcoholism (Pitel et al. 2015). Memory functions are crucial in decision-making, which is skewed in multiple ways in alcoholism. For example, the compulsive state of addiction is thought to result from changes in the glutamatergic projections from the frontal cortex and anterior cingulate cortex, areas involved in decision-making, to the ventral striatum (Kalivas and Volkow 2005), and goal-directed behavior requiring active decision-making has been suggested to be actively suppressed during compulsive addiction behavior (Barker and Taylor 2014).

In the experimental part of this thesis, we have studied neurotransmitter receptor binding density in the brain areas involved in reward, habit formation, memory and decision-making. These areas include ventral and dorsal striatum, frontal and entorhinal cortical areas, and hippocampal areas.
2.2 NEUROTRANSMITTER SYSTEMS STUDIED IN THIS THESIS: GABA, GLUTAMATE AND OPIOIDS

In this chapter, I shall provide some details of the three neurotransmitter systems of the CNS, which were studied in the experimental part of this thesis by using human post-mortem whole hemisphere autoradiography. These neurotransmitters are GABA, glutamate and opioids.

2.3 GABA

GABA, \( \gamma \)-aminobutyric acid, is the primary inhibitory neurotransmitter in the CNS. It is released from the presynaptic neuron into the synaptic cleft, when the presynaptic GABAergic neuron fires an action potential (Liang and Olsen 2014). It is known that alcohol can modulate GABA release in certain areas of the brain (Tabakoff and Hoffman 2013). The potentiating effect of alcohol on GABA release from the presynaptic stores involves presynaptic modulation via G-protein coupled receptors (GPCRs) such as GABA\(_\beta\) \( \gamma \), serotonine 2C-, cannabinoid type 1- and corticotropin-hormone releasing type 1- receptors (Kelm et al. 2011). The inhibitory GABA\(_A\) receptor is one of the main targets of alcohol in the CNS. Acute exposure to alcohol enhances GABA\(_A\) function, whereas chronic alcohol causes alterations in GABA\(_A\) receptor, which are important in the appearance of the withdrawal symptoms when alcohol intake ceases.

2.3.1 GABA\(_A\) receptor function and rewarding effects of alcohol – connections between GABAergic and dopaminergic neurotransmission

GABA-driven neurochemistry is an important component of the production of the rewarding and reinforcing effects of alcohol, and therefore its altered function is one of the driving forces of alcoholism (Koob 2004). Alcohol’s ability to alter GABA\(_A\) receptor function has been demonstrated in rodent studies. An inverse agonist of GABA\(_A\) receptor, RO15-4513, dose-dependently decreased alcohol administration without causing any behavioral signs of seizure activity in rats (Samson et al. 1987). RO15-4513 can reverse the anti-conflicting and anxiolytic actions of alcohol in rodents (Britton et al. 1988). However, also seizure activity was observed in the electroencephalogram with similar doses in this study. A GABA\(_A\) receptor antagonist, picrotoxin, was demonstrated to block the anti-conflict actions of alcohol without any external signs of seizure activity in rats (Liljequist et al. 1983). Based on these in vivo studies, it has been suggested that at the normally consumed concentrations, alcohol does not directly induce dopamine release in the CNS, but induces the dopamine release in the NAc in an indirect manner instead (Yim et al. 1998). Several research groups have concluded that alcohol can enhance the release of opioid peptides in the CNS (Cowen and Lawrence 1999). The opioid peptides, in turn, are able to inhibit GABAergic interneurons of the VTA (Di Chiara and North 1992). In a normal situation, these GABAergic interneurons provide tonic inhibition of dopaminergic neurons of the VTA. In the presence of alcohol, there is a suppression of the tonic GABAergic inhibition of dopaminergic neurons of the VTA and thus, dopamine is released from the neurons projecting to the NAc. This pathway explains why opioid-antagonists like naltrexone and nalmefene possess the ability to inhibit alcohol-induced dopamine release in the NAc and thereby cut down heavy drinking and reduce the pleasurable effects of alcohol (Tabakoff and Hoffman 2013). In addition to the indirect effect on opioid receptors, acute alcohol potentiates the function of GABA\(_A\) receptors. However, the net effect of alcohol in the VTA is the inhibition of GABAergic function. One reason for the refractory nature of GABA\(_A\) receptors in the VTA to resist the effects of alcohol may be attributable to their subunit composition. Work conducted by Okada and colleagues (Okada et al. 2004) suggested that
the majority of dopaminergic neurons of the VTA do not express the δ-subunit, which appears to be essential in the ability of alcohol to enhance the sensitivity of the GABA<sub>A</sub> receptor (Wallner et al. 2003).

### 2.3.2 GABAA receptors and anxiety spectrum disorders

Impaired or altered GABAA receptor binding has been associated in several studies with anxiety spectrum disorders. Imaging studies have revealed altered benzodiazepine receptor binding densities in patients with anxiety spectrum disorders. Geuze and colleagues observed reduced [11C]flumazenil binding in the cortical areas, the hippocampus and the thalamus, and Bremner and co-workers detected reduced [123I]iomazenil binding in the prefrontal cortex of patients with post-traumatic stress disorder (Bremner et al. 2000, Geuze et al. 2008). There are reports of reduced [11C]flumazenil binding in the insular, frontal, temporal and parietal cortices, but increased binding in the hippocampus and parahippocampal region in panic disorder patients have been reported (Cameron et al. 2007, Hasler et al. 2008). These findings demonstrate the crucial role of GABAergic neurochemistry in the pathogenesis of anxiety spectrum and mood disorders, which in turn, considering the alcohol’s ability to modulate GABAergic function, elucidate the fundamentals of the link between these psychiatric disorders and alcoholism (Fein 2015).

Due to its crucial role in different states and symptoms of alcoholism and in associated psychiatric disorders like mood disorders, GABAA receptor binding was studied in Cloninger type 1 and 2 alcoholics and controls in the study 1 of this thesis. Therefore, GABAA receptor and its interactions with alcohol will be discussed below.

### 2.3.3 Structure and function of GABAA receptors

GABAA receptors are located both synaptically and extrasynaptically. GABAA receptors are ionotropic receptors (Nayeem et al. 1994), which adjust the rate of excitation and inhibition in a rapid manner (Liang and Olsen 2014). The GABAA receptor forms a pentameric ion channel (Nayeem et al. 1994), through which chloride (Cl) ions can enter the cell. GABAA receptor has several isoforms; in fact as many as 19 different types of GABAA receptor subunits have been identified (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) (Olsen and Sieghart 2009). Each GABAA receptor consists of different combinations of five of these subunits. Neurotransmitter GABA, released from the presynaptic GABAergic neurons, binds to the GABAA receptors either within the synapse or present in extrasynaptic locations. Most of the synaptic GABAA receptors have a composition of two α-, two β- and one γ-subunits, the γ-subunit located between α- and β-subunits (Liang and Olsen 2014). These receptors mediate the phasic GABAergic inhibition (See Figure 2). Extrasynaptic GABAA receptors regulate the tonic GABAergic inhibition. They mediate the persistent hyperpolarization of a cell which they reside as a reaction to GABA spilt over from the synaptic cleft (Semyanov et al. 2004). These extrasynaptic GABAA receptors are usually composed of α4/6-subunits with a δ-subunit, which is a subunit only present in these extrasynaptic receptors (Liang and Olsen 2014, Wei et al. 2003). GABAA receptors are translocated between the cell membrane and intracellular sites, thereby regulating the excitation and inhibition rate of the CNS (Kittler et al. 2000).
2.3.4 Alcohol affects GABA\textsubscript{A} receptor function

GABA\textsubscript{A} receptors play a central role in the regulation of the effects of alcohol on the balance between excitation and inhibition of the CNS and as a consequence, in the emergence of the withdrawal symptoms after cessation of alcohol drinking (Liang and Olsen 2014). GABA\textsubscript{A} receptors are very sensitive to alcohol, as very low doses of alcohol are able to alter GABA-gated currents of the CNS (Sundstrom-Poromaa et al. 2002). Furthermore, alcohol can potentiate the function of GABA\textsubscript{A} receptor by stabilizing the “open-form” of this pentameric ion channel, which in turn permits the ion influx into the cell and thereby alters neuronal excitability (Sauguet et al. 2013, Tabakoff and Hoffman 2013).

2.3.5 Effect of acute alcohol on GABA\textsubscript{A} receptors

Acute alcohol intoxication causes alterations in the GABA\textsubscript{A} receptor system which contribute to the acute effects of alcohol in the CNS. The effects are dependent on the amount of alcohol consumed (Liang and Olsen 2014). In a CNS concentration ranging from 5-10 mmol/L, which is achieved by having less than three normal-sized drinks, one can detect potentiation of GABA\textsubscript{A} receptor function and suppression of the excitation rate of the CNS. Behaviorally, this is demonstrated as anxiolysis, sedation and alterations in mood and memory function (Davies 2003).

A single dose of alcohol potentiates the GABA\textsubscript{A}–regulated influx of Cl-ions into the cell (Harris and Allan 1989). In addition, the internalization and expression of the GABA\textsubscript{A} receptors have been shown to change in response to a single dose of alcohol. Different GABA\textsubscript{A} receptor subtypes react differently to acute alcohol, having different reaction times (ranging from almost instantaneous changes occurring within 5-15 minutes to changes appearing in hours or even days). The instantaneous changes sum to cause a decrease in
GABA<sub>A</sub> receptor availability/reactivity. The normalization of GABA<sub>A</sub> receptor expression after a single alcohol intoxication takes two weeks (Liang and Olsen 2014, Liang et al. 2007).

### 2.3.6 Effect of chronic alcohol on GABA<sub>A</sub> receptors

Chronic alcohol causes a long-lasting downregulation of GABA<sub>A</sub>–mediated inhibition of the CNS (Liang et al. 2009). These changes induce various alcohol withdrawal symptoms. If alcohol is present chronically, the α4 subunit containing GABA<sub>A</sub> receptors are upregulated, whereas α1 and α3 subunit containing receptors are downregulated (Cagetti et al. 2003). These changes in receptor function evoke symptoms like dysphoria, anxiety, sleep disturbances and convulsions should alcohol use be terminated (Kumar et al. 2009). However, it is important to remember that alcohol withdrawal symptoms do not result from a dysfunction in a single neurotransmitter system. Several dimensions of alcohol use, like reward, craving, tolerance and compulsive behavior, are attributable to changes in many neurotransmitter system, including the GABAergic, the glutamatergic, the serotonergic, the opioidergic and the dopaminergic systems, acting in concert.

### 2.3.7 GABA<sub>A</sub> subunits, their encoding genes and modulation of alcohol consumption

The subunit composition of GABA<sub>A</sub> receptor modulates not only properties like alcohol consumption and preference but also is involved in mediating many of the effects of alcohol like anxiolysis, sedation, motor impairment and hypnosis. This has been demonstrated in several works conducted in rodents e.g. either knockout (KO) and knock-in studies, in which the expression of a certain GABA<sub>A</sub> receptor subunit type is altered (Liang and Olsen 2014, Tabakoff and Hoffman 2013). Based on their work, Wallner and coworkers suggested that GABA<sub>A</sub> receptors with a δ-subunit, the so-called extrasynaptic GABA<sub>A</sub> receptors, are sensitive to the alcohol concentrations normally used by humans during so-called “social-drinking” and these extrasynaptic GABA<sub>A</sub> receptors may be the primary target of alcohol (Wallner et al. 2003). The alcohol sensitivity also appeared to depend on the subtype of the β-subunit. Considering the amount of subunit types (19) and the vast variety of subunit combinations that form GABA<sub>A</sub> receptor pentamers, it is obvious that the exact contribution of the characteristic subunit combinations in specific brain regions to the sedative, anxiolytic, anticonvulsant, motor-impairing and several other effects of alcohol have still to be fully defined. More research will be required to disentangle the relevance of different subunit compositions in the actions of alcohol (Liang and Olsen 2014, Tabakoff and Hoffman 2013).

In support of the relevance of GABA<sub>A</sub> receptor subunit composition to alcohol’s effects in the CNS, the genes coding the compositions of the GABA<sub>A</sub> receptor subunits have been linked to alcoholism in several human studies (Enoch et al. 2006, Enoch et al. 2009, Loh and Ball 2000, Long et al. 1998). The genes coding α2 and γ1 subunits of GABA<sub>A</sub> receptor (GABRA2 and GABGR1, respectively) have been shown to be downregulated in post-mortem brains of human alcoholics, probably as a sign of tolerance (Enoch et al. 2012). The risk of alcoholism may be modulated by genetic polymorphisms in the α2 subunit of the GABA<sub>A</sub> receptor, which is encoded by the GABRA2 gene. The GABA<sub>A</sub> receptor subunit composition in the mesolimbic dopamine reward pathway is α2β1γ1, and minor SNPs of GABRA2 have been repeatedly associated with alcoholism (Enoch 2014). The correlation between different GABRA2 minor SNPs and alcoholism has been suggested to exist via personality traits, which predispose to alcoholism (Dick et al. 2009, Dick et al. 2013). A polymorphism in GABRA2 might play a role in the anxiety, stress response and reward circuitry of the brain (Covault et al. 2008, Edenberg et al. 2004, Enoch et al. 2006, Fehr et al. 2006, Freund and Ballinger 1989a, Lappalainen et al. 2005). Fehr and co-workers found that the prevalence of a GABRA2 gene risk haplotype is higher among alcoholic patients with a family history of alcoholism and an early onset of dependence, and the functional imaging studies of Kareken and co-workers also point to an an association between GABRA2 haplotype, familial alcoholism and responsiveness to alcohol-related cues (Fehr et al. 2006,
Kareken et al. 2010a, Kareken et al. 2010b). One recent study revealed an impact of the GABRA2 genotype on the neurocircuitry of incentive-motivation during adolescence (Heitzeg et al. 2014). In addition, Uhart and colleagues demonstrated an association between minor alleles of several GABRA2 SNPs and attenuated negative responses to alcohol (Uhart et al. 2013), whereas Arias and co-workers reported an association between a GABRA2 SNP and increased stimulation and feelings of a “high” during alcohol intake (Arias et al. 2014). A genetic variation of GABRA2 has been associated with a tendency to externalizing behaviors during adolescence and adulthood, and extraversion and sensation-seeking during early adolescence (Dick et al. 2009, Dick et al. 2013). If one considers the differences between the late-onset, anxious Cloninger type 1 alcoholics with high harm-avoidance, who are believed to use alcohol for its anxiolytic properties, and the early-onset, impulsive and socially hostile Cloninger type 2 alcoholics in whom alcoholism is a strongly inherited trait and who use alcohol for its euphoric effect, the above genetic data might indicate that there may well be gene-derived differences in the GABA$\alpha$ receptor composition between these alcoholic subtypes.

A polymorphism in GABRG1 gene coding $\gamma$1 subunit of GABA$\alpha$ receptor has been linked to alcoholism. According to the genetic studies conducted in various populations, polymorphism of GABRG1 seems to affect the direct effects of alcohol in the CNS and possibly the level of response to alcohol (Covault et al. 2008, Enoch et al. 2009, Ray and Hutchison 2009). It is notable, that GABRA2 and GABRG1 genes are tightly linked and closely adjacent, which may confound the interpretation of the research findings (Enoch 2014).
2.4 GLUTAMATE

Alcohol dependence is a progressive disease. The gradual escalation from unproblematic social drinking to continuous compulsive alcohol use highlights alcohol’s ability to impact on plasticity, learning and memory – processes that strongly involve glutamatergic neurotransmission (Holmes et al. 2013). It has been proposed that the development of an addictive behavior requires the participation of glutamatergic synaptic plasticity in the mesolimbic dopamine system (Eisenhardt et al. 2015). An imbalance in glutamate homeostasis has been suggested to underlie the relapse propensity in alcoholism (Everitt and Robbins 2005, Kalivas 2009). A recent report demonstrated that a blockade of either NMDA- or AMPA-receptor function on the dopaminergic neurons of the VTA or NAc could inhibit alcohol-relapse in an animal model of this condition. Surprisingly, a knockout of either GluN1 subunit of NMDA receptor, or GluA1 subunit of AMPA receptor of these neurons did not affect voluntary alcohol-intake, which does not point to a significant role of the glutamatergic function in this aspect of the rewarding process (Eisenhardt et al. 2015). A hyperfunctional glutamatergic system has been suggested to be strongly involved in the compulsive behavior seen in chronic alcoholism (Vengeliene et al. 2008). The compulsive (also known as the late) state of substance dependence is thought to result from changes in the glutamatergic projections from frontal cortex and anterior cingulate cortex to the ventral striatum, which have changed during repeated exposure to the substance (Kalivas and Volkow 2005), for example, alcohol. The second publication of this thesis examined mGluR2/3 binding, which is presumed to be altered in alcoholism (Kufahl et al. 2011, Meinhardt et al. 2013), although there may well be other changes in the glutamate system. The function of the glutamatergic system of the CNS and how it is altered by alcohol will be discussed below.

2.4.1 Glutamate is the major excitatory neurotransmitter in the brain

Glutamate is the most abundant neurotransmitter in the CNS. It is the brain’s major excitatory neurotransmitter, which controls basal neuronal activity and governs synaptic plasticity (Olive 2009). Many fundamental neuronal processes like basic neuronal communication and consequently mood regulation, memory and learning are widely based on glutamatergic neurotransmission (Hovelsø et al. 2012, Mao et al. 2013).

After the release into the synapse from the vesicles that fuse into the cell membrane of the presynaptic neuron, glutamate binds to the glutamate receptors located pre- and postsynaptically and in the glia (Pomierny-Chamiolo et al. 2014). These receptors are subdivided into two main categories: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). The co-operation of the entire glutamatergic neurotransmission, including the excitation rate set and maintained by the function of iGluRs and tuned by the mGluRs, is required to optimize the function of the CNS to adapt to changing environments and, finally, to sustain homeostasis.

iGluRs are ligand-gated ion channels, which mediate the fast synaptic neurotransmission, whereas mGluRs are GPCRs that modulate the rate of excitation governed by the iGluRs (Hovelsø et al. 2012, Olive 2009). There are three types of iGluRs: N-methyl-d-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isozolepropionic acid (AMPA) and kainate receptors. There are eight mGluRs which have been cloned (mGluR1-8), and grouped into three separate groups according to their sequence homology (Ferraguti and Shigemoto 2006, Hovelsø et al. 2012). iGluRs and mGluRs co-localize in the same synapses (Pin and Duvoisin 1995), but mGluRs are found also in synapses of other neurotransmitters (Hovelsø et al. 2012). The structure and function of mGluRs will be discussed in more detail below, since the binding density of group II mGluRs was studied in the experimental part of the thesis (study 2).
2.4.2 mGluRs modulate glutamatergic excitation

mGluRs belong to the family of GPRCs. They consist of seven transmembrane hydrophobic segments, which are closely located. Three extra- and three intracellular loops separate these segments. The extracellular N-domain contains a binding site for glutamate. The intracellular C-terminal domain and the loops are involved in the regulation of the receptor. C-terminal interacts with various intracellular proteins including cytoskeletal, scaffolding and signaling proteins, as well as receptors bound to the cell membrane (Pomierny-Chamiolo et al. 2014) (See Figure 3). mGluRs may function as homo- or heterodimers (Doumazane et al. 2011, Xi et al. 2002).

![A basic structure of metabotropic glutamate receptor.](image)

Group I mGluRs (mGluR1, mGluR5) are positively coupled to phosphoinositide hydrolysis by Gq protein, which increases the levels of the second messenger, phosphoinositide. They enhance the neuronal excitation set by the iGluRs. Group II mGluRs (mGluR2, mGluR3) and group III mGluRs (mGlur4, mGluR6-8) are negatively coupled to adenylate cyclase through the Gi protein. Their activation inhibits glutamate release of the synapse and reduces glutamatergic tone in the CNS (Ferraguti and Shigemoto 2006, Pomierny-Chamiolo et al. 2014). Since mGluRs are expressed also in synapses releasing other neurotransmitters than glutamate, they are able to regulate GABAergic neurotransmission and extracellular dopamine levels in the NAc (Bradley et al. 1999, Hu et al. 1999, Stefani et al. 1994).

2.4.3 Group I mGluRs promote excitation

Group I mGluRs are located mainly postsynaptically, but also a presynaptic localization has been suggested (Pilc et al. 2008, Pomierny-Chamiolo et al. 2014). Distribution of mGluR1 and mGluR5 in the CNS is different, indicative of different roles for these
receptors. In humans, a dense distribution of mGluR1 is seen in the cerebellum and thalamus, followed by the hippocampus and the FC (Toyohara et al. 2013). The mGluR5 has the most intense expression in the anterior cingulate cortex (ACC), PFC, and temporal cortex, dorsal and ventral striatum, hippocampus and thalamus (Kagedal et al. 2013).

The main outcome of group I mGluR activation can be summarized as a promotion of excitability, which occurs via several mechanisms. The activation of group I mGluRs stimulates phospholipase C and enhances calcium (Ca$^{2+}$) -release from the intracellular stores. In addition, group I mGluR function potentiates Ca$^{2+}$-channel function and decreases potassium (K$^+$) -channel permeability on the cell membrane. Group I mGluRs are functionally connected to NMDA receptors via Homer and scaffolding proteins and their activation potentiates iGluR function (Ferraguti and Shigemoto 2006, Francesconi and Duvoisin 2000, Pomieri-Chamiolo et al. 2014, Sheng 2001).

Due to the very dense distribution of mGluR5 and the moderate distribution of mGluR1 in limbic structures, it is not surprising that group I mGluR function is linked to mood regulation, including anxiety and depression. The group I mGluR negative allosteric modulators and antagonists are known to reduce anxiety-related behavior in different test paradigms of anxiety in animals (Pomierny-Chamiolo et al. 2014). The function of group I mGluRs has a profound impact on the effects of alcohol, and, the inhibition of group I mGluRs has proven effective in the treatment of signs of addiction in animal studies (Pomiery-Chamiolo et al. 2014), however with some major problems which will be discussed in chapter 2.10.2.

2.4.4 Group II mGluRs inhibit glutamate neurotransmission

mGluR2 and mGluR3 serve as the inhibitory autoreceptors of the glutamatergic system. They inhibit presynaptic glutamate release and reduce postsynaptic excitability (Ferraguti and Shigemoto 2006). These receptors are located mainly presynaptically on glutamate terminals, but are expressed also on postsynaptic neurons and glia (Pomiery-Chamiolo et al. 2014). Group II mGluRs activate G$\text{-}$protein and are thereby negatively coupled to the activity of adenylyl cyclase. The stimulation of group II mGluRs results in reduced levels of intracellular cyclic adenosine monophosphate (cAMP) (Olive 2009). Group II mGluRs potentiate K$^+$-channel function and suppress Ca$^{2+}$-channel function. These alterations promote the polarity and suppress the excitation of the nerve cell (Ferraguti and Shigemoto 2006, Pomieri-Chamiolo et al. 2014). By regulating glutamatergic function and the amount of extracellular glutamate, group II mGluRs are involved in the long-term potentiation (LTP) and long-term depression (LTD) as well as other processes involving synaptic plasticity (Kahn et al. 2001, Nicholls et al. 2006).

In humans, mGluR2 is most densely distributed in the cortical areas, above all, in the orbitofrontal cortex, followed by parietal cortex, ACC and PFC. Moderate distribution is seen in the hippocampus, NAc and caudate, as reported by a mRNA study among schizophrenia patients and controls (Ghose et al. 2008). Studies conducted in rodents suggest the most intense distribution of mGluR2/3 in the hippocampus and olfactory bulb, and moderate expression in the striatal and cortical areas (Olive 2009). mGluR3 is most densely expressed in the neocortex, caudate, putamen and substantia nigra in humans, followed by the hippocampus, amygdala and thalamus (Pomiery-Chamiolo et al. 2014).

Group II mGluRs are involved in a variety of functions of the CNS ranging from the control of REM sleep to the regulation of mood and cognitive processes (Pomiery-Chamiolo et al. 2014). Therefore, the modulation of mGluR2/3 function has been the focus of much research interest with the goal of finding new treatment options for several psychiatric and neurologic illnesses (reviewed e.g. in Swanson et al. 2005). Potentiating either mGluR2, mGluR3 or mGluR2/3 function has been postulated as a possible future treatment for schizophrenia, especially its negative and cognitive symptoms (Ellaithy et al. 2015, Li et al. 2015). mGluR2/3 antagonists have shown evidence of promising antidepressant-like effects in animal studies. Several drug companies have on-going clinical
trials of mGluR2/3 negative allosteric modulators (NAM) in the treatment of depression (Hashimoto et al. 2013). Preclinical studies of mGluR2 positive allosteric modulators (PAM) suggest efficacy on anxiety and sleeping disturbances with these compounds (Sheffler et al. 2011). mGluR2/3 modulating compounds also hold promises for addiction treatment, which will be discussed in chapter 2.10.2.

### 2.4.5 Group III mGluRs are presynaptic inhibitory receptors

Similar to the group II mGluRs, group III mGluRs serve as presynaptic inhibitory autoreceptors of the glutamate neurochemistry of the CNS (Ohishi et al. 1995). In addition to the regulation of glutamate trafficking, they also inhibit GABA-release in GABAergic synapses (Kogo et al. 2004, Semyanov and Kullmann 2000). As well as group II mGluRs, group III mGluRs are negatively coupled to G-proteins and inhibit intracellular cAMP-cascade and interact with several intracellular proteins (Pomierny-Chamiolo et al. 2014).

Evolving from their function as modulators of the main neurotransmitter systems of the CNS, mainly curbing excessive glutamatergic tone, group III mGluRs are involved in fundamental processes of the CNS, like neuroprotection. Group III activation in the neurons and glia prevent excitotoxicity and cell death (Bruno et al. 1996, Gasparini et al. 1999, Xi et al. 2003). Group III mGluRs participate in memory formation and regulation of anxiety and pain (Altinbilek and Manahan-Vaughan 2007, Marabese et al. 2007, Palazzo et al. 2008).

mGluR6 is expressed only in the retina (Nakajima et al. 1993), whereas mGluR4, mGluR7 and mGluR8 are either densely or moderately expressed in the striatal areas like NAc, caudate and putamen (Wu et al. 1998). Of the three latter receptor subtypes, mGluR7 is more intensely expressed in the basal ganglia compared to the two other group III mGluRs (Mao et al. 2013). The pharmacological modulation of all three mGluR subtypes affects alcohol-related behavior in animals, as will be discussed in chapter 2.10.2.

### 2.4.6 Acute alcohol inhibits glutamate neurotransmission via NMDA receptors

Acute exposure to alcohol inhibits NMDA receptor function (Lovinger et al. 1989, Lovinger et al. 1990). Therefore, in addition to the GABA receptor, the NMDA receptor is considered as one of the main molecular targets of alcohol in the CNS (Gass and Olive 2008, Vengeliene et al. 2008). The intoxicating potency of several alcohols, i.e. not only ethanol, correlates linearly with their potency to inhibit the activity of the NMDA receptor (Vengeliene et al. 2008). As a result of NMDA receptor inhibition, alcohol interferes with crucial processes of synaptic plasticity, i.e. LTP and LTD, in several brain areas, for example in the hippocampus and dorsal striatum. Alterations in the NMDA-driven LTP and LTD processes have been suggested to underlie typical effects of acute alcohol, like disturbances of memory formation, but also drinking behaviors, which are typical features of chronic alcoholism (Blitzer et al. 1990, Morrissett and Swartzwelder 1993, Wang et al. 2007, Yin et al. 2007).

AMPAR and kainate receptors are not among the primary targets of alcohol at the concentrations normally consumed, but their action is clearly affected by acute alcohol (Vengeliene et al. 2008), which inhibits AMPAR and kainate receptors. The sensitivity of these receptors to alcohol is not as high as that of NMDA receptors (Frohlich et al. 1994, Gass and Olive 2008, Nie et al. 1994, Wirkner et al. 2000).

mGluRs are not among the primary targets of alcohol. Alcohol-related changes in the function of mGluRs occur via its effects on its primary targets, for example GABA and ionotropic glutamate receptors.

### 2.4.7 Chronic alcohol induces a hyperglutamatergic state

Chronic alcohol use leads to a hyperglutamatergic state, during which the level of extracellular glutamate is elevated and the function of the receptors involved in glutamatergic neurotransmission becomes altered (Holmes et al. 2013). Enoch and her co-
workers detected an upregulation of several glutamatergic genes in the hippocampus of alcoholics (Enoch et al. 2014), which presumably applies to the other brain areas involved in alcoholism. Tsai and colleagues (Tsai et al. 1998) reported elevated levels of excitatory neurotransmitters, including glutamate, in the cerebrospinal fluid (CSF) of alcohol dependent subjects during withdrawal, and CSF glutamate levels correlate positively with the severity of alcoholism (Umhau et al. 2010). The following chapter will summarize the effects of chronic alcohol to iGluRs and mGluRs.

2.4.8 Effects of chronic alcohol on iGluRs and mGluRs
Since it can inhibit the function of the NMDA receptor, chronic alcohol induces an upregulation of NMDA receptor subunits in several brain areas including the cortex and the hippocampus (Chandler et al. 1999). Chronic exposure to alcohol also increases NMDA receptor functionality, like conductance, cation influx and clustering of the receptor (Gass and Olive 2008). The effect of chronic alcohol in AMPA receptor function has been studied using several research methods. When alcohol use becomes chronic, it enhances AMPA receptor function in several brain areas. There are cell culture studies and rodent studies pointing to an upregulation of AMPA receptor function and subunit protein expression in the cortex and basolateral amygdala (Chandler et al. 1999, Lack et al. 2007). The binding density of AMPA receptors has been reported to increase during withdrawal period in rats (Haugbol et al. 2005). Rather few studies have examined the chronic effect of alcohol on kainate receptors. In the study of Chandler and colleagues (Chandler et al. 1999), chronic alcohol had no effect on kainate receptor subunit expression. However, administration of the AMPA/kainate receptor antagonist has been shown to inhibit alcohol seeking in rodents (Bäckström and Hyytiä 2004, Czachowski et al. 2012).

In addition to the altered state of iGluR neurochemistry, chronic exposure to alcohol induces clearly detectable changes in mGluR function of the brain. The most convincing of these changes is an increased group I mGluR function and decreased function of group II and III mGluRs.

The protein levels of group I mGluRs mGluR1 and mGluR5 increase as a result of chronic treatment with alcohol in several brain areas that are essential for reward, craving, learning and memory, including the NAc and dentate gyrus (Cozzoli et al. 2012, Galindo et al. 2004). Meinhardt and coworkers (Meinhardt et al. 2013) reported reduced transcript levels of GRM2, the gene coding for mGluR2 in the ACC of human alcoholics. They also found a loss of mGluR2 in the infralimbic cortex of post-dependent rats exposed to chronic intermittent ethanol-vapor and withdrawal. These rats did not respond to the mGluR2/3-agonist therapy by decreasing glutamate levels, in contrast to their controls, suggesting impaired glutamate autoreceptor function as a result of chronic alcohol. Meinhardt and coworkers (Meinhardt et al. 2013) were able to restore the mGluR2-function of the medial prefrontal cortex (mPFC) in rats by using lentiviral therapy. The normalization of the mGluR2 expression led to the abolishment of the alcohol-seeking behavior of these rats, which points to a strong role of mGluR2 receptor function of the ACC in the regulation of alcohol-seeking and relapse-related behaviors. Although there are no reports about chronic alcohol’s effects on group III mGluR function or density, the results from pharmacological studies suggest that enhancement of group III mGluR function could prevent addiction-related behaviors, as the mGluR7 allosteric agonist, AMN082, has suppressed alcohol, cocaine and heroin intake and alcohol and cocaine seeking, and mGluR8 agonist DCPG has prevented alcohol self-administration and cue-induced reinstatement in rodents (Bäckström and Hyytiä 2005, Bahi et al. 2012, Li et al. 2013, Salling et al. 2008). Presumably, the changes of group III mGluR function induced by chronic alcohol intake are involved in the formation of behavioral characteristics of addiction, for example, compulsive alcohol intake, loss of control over intake, depression and anxiety.
2.5 OPIOID SYSTEM OF THE CNS

There are four types of opioid receptors in the CNS. Three of them are called “classical” opioid receptors, namely the µ- (mu-), δ – (delta-) and κ- (kappa-) receptors (MOR, DOR and KOR, respectively). The fourth is the less studied nociceptin-orphanin receptor, which is not considered as an opioid receptor from the pharmacological point of view (Nutt 2013). Therefore, its properties will not be further reviewed here.

Opioid receptors belong to a family of GPCRs, and their nature is inhibitory. They trigger intracellular signaling via G-protein-dependent pathways. An internalization of the receptor is a typical response to receptor agonism (Pradhan et al. 2011). A stimulation of these receptors by either endo- or exogenous ligands triggers the closure of voltage-sensitive Ca²⁺-channels with the result of hyperpolarization, caused by K⁺-efflux. Therefore, the activation of the opioid receptors results in an inhibition of the target cell, such that neurotransmitter release and neuroimpulse conductance are inhibited. The natural ligands of opioid receptors are endogenous opioids synthetized from their precursor-peptides in the CNS in a time-dependent, on-demand manner.

The individual distribution profile of the three opioid receptors in the CNS reveals that these receptors are densely expressed in the brain areas involved in sensory and motor functions like thalamus, and in particular in those brain areas integrating and perceiving sensory information like amygdala and cortical areas (Nutt 2013). In addition, a dense distribution is present in the mesolimbic and mesocortical pathways, which are crucially involved in the core functions of addiction (Nutt 2013). In most brain areas, there is an overlap in the expressions of MOR, KOR and DOR. Specifically, the coexistence of MOR and KOR is common in many structures of the brain (Le Merrer et al. 2009).

In addition to the broad role of the opioid system in the regulation of pain and mood, the function of the opioid system of the CNS contributes to the production of the effects of alcohol, and is densely involved in alcohol addiction (Nutt 2013). The MOR binding density of Cloninger type 1 and 2 alcoholics was explored in study 3 of this thesis. In this chapter, I intend to cover the endogenous ligands and receptors of the opioid system. In addition, their involvement in alcohol related phenomena in the CNS is discussed. Finally, an overview of opioid system modulating agents in the current treatment of alcoholism will be provided.

2.5.1 Endogenous opioids are natural neurotransmitters of opioid receptors

In mammalian species, there are three precursors, namely pro-opiomelanocortin, pro-enkephalin and pro-dynorphin, from which the endogenous opioids are derived. Pro-opiomelanocortin (POMC) is the precursor of β-endorphin, which has high affinity for both MOR and DOR, but also low affinity to KOR. Met- and Leu-enkephalins are derived from β-endorphin. These enkephalins express some affinity for the MOR, but a tenfold higher affinity to DOR and they have much less KOR-affinity (Lord et al. 1977, Nutt 2013, Zadina et al. 1997). In contrast, dynorphin-A, dynorphin-B and neo-endorphin display a strong affinity for KOR and lower affinity for the MOR and DOR (Nutt 2013). Endomorphin-1 and endomorphin-2 are highly MOR-selective ligands (Zadina et al. 1997).

The endogenous ligands of MOR and DOR are experienced as rewarding. Rodents will self-administer β-endorphin (van Ree et al. 1979). Furthermore, in work conducted in experimental animals, it has been shown that alcohol has the ability to increase the release of β-endorphin and POMC in several brain areas, including the NAc. The release has been postulated to be greater with lower concentrations of alcohol (Nutt 2013). Interestingly, there are innate differences in the alcohol-induced release of endogenous opioids between alcohol-preferring and non-preferring animals, which suggest that there might be
differences in opioidergic neurotransmission between these animal lines, possibly linked to their alcohol consumption (de Waele and Gianoulakis 1993).

In addition to β-endorphin, also enkephalins and dynorphins are affected by alcohol. A moderate intraperitoneal dose of alcohol (i.e. acute treatment) triggers Met-enkephalin release in the NAc shell of rats whereas no response is seen in central amygdala (Lam et al. 2008, Marinelli et al. 2005, Marinelli et al. 2006). The differences in the release of these endogenous opioids in different brain areas presumably reflect the distinct functional roles of these areas. In contrast to the acute effects of alcohol, animal studies have found evidence of a reduction in the release of Met-enkephalin after chronic alcohol in the striatum and hypothalamus (Seizinger et al. 1983). Interestingly, innate differences in baseline Met-enkephalin levels of the striatum between alcohol preferring and non-preferring animal lines have been found (Blum et al. 1987). In addition, these findings point to an involvement of the inherited composition of the opioid neurotransmission in alcohol preference, and possibly in the vulnerability to alcoholism.

The acute exposure to alcohol affects also dynorphin release, since a high dose of acute alcohol increases the release of dynorphin (Lam et al. 2008, Marinelli et al. 2005, Marinelli et al. 2006). Dynorphin release is possibly linked to the aversive properties of high alcohol doses.

2.5.2 Opioid receptors
The three opioid receptors have distinct functions and distributions in the CNS. The functions of MOR will be described thoroughly, since its binding density was studied in the study 3 of the thesis but also DOR and KOR will be discussed briefly.

2.5.3 MOR function is essential in addiction
MOR is the most important receptor in the opioid receptor family in terms of alcohol use and addiction, although also KOR and DOR play a role. MOR is located both pre- and postsynaptically and is widely distributed throughout the human CNS (Cross et al. 1987). The highest MOR binding has been detected in the thalamus, caudate nucleus, putamen and cortex, where it was concentrated in the superficial layer (Cross et al. 1987, Gross-Isseroff et al. 1990). Although the opioid receptor distribution in the whole CNS is sparse compared to the receptor density of the main inhibitory and excitatory neurotransmitters, GABA and glutamate, MOR has a dense distribution in several brain areas implicated in alcoholism and addiction in general, and it has a crucial role in the regulation of several essential processes characterizing alcoholism.

MOR activation is experienced as rewarding, i.e. MOR agonism produces conditioned place preference (CPP) and self-administration of drugs in animal models (Devine and Wise 1994, Shippenberg et al. 1992). The extent of dopamine activation of the NAc of this MOR-agonism driven reward is unclear, but dopamine is believed to play a role, since MOR and DOR agonist administration produces an elevation of the dopamine levels in the VTA (Devine et al. 1993). Several KO studies in mice have revealed the essential role of MOR in the mediation of the rewarding effects of all drugs of abuse, including alcohol, whereas the role of DOR function in this process is modulatory, facilitating the emotional states and memories related to the drug use (Charbogne et al. 2014).

MOR activation has the ability to promote disinhibition in the CNS, since inhibitory GABA-containing interneurons are among its primary targets (Nicoll et al. 1980). The MORs of the VTA are located in the inhibitory GABAergic neurons (Nutt 2013), which regulate dopaminergic input into the NAc in a tonic manner. The activation of the MORs inhibits GABAergic neurons in the VTA, which in turn results in increased dopaminergic firing to the NAc, as dopaminergic efferents from the VTA are “unleashed” from the control of GABAergic interneurons (Di Chiara and North 1992). This increased dopaminergic input into the NAc contributes to the rewarding effects of alcohol.
2.5.4 Alcohol affects MOR function

Several human and animal study methods have demonstrated the profound effect of alcohol on MOR function. According to human PET studies conducted with $[^{11}C]$carfentanil, acute alcohol consumption induces a decrease in $[^{11}C]$carfentanil binding, i.e., increased release of endogenous opioids with an affinity to MOR in the orbitofrontal cortex (OFC) and NAc. In heavy drinkers, the intensity of this change correlates with the severity of alcohol misuse and reported subjective feelings of high (Mitchell et al. 2012). These findings provide evidence for alcohol-induced release of endogenous opioids, and for the involvement of the opioidergic neurotransmission in reward and addiction.

In the PET studies of Heinz and colleagues and Weerts and co-workers, $[^{11}C]$carfentanil binding to MOR was increased in abstinent alcoholics when compared to controls (Heinz et al. 2005, Weerts et al. 2011). In the study of Heinz and colleagues (2005), this increase correlated positively with the reported alcohol craving, whereas in the study of Weerts and colleagues, the correlation between $[^{11}C]$carfentanil binding and craving was inverse. The observed increased $[^{11}C]$carfentanil binding in alcoholics could be due to increased density or affinity of the MOR. It could also be a reflection of decreased release of MOR-sensitive endogenous opioids.

The immunohistochemical study of Saland and colleagues in rodents after two weeks of chronic alcohol exposure found some evidence of decreased MOR expression in several brain areas indicated in addiction, like the cortex, hippocampus, NAc and striatum, but elevated DOR in CA1 area of the hippocampus (Saland et al. 2005). Another recent rodent study reported decreased expression of the MOR encoding gene Oprm in NAc as a result of moderate long-term alcohol exposure (Jonsson et al. 2014). These potentially contradictory results between the human and animal studies may be due to different research techniques, a different state of alcoholism (chronic intake or abstinence after detoxification), but also possibly due to differences between the species.

Due to the important role of MOR in reward, the manipulation of MOR system is a possible avenue of addiction drug development. MOR KO animals do not self-administer alcohol, and they even seem to assess the substance as aversive (Roberts et al. 2000), which emphasizes the profound role of MORs in alcohol reward and development of alcoholism. For years it has been known that MOR antagonism reduces alcohol consumption and suppresses cue-induced reinstatement of alcohol seeking in rodents (Hyytiä 1993, Ciccocioppo et al. 2002). Although currently no MOR specific compounds are available for the treatment of addiction, MOR is the main target of naltrexone and nalmefene, the non-selective opioid antagonists used in the treatment of alcoholism (Emmerson et al. 1994).

2.5.5 MOR function plays a role in mood regulation and impulsivity

The structure and function of MOR has been linked to the regulation of mood and impulsivity. There are several studies which have investigated a single nucleotide polymorphism A118G of OPRM1 gene encoding human MOR suggesting that this polymorphism links to certain aspects of impulsivity and impaired self-control, as well as to enhanced sensitivity to alcohol’s effects. Recently, a report appeared of greater activation of functional connectivity of the ventral and dorsal striatum in the BOLD fMRI during the presentation of alcohol-related cues among alcoholic G-allele carriers of A118G when compared to alcoholic A-allele homozygotes (Ray et al. 2014). The authors suggested this increased functionality was related to greater sensitivity to reward and limited self-control. G-allele carriers of A118G mutation may display increased sensitivity to the rewarding effects of addictive substances. The G-allele carriers reported increased liking in an initial study among adolescents experiencing their first dose of nicotine (Schuck et al. 2014). Furthermore, it has been postulated that the enhanced reactivity of the A118G G-allele carriers to alcohol-induced stimulation and positive mood would be regulated by dopamine transporter genotype (Ray et al. 2014). Gross-Isseroff and colleagues (Gross-Isseroff et al. 1990) detected elevated MOR binding density in their post-mortem
autoradiography study of young suicide victims. This finding was speculated to possibly link to the depression. On the other hand, in the light of the aforementioned genetic studies, the elevated MOR binding could also link to impulsivity, which often characterizes young suicide victims (Apter 2010).

2.5.6 DOR activation is experienced as rewarding
The DOR distribution in the human CNS is sparse with the highest distribution being found in the putamen and thalamus (Cross et al. 1987). In fact, there is a growing pharmaceutical research interest in the DOR as a possible modulator of anxiety and addiction. Although its contribution to the reward process is still a matter of debate, DOR plays a role in addiction (Pradhan et al. 2011). DOR activation has rewarding properties, since DOR agonists induce self-administration and CPP in animals (Devine and Wise 1994, Shippenberg et al. 1992), but DOR KO studies and experiments where DOR function has been antagonized, both point to a modulating rather than a crucial role for DOR in the reward process (Chu Sin Chung and Kieffer 2013). Both human and rodent studies indicate that alcohol can alter DOR function. One preliminary human PET study of Weerts and colleagues using DOR-specific ligand \[^{11}C\]methylnartindole reported a trend of increased DOR binding in the ventral striatum of abstinent alcoholics (Weerts et al. 2011). In line with these findings, it has been reported that there is an increase in DOR binding in the hippocampus of rodents after chronic alcohol intake (Saland et al. 2005).

Several studies suggest that DOR manipulation can modulate alcohol use–related phenomena. DOR blockade inhibits alcohol seeking in animal models (Shippenberg et al. 2008, Marinelli et al. 2009), however, many of these studies used concentrations of DOR antagonists that display activity also at MOR and other neurotransmitter receptors, which may confound the interpretation of the results (Shippenberg et al. 2008). DOR-agonism alleviates alcohol withdrawal-induced anxiety (van Rijn et al. 2010). Surprisingly, in a KO animal study, DOR KO mice self-administered more alcohol than wild type animals (Roberts et al. 2001). The authors speculated that the increased alcohol consumption could be a compensatory mechanism linked to the increased anxiety observed in these animals. This emphasizes the potential role of DOR neurochemistry in the development of alcoholism but also, by demonstrating the regulatory role of DOR both in anxiety and in alcohol intake, the entangled nature of the functions of the neurotransmitter receptors in the CNS.

Animals subjected to genetic deletion of either DOR or preproenkephalin, the endogenous precursor ligand of DOR, show symptoms of anxiety and depression (Filliol et al. 2000, König et al. 1996). These results and DOR-agonism studies point to DOR as a potential target in the treatment of mood and anxiety-spectrum disorders in the future. The marked role of DOR in pain perception suggests that DOR agonists could be especially useful in combatting mood disorders induced by chronic pain. The neuroprotective properties of DOR-agonism also hint at a role for these ligands in the treatment of neurological diseases (Pradhan et al. 2011).

2.5.7 KOR activation is aversive
KOR is involved in the perception of pain, neuroendocrinological responses, mood regulation and cognition (Simonin et al. 1995). According to Cross and co-workers, the highest KOR-binding in humans is seen in the deep layers of the cortex, claustrum and caudate nucleus (Cross et al. 1987), whereas Simonin and colleagues reported that the highest distribution existed in the cortex, basal ganglia, amygdala, olfactory bulb and thalamus. In the VTA, the distribution of KOR was moderate (Simonin et al. 1995). It is assumed that KOR regulates the level of the basal dopamine release in the NAc in a limiting manner, and therefore represents an opposing force to MOR-driven actions (Pan 1998, Spanagel et al. 1992). Indeed, KOR agonists produce place aversion and dysphoric effects in laboratory animals (Wee and Koob 2010). The usefulness of KOR-agonists for
example in the treatment of pain is limited, since these compounds are hallucinogenic and highly aversive.

The pharmacological blockade of KOR holds promises to moderate stress response and drug craving as well as depression (Pradhan et al. 2011). For example, KOR KO animals administer less alcohol than their wild type counterparts (Kovacs et al. 2005). KOR antagonism reduces alcohol intake in rodents (Cashman and Azar 2014, Walker and Koob 2008). In addition, a knockdown study of prodynorphin, the precursor of the endogenous ligand of KOR, in the NAc of rats, demonstrated decreased depressive behavior and a more rapid attenuation of a locomotor sensitization to cocaine (Cohen et al. 2014), which point to a role for KOR both in the regulation of mood and in the development of addiction.

2.5.8 Non-selective opioid antagonists can reduce relapse to heavy drinking
A specific blockade of any of the three opioid receptors, i.e. MOR, DOR and KOR, attenuates alcohol self-administration in animals (Cashman and Azar 2014, Ciccocioppo et al. 2002, Hyytiä 1993, Shippenberg et al. 2008). However, currently, only a non-selective opioid receptor blockade is available for the treatment of alcoholism in humans. Taking into account the central role of opioidergic neurotransmission in reward, one could postulate that a non-selective blockade of opioid responses would lead to lack of reward and therefore escalate craving and promote relapse. However, this is not the case. Non-selective opioid antagonists, naltrexone and nalmefene, are among the few drugs clinically used in the treatment of alcoholism (Wackernah et al. 2014). One explanation for the efficacy of these pharmacological agents in alcoholism is their ability to prevent the endogenous opioids from exerting their action. Possibly, these compounds are “overdosed” and therefore, they block all opioid receptors in a non-selective manner (Nutt 2013). Thus, both craving for alcohol and reward from alcohol, mediated by endogenous opioids and elevated by an imbalance of the opioid system caused by chronic exposure to alcohol, are blocked.

2.5.9 Naltrexone inhibits reward and reduces craving
Of the two opioid antagonists used in the treatment of alcohol dependence, naltrexone has been longer on the market. Naltrexone is a non-specific antagonist of opioid receptors, which has its highest affinity for MOR, but also some affinity for KOR and only minor affinity for DOR (Codd et al. 1995, Emmerson et al. 1994). Human fMRI studies with [11C]carfentanil and [11C]methylartindole have demonstrated an almost complete occupancy of MOR, but only about 20% occupancy of DOR although with extensive variance between subjects after administration of oral naltrexone (50 mg, the recommended daily dose) (Weerts et al. 2008). Due to the lack of a specific, tritium-labeled ligand for clinical human use, the occupancy of KOR remains to be resolved. A recent meta-analysis has found evidence that naltrexone has efficacy in the prevention of relapse and heavy drinking (Jonas et al. 2014). It is believed that the efficacy of naltrexone, although modest and heterogeneous (Spanagel and Kiefer 2008), may be based on its ability to reduce craving and modulate the reward experienced while drinking (Chick et al. 2000, Drobes et al. 2003, Drobes et al. 2004, Nutt 2013).

2.5.10 Nalmefene resembles naltrexone
Like naltrexone, nalmefene is a non-specific antagonist of opioid receptors and its clinically relevant properties are very similar to naltrexone. The modest differences presumably originate from its slightly different opioid receptor binding profile. Similarly to naltrexone, nalmefene has the highest affinity for MOR, followed by KOR and DOR. Its affinity for KOR and DOR is higher than that of naltrexone (Emmerson et al. 1994). Its bioavailability and half-life are longer than the corresponding values for naltrexone (Nutt 2013), which may be beneficial in the clinical reality. In rodents, nalmefene decreases alcohol intake in a dose-dependent manner (June et al. 1998). In vitro cell culture studies suggest that
nalmefene acts as a partial agonist of KOR (Remmers et al. 1999), which may have clinical importance. Human binding studies with [11C]carfentanil point to a high occupation of MOR with nalmefene, that persists longer than can be achieved with naltrexone (Ingman et al. 2005). In the meta-analysis of Jonas and colleagues, nalmefene was shown to reduce the number of heavy drinking days and drinks per day (Jonas et al. 2014).

**2.5.11 Efficacy of non-selective opioid-antagonists in the treatment of alcoholism**

Despite the promising results of animal studies, non-selective opioid-antagonists have displayed only a modest efficacy in humans and there is extensive variation in the clinical response e.g. only about 20-30% of patients respond to naltrexone (Spanagel and Kiefer 2008). Several researchers have suggested that opioid receptor function modulating agents may not benefit all alcoholics, but may instead exert efficacy among a subset of alcoholics, as reviewed below.

Kiefer and colleagues (Kiefer et al. 2008) suggest that a division of alcoholics according to Cloninger’s typology would predict treatment response and help clinicians to identify those patients who would most likely benefit from opioidergic medications. In their analysis of two large-scale European clinical studies, the subset of Cloninger type 2 early onset alcoholics benefited from naltrexone more than Cloninger type 1 alcoholics (Kiefer et al. 2008). The study of Monterosso and co-workers (Monterosso et al. 2001) demonstrated that patients with a strong family history of alcoholism and high intensity of craving benefit more from naltrexone-treatment. Oslin and colleagues found that the difference in naltrexone treatment outcome depended on the A118G polymorphism of OPRM1, which has been shown to cause variation in the endorphin binding properties of MOR and the altered HPA-axis activation to naloxone (Oslin et al. 2003). Taken together, these studies suggest that some typical characteristics of Cloninger type 2 alcoholics, such as early-onset of alcoholism and high heritability predict a better treatment-outcome with a non-selective opioid antagonist naltrexone. In consideration of the finding of Oslin and colleagues (Oslin et al. 2003), one could speculate that the variation in MOR receptor function, possibly due to a genetic background, could explain the very different responses to opioid antagonists between the various alcoholic subgroups. Interestingly, polymorphism of A118G has been linked to impulsivity, as discussed above. In the study of Ray and colleagues polymorphism of A118G was associated with increased fronto-striatal negative functional connectivity in fMRI suggested to reflect impaired balance between frontal control over reward and habit-driven input (Ray et al. 2014). In addition, A118G polymorphism has been linked with greater liking of first-experience nicotine among adolescents (Schuck et al. 2014), possible implying that these individuals have a greater sensitivity to reward. Impulsivity is one of the fundamental characteristics of Cloninger type 2 alcoholics. There is a possibility that the characteristic differences between Cloninger type 1 and 2 alcoholics may stem, at least in part, from the differences in opioid receptor function between these alcoholic subgroups.
2.6 PHARMACOLOGICAL TREATMENT OF ALCOHOLISM

There is no treatment of choice for alcoholism. No single pharmacological or non-pharmacological therapeutic approach is effective for every patient (Wackernah et al. 2014). Although not only the efficacy but also the application of current pharmacotherapies is limited, they still have proven efficacy in many measures and should perhaps be administered more often than is currently the case (Holmes et al. 2013). Different medications target different aspects of alcoholism, like reward, craving, relapse-propensity or withdrawal.

Considering the pharmacological treatment of alcoholism, it must be borne in mind, that combining psychotherapy, supportive therapy or supervision to pharmacological treatment of alcoholism often improves treatment outcomes and is therefore the recommended treatment approach. For example, treatment results with disulfiram improve significantly with supervision or therapy (Azrin 1976, Jorgensen et al. 2011) and according to several studies, naltrexone is effective when combined to psychosocial therapies or interventions (Agosti et al. 2012, Heinala et al. 2001). Several researchers have emphasized the regrettable truth about the ambivalent commitment towards treatment among patients and distrust of the abstinence-focused programs among both patients and clinicians (Aubin and Daeppen 2013). The best treatment results have been reported with multimodal treatments combining pharmacological and therapeutic approaches (Miller et al. 2011).

2.7 CURRENTLY APPROVED MEDICATIONS FOR THE TREATMENT OF ALCOHOLISM

Currently, there are five medications for the treatment of alcoholism approved by the European Medications Agency (EMA): Disulfiram, oral naltrexone, extended-release injectable naltrexone, acamprosate, and nalmefene. The first four of these drugs are approved also by the US Food and Drug Administration (Swift and Aston 2015). In addition, there are several other compounds that are used off-label or are under investigation for the treatment of alcoholism, i.e. aripiprazole, baclofen, bupropion, gabapentin, memantine, metadoxine, olanzapine, ondansetron, prazosin, quetiapine, rimonabant, SSRIs, topiramate, varenicline and zonisamide (Swift and Aston 2015). In this chapter, I will discuss the medications approved by the EMA followed by a brief presentation of two off-label compounds, topiramate and baclofen. These last two were chosen from the aforementioned wide variety of off-label compounds, since topiramate affects both glutamatergic and GABAergic neurochemistry, and baclofen has its main effect on GABA\textsubscript{B} receptors. The alterations of glutamatergic and GABAergic systems were the focus in the experimental part of this thesis (studies 1 and 2). In addition, there are reports suggesting good patient adherence to these compounds, since topiramate is widely prescribed for the treatment of alcoholism in the US, whereas in France, there has been a widespread lobbying by patients and some physicians for baclofen to receive official approval for this indication (Del Re et al. 2013, Rolland et al. 2012). Finally, I will try to have a look at the future by covering the current state of mGluR agents under investigation for the treatment of alcoholism.

These medications alter different neurobiological aspects of drinking and alcoholism and they may change the subject’s preference for or propensity to alcohol intake. Two of the main mechanisms of action are reward inhibition and reduction of relapse propensity, both of which result in reduced alcohol consumption. For example, disulfiram increases the aversive effects of alcohol drinking and thereby prevents drinking. The opioid antagonist naltrexone inhibits the rewarding and pleasurable effects of alcohol and reduces the intake...
of alcohol through this mechanism. Some of the medications act as a substitute for alcohol in specific receptors like glutamate receptors, GABA receptors and other ion channels and prevent withdrawal caused by the altering the properties of these receptors - a result of chronic alcohol intake (Swift and Aston 2015). A summary of the characteristics of the pharmacotherapies of alcoholism treatment approved by the EMA can be found in Table 1.

Table 1. Characteristics of the pharmacotherapies approved by the EMA for the treatment of alcoholism.

<table>
<thead>
<tr>
<th>Pharmacotherapy</th>
<th>Binding / Action site</th>
<th>Dosage</th>
<th>Safety</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disulfiram</td>
<td>• Inhibition of aldehyde dehydrogenase</td>
<td>• 200mg daily or 400mg twice a week</td>
<td>• May be fatal with alcohol, Hepatic damage</td>
<td>• Aversion to alcohol → Risk of reinstatement ↓</td>
</tr>
<tr>
<td>Acamprosate</td>
<td>• Not fully understood • mGluR5 inhibition • NMDA-receptor binding • Ca²⁺ the active component?</td>
<td>• 666mg 3 times a day</td>
<td>• Safe</td>
<td>• Relief of withdrawal → Craving ↓ → Negative reinforcement ↓</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>• Non-selective opioid receptor antagonist • Main target MOR</td>
<td>• 50mg daily or 380mg i.m. every 4 weeks</td>
<td>• Hepatic damage</td>
<td>• Reward ↓ → Craving ↓ → Relapse propensity ↓</td>
</tr>
<tr>
<td>Nalmefene</td>
<td>• Non-selective opioid receptor antagonist • Main target MOR</td>
<td>• 18mg daily or as needed</td>
<td>• Hepatic damage</td>
<td>• Reward ↓ → Craving ↓ → Relapse propensity ↓</td>
</tr>
</tbody>
</table>

2.7.1 Disulfiram causes aversion to alcohol
Disulfiram has been used for the treatment of alcoholism for decades. It inhibits aldehyde dehydrogenase, which leads to an accumulation of acetaldehyde in the circulation if alcohol is consumed. The symptoms of accumulating acetaldehyde include flushing, tachycardia, nausea and sweating (Suh et al. 2006, Swift and Aston 2015). In fact, the combination of alcohol and disulfiram may be fatal (Suh et al. 2006), which needs to be emphasized when treating patients. The aversive effects of disulfiram are thought to remove the drive to use alcohol. The efficacy of disulfiram is still under a debate due to mixed results of clinical trials. Good supervision of the pill taking seems to improve treatment results, as well as information about the treatment regimen and therapeutic goal (Suh et al. 2006). In a randomized multicenter study combining CBT and supervised medication, disulfiram was superior to naltrexone and acamprosate (Laaksonen et al. 2008).

2.7.2 Naltrexone and nalmefene attenuate reward and craving
Naltrexone is a non-selective opioid antagonist of MOR, DOR and KOR, with highest affinity for MOR. Its function on the opioid receptors has been described more extensively in chapter 2.5.9. Opioid antagonists reduce alcohol intake by attenuating the rewarding effects of alcohol. They occupy the binding sites of endogenous opioids on opioid receptors and prevent the release of dopamine in the NAc. They also prevent craving during both alcohol intake and in abstinence (Swift and Aston 2015). The efficacy of naltrexone is
modest (Swift and Aston 2015), but it seems to cut down heavy drinking and reduce the number of drinking days (Rosner et al. 2010b).

Nalmefene is a compound very similar to naltrexone, with its partial agonist activity at KOR and decreased risk of hepatotoxicity being the most prominent differences between it and naltrexone (Swift and Aston 2015). Nalmefene treatment should be combined with a psychosocial intervention. As the goal for nalmefene treatment is a reduction in alcohol consumption, psychosocial support must be available to improve treatment adherence and ensure the reduction in consumption (EMA 2013). One possible way of dosing nalmefene is to use it as-needed, during times of heightened risk for heavy drinking (Swift and Aston 2015). In several trials, nalmefene has been efficient in reducing alcohol consumption and the number of drinking days, but also opposite results i.e. no efficacy, have been reported (Anton et al. 2004, Gual et al. 2013, Mason et al. 1994, Mason et al. 1999).

2.7.3 Acamprosate promotes abstinence
Acamprosate, calcium N-acetylhomotaurinate, is able to reduce craving and withdrawal symptoms like neuronal hyperexcitability. It is thought that acamprosate is able to suppress negative reinforcement. Compared to other withdrawal medications like benzodiazepines, acamprosate is devoid of addictive potential and it does not cause sedation or anxiolysis (Littleton 1995). The mechanism of action of acamprosate is not fully understood. Some studies suggest that the drug can bind to mGluR5 and NMDA receptors (Harris et al. 2002, Naassila et al. 1998). Recently, the activity of N-acetylhomotaurinate on the glutamate system has been questioned, and it has been proposed that N-acetylhomotaurinate itself is not the active component in the CNS. For example, Spanagel and his co-workers have suggested that calcium is the active component of acamprosate (Spanagel et al. 2014).

Despite the confusion about the mechanism of action, acamprosate seems to have modest efficacy in abstinence promotion. According to a Cochrane database review conducted by Rösner and co-workers (Rosner et al. 2010a), acamprosate reduces the risk of any drinking and increases the duration of abstinence. Due to its favourable side-effect profile and the lack of addiction potential, it is the most commonly used medication for the treatment of alcoholism in the US (Spanagel et al. 2014). Acamprosate is approved as one of the five medications for the treatment of alcoholism by EMA (EMA 2013). Along with naltrexone, it is recommended as a first-line therapy in the treatment of alcoholism by National Institute for Health and Care Excellence (NICE) in the United Kingdom (NICE 2011). According to the Finnish treatment regimen, acamprosate is an option for the treatment of alcoholism (Käypä hoito -suositus, 2015).

2.8 OFF-LABEL TREATMENT OF ALCOHOLISM: TOPIRAMATE AND BACLOFEN

Although topiramate is not officially approved for the treatment of alcoholism in either Europe or the US, it is more often prescribed for alcoholism than acamprosate and naltrexone combined in the Veterans Health Administration system of the US, which hints at its efficacy in the treatment of alcoholism (Del Re et al. 2013). Topiramate is an antiepileptic medication that acts by decreasing the activity of the Ca²⁺-channels, inhibiting glutamatergic excitation and enhancing GABAergic inhibition of the CNS (Johnson 2005, White et al. 2000, Zhang et al. 2000). According to a recent meta-analysis conducted by Jonas and co-workers (Jonas et al. 2014), topiramate has efficacy in decreasing the number of heavy drinking days and in reducing the number of drinks consumed on a drinking day. The side effects of topiramate include anhedonia and increased risk of suicide (Swift and Aston 2015), which should be cautiously monitored when treating this group of patients. Interestingly, a common side effect of topiramate is anorexia, which has as its part in paving the way for novel treatment approaches to alcoholism treatment research based on
the appetite control mechanisms of the body, like ghrelin antagonists (Swift and Aston 2015).

Baclofen is used as an off-label treatment of alcoholism in some European countries. It is a GABA\textsubscript{B} receptor agonist, which is thought to reduce craving and the anxiety associated with alcoholism, especially during withdrawal (Brennan et al. 2013). The results assessing its efficacy in promoting abstinence are mixed with more research needed to reliably assess the efficacy and safety of baclofen in the treatment of alcoholism (Brennan et al. 2013, Liu and Wang 2015).

2.9 EFFICACY OF THE CURRENT MEDICATIONS

Unfortunately, the efficacies of the pharmacotherapies of the alcoholism are still only modest, which may in part explain the low utilization rates of these compounds in comparison with the prevalence of alcoholism (Swift and Aston 2015, Spanagel and Kiefer 2008). According to a meta-analysis of Jonas and co-workers, the number needed to treat to prevent any drinking is 12 for acamprosate and 20 for oral naltrexone. In this analysis, disulfiram was not effective (Jonas et al. 2014). The wide heterogeneity of alcoholism and different symptoms depending on the stage of the illness are factors that signify the need for individual treatment protocols (Heilig and Leggio 2016). Due to the lack of clinical measurement tools about the neurotransmission profile of the patient, excluding the hints provided by the character of the patient him/herself, it is currently impossible to devise an individually tailored treatment, which probably explains the low efficacy of these compounds. Therefore, there is a great need for measurement tools that could predict the individual response to a certain compound. Imaging technologies and genetics can provide this information (Heilig and Leggio 2016, Spanagel and Kiefer 2008), but relevant clinical exploitation of these techniques still has to be performed.

2.10 FUTURE OF THE TREATMENT OF ALCOHOLISM: GLUTAMATERGIC MEDICATION

As discussed previously in chapter 2.4, numerous studies in several fields have revealed the extensive effect of glutamate signaling on the rewarding and intoxicating effects of alcohol. In addition, the function of glutamate neurochemistry undergoes marked adaptations during chronic alcohol intake, and these changes presumably underlie at least part of the withdrawal, craving and compulsive alcohol use, which are characteristics of alcohol addiction (Holmes et al. 2013). Therefore, the manipulation of the function of the glutamatergic neurochemistry holds promises for the future treatment of alcoholism, despite several problems associated so far with the glutamatergic compounds in the preclinical and clinical testing (Holmes et al. 2013). Furthermore, glutamatergic compounds are being currently evaluated in the treatment of other psychiatric disorders like anxiety, schizophrenia and depression (Gerhard et al. 2016, Raber and Duvoisin 2015, Wieronska et al. 2016). Glutamate neurochemistry offers several possible targets for medication. Below, the effects of the manipulation of the iGluRs, mGluRs and glutamate transporters on alcohol related behaviors are discussed.

2.10.1 Manipulation of the iGluR function may induce problematic side-effects

Agents that affect iGluR function have a clear effect on alcohol-related behavior in animal models. In brief, NMDA-agonists attenuate oral alcohol consumption in rats and ameliorate several other alcohol-related behaviors (Gass and Olive 2008). AMPA- and kainate-antagonism produces similar results (Gass and Olive 2008). Partial NMDA-agonists facilitate extinction (Kalivas and Volkow 2011).
Administration of NMDA receptor antagonists has been found to modulate several alcohol related behaviors in animal models. These compounds are able to reduce the self-administration, withdrawal symptoms and neurotoxicity of alcohol (Rassnick et al. 1992, Stepanyan et al. 2008). A NMDA receptor antagonist, memantine, has been clinically tested among alcoholics. There were positive results suggestive of an alleviation of withdrawal symptoms and reduced cue-induced craving (Krupitsky et al. 2007a, Krupitsky et al. 2007b). It has been postulated that targeting specific subunits of NMDA receptors could be one way to bypass the side-effects of NMDA receptor antagonists. Another option would be to devise drugs that would be able to allosterically modulate NMDA receptor function. These approaches could offer better tolerated ways to affect glutamate neurotransmission (Holmes et al. 2013).

As mentioned above, topiramate is already used off-label in the clinical treatment of alcoholism. This drug has a complicated neurochemistry, e.g. it inhibits the release of glutamate and antagonizes AMPA and kainate receptors (Holmes et al. 2013). The efficacy of topiramate in the treatment of alcoholism suggests that modulation of AMPA and kainate receptors could be another way to influence alcohol related behaviors, although AMPA and kainate receptors presumably do not mediate the effects of alcohol at normally consumed levels of alcohol (Holmes et al. 2013). Animal studies suggest that AMPA and kainate receptor antagonists are able to reduce alcohol seeking behavior (Bäckström and Hyytiä 2004).

The side-effects of the iGluR antagonists would be the main concern associated with the clinical use of these compounds. iGluR antagonists cause cognitive impairments like disorientation and loss of memory, as well as psychoactive effects like hallucinations and depersonalization. The agonism and antagonism of iGluRs leads to neurotoxic processes (Pomierny-Chamiolo et al. 2014). Due to these problems, there has been a growing research interest towards modulation of the mGluR system in the treatment of alcoholism (Holmes et al. 2013, Olive 2009).

2.10.2 Manipulation of the mGluR function offers several possible targets
The allosteric modulation of the metabotropic glutamate neurotransmission could offer tools for the treatment of addiction, but also to a wider variety of impaired CNS functions including chronic pain, neurological disorders like Parkinson’s disease, neurodegenerative disorders and epilepsy, as well as psychiatric disorders like anxiety, depression and schizophrenia (Olive 2009).

Inhibition of mGluR1 is able to reduce alcohol consumption in animal models, although this effect might have been due to decreased locomotion and general inhibitory properties of mGluR1 NAMs (Besheer et al. 2008a, Lominac et al. 2006). Similarly, inhibition of mGluR5 has been reported to reduce alcohol drinking, CPP, withdrawal and cue-induced reinstatement of alcohol administration in animal models (Holmes et al. 2013). Animal studies have found evidence for reduced responding to natural rewards with high doses of mGluR5 NAMs. This blurs the picture and suggests possible unwanted side-effects in clinical use (Holmes et al. 2013, Olive 2009).

Agonism of mGluR2 and mGluR3 receptor subtypes results in reduced release of glutamate from the presynaptic terminals. In animal models, this attenuates the reinforcing effects of alcohol and inhibits the reinstatement of alcohol use (induced by cue, stress or priming). mGluR2/3 agonist attenuated cue- and stress-induced reinstatement of alcohol use and prevented relapse in rodents (Rodd et al. 2006, Zhao et al. 2006). As mentioned above, restoring mGluR2 function in the mPFC by lentiviral delivery of mGluR2 expression was able to prevent alcohol seeking behavior in dependent rats (Meinhardt et al. 2013). Interestingly, animal models suggest most profound effects in addicted subjects (Bäckström and Hyytiä 2005, Kufahl et al. 2013, Rodd et al. 2006, Sidhpura et al. 2010, Zhao et al. 2006). Possibly, these compounds could act simultaneously by suppressing intake and preventing neuronal damage caused by the hyperglutamatergic state of chronic alcoholism (Holmes et
al. 2013). However, in animal models, higher doses of group II mGluR agonists have been reported to decrease locomotion and interest for the natural rewards, which warrants further study (Olive 2009).

An allosteric agonist of mGluR7 has been reported to reduce alcohol consumption and alcohol-evoked CPP in rodents, whereas a selective mGluR7 inhibition enhances alcohol-intake (Bahi 2012, Bahi et al. 2012). A systemic administration of mGluR8 agonist attenuated alcohol reinforcement, self-administration and cue-induced drug seeking (Bäckström and Hyytiä 2005). Currently, there are no reports about the involvement of the mGluR4 receptor subtype in the consumption of alcohol. However, the function of this receptor is implicated in depression, anxiety and convulsions (Pomier-Chamiolo et al. 2014), all of which are typical symptoms of alcohol withdrawal. Therefore, modulation of mGluR4 function might have potential benefits in the treatment of alcoholism. There are reports about decreased sucrose intake after treatment with an mGluR7 agonist and decreased spontaneous locomotion with an mGluR8 agonist (Mao et al. 2013), interesting results, which need to be confirmed. Considering the mechanisms of actions of these compounds, group III mGluR modulation holds promises in the treatment of several psychiatric conditions including addiction and alcoholism.

2.10.3 Manipulation of the glutamate transporter function could confer neuroprotection
Glutamate transporters remove glutamate from the synaptic cleft back into the intracellular space. Therefore, their function is of interest in alcoholism, which is characterized by a hyperglutamatergic state. Post-mortem studies suggest reduced expression of GLAST and GLT-1 in human alcoholics (Kryger and Wilce 2010). Boosting of this system could offer protection against the neurotoxic effects of alcoholism. The effectiveness of enhancement of glutamate transporter function has been demonstrated in several preclinical and also some clinical studies conducted with N-acetylcysteine. N-acetylcysteine has prevented reinstatement of cocaine, heroin and nicotine use in animal models, whereas there are some clinical studies suggesting that N-acetylcysteine can reduce craving (Roberts-Wolfe and Kalivas, 2015).

2.10.4 Conclusions from the findings of glutamatergic compounds
In summary, the modulation of the glutamatergic system is a promising avenue in the treatment of alcoholism and its negative effects to the CNS. However, since it is such a major neurotransmitter system of the CNS, glutamate is crucially involved in the critical functions of cognition, emotion and general functioning of the subject. The manipulation of this system needs to be subtle and targeted, and if this can be achieved, it may offer innovative approaches for the treatment of addiction as well as other CNS diseases in the future. In the light of current knowledge, manipulation of the group II mGluRs could serve as medications for the treatment of alcoholism in the near future.
3 Aims

The aim of this thesis is to provide information about neurotransmitter changes associated with alcoholism. The research method applied was human whole-hemisphere autoradiography in a study sample of ten Cloninger type 1 alcoholics, eight Cloninger type 2 alcoholics and ten control subjects. It was hoped that by examining possible binding density differences between these groups, it would be possible to reveal alterations in neurotransmitter composition, which either underlie alcoholism or are its consequence. The focus was on neurotransmitters that contribute to the manifestation of the characteristic symptoms of alcoholism and other phenomena associated with alcoholism. This knowledge can help the clinicians to choose effective treatments and be of benefit to the research community in the development of new medications for alcoholism.

The specific aims of the three publications were:

1. To study alterations in [³H]flunitrazepam binding site density, indicating GABA_A receptor benzodiazepine binding site density, in Cloninger type 1 and 2 alcoholics when compared with controls using post-mortem whole-hemisphere autoradiography.

2. To investigate differences in mGluR2/3 binding density between Cloninger type 1 and 2 alcoholics and control subjects using [³H]LY341495, a highly-selective group II mGluR antagonist in a post-mortem whole-hemisphere autoradiography.

3. To study opioid receptor binding densities in Cloninger type 1 and 2 alcoholics in comparison with controls using [³H]naloxone and [³H]DAMGO (2-Ala-4-meph-5-gly-enkephalin) in a post-mortem whole-hemisphere autoradiography.
4 Methods

All three studies of the thesis were conducted with human post-mortem whole-hemisphere autoradiography to study binding density of [³H]flunitrazepam (study 1), [³H]LY341495 (study 2), and [³H]naloxone and [³H]DAMGO (study 3) in Cloninger type 1 and 2 alcoholics and controls. The goals were to explore differences in GABA A receptor (study 1), mGluR2/3 (study 2) and MOR (study 3) binding densities between three study groups.

4.1 Collection of the study sample
The study sample consisted of the post-mortem left hemispheres of 28 human study subjects. The sample was obtained from clinical autopsies conducted in the Department of Forensic Medicine, University of Oulu, Oulu, Finland and the Department of Forensic Medicine, University of Eastern Finland, Kuopio, Finland during years 1997-1998. This part of the study received approval from the National Institute of Medicolegal Affairs, Helsinki, Finland. During the autopsy, the brains were removed, cleaned of the dura and divided into two hemispheres at the midsagittal plane. None of the hemispheres were damaged or had any macroscopic neuroanatomical abnormalities, and subsequent Nissl staining revealed no damage or abnormalities. The left hemispheres were placed on a glass-plate and frozen (-75°C), after which they were sectioned into 100 µm thick canto-meatal slices and air-dried before storage with dehydrating agents at -25°C, until used in autoradiography. The cryosectioning was performed in the School of Pharmacy, University of Eastern Finland, Kuopio, Finland (for details of the cryosectioning, see Mantere et al. 2002). Each cryosection was coded prior to the subsequent blind analysis of data.

A normal post-mortem analysis for drugs (including alcohol) was performed in the Department of Forensic Medicine, University of Helsinki, Helsinki, Finland as a part of the autopsy protocol. Medical records of the causes of death, medical treatments and diagnosed diseases were collected and available from all the study subjects, as well as their criminal record, if any. The data from the records was collected with the permission of the Ethics Committee of the University of Oulu, Oulu, Finland.

4.2 Diagnostics
Two physicians set the diagnoses for alcohol dependence independent of each other. Mental disorders were encoded according to DSM-IV (APA 1994). The diagnosis for alcohol dependence (DSM-IV) was based on the results of the autopsy, the causes of death and data from the medical records. Alcoholics were sub-classified as type 1 or 2 according to Cloninger (Cloninger et al. 1988). In the sub-classification of alcoholism, an early onset of alcohol abuse (before 25 years of age) and documented severe antisocial behavior were the main inclusion criteria for Cloninger type 2 alcoholics. The kappa coefficient for the diagnostic agreement was 0.9, i.e., one Cloninger type 2 alcoholic was diagnosed as a type 1 alcoholic by the second physician. Otherwise the diagnoses were concordant. Subjects with a psychotic disorder or any neurological diseases, as well as subjects taking any medication that could affect the CNS (such as antipsychotics or antidepressants), were excluded.

4.3 Study subjects
The study sample consisted of Finns. All of them had died of sudden causes. The sample included ten control subjects (eight males and two females; age: mean= 53.5 years, standard deviation (SD)= 10.6; post-mortem interval (PMI): mean= 14.9 hours, SD= 9.2), 10 Cloninger type 1 alcoholics (eight males and two females; age: mean= 51.8 years, SD= 12.0; PMI: mean= 12.9 hours, SD= 5.4) and eight type 2 alcoholics (all males; age: mean= 34.6 years,
SD= 12.2; PMI: mean= 14.1 hours, SD= 3.4). For the detailed information about the study subjects, please see Table 2.

Table 2. Details of the study subjects. Legends: PMI, post-mortem-interval; BAC, blood alcohol content.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Age</th>
<th>PMI</th>
<th>BAC</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-alcoholic controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Male</td>
<td>55</td>
<td>5.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>45</td>
<td>9.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>77</td>
<td>7.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>57</td>
<td>11.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>50</td>
<td>18.5</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>60</td>
<td>12.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>49</td>
<td>33.0</td>
<td>0.4</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>53</td>
<td>29.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>53</td>
<td>11.0</td>
<td>0.0</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>36</td>
<td>11.0</td>
<td>0.0</td>
<td>Dissection of aorta</td>
</tr>
<tr>
<td>Type 1 alcoholics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Male</td>
<td>39</td>
<td>12.5</td>
<td>0.0</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>48</td>
<td>4.5</td>
<td>0.1</td>
<td>Acute pancreatitis</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>45</td>
<td>12.0</td>
<td>1.5</td>
<td>Suicide by hanging</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>42</td>
<td>14.8</td>
<td>0.8</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>76</td>
<td>10.5</td>
<td>3.2</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>56</td>
<td>19.0</td>
<td>4.1</td>
<td>Ethanol intoxication</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>48</td>
<td>6.5</td>
<td>1.5</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>69</td>
<td>16.0</td>
<td>4.7</td>
<td>Ethanol intoxication</td>
</tr>
<tr>
<td>9</td>
<td>Female</td>
<td>57</td>
<td>11.0</td>
<td>2.0</td>
<td>Right subdural hemorrhage</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>45</td>
<td>23.5</td>
<td>3.1</td>
<td>Drowning</td>
</tr>
<tr>
<td>Type 2 alcoholics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Male</td>
<td>49</td>
<td>12.0</td>
<td>2.7</td>
<td>Fibrotic degeneration of myocardium</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>37</td>
<td>9.5</td>
<td>3.0</td>
<td>Gunshot wound</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>47</td>
<td>15.5</td>
<td>3.0</td>
<td>Knife wound</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>20</td>
<td>14.5</td>
<td>1.3</td>
<td>Knife wound</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>46</td>
<td>18.0</td>
<td>0.0</td>
<td>Suicide by hanging</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>18</td>
<td>9.5</td>
<td>1.5</td>
<td>Heart rupture (car accident)</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>32</td>
<td>16.5</td>
<td>3.6</td>
<td>Suicide by hanging</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>28</td>
<td>17.5</td>
<td>0.0</td>
<td>Suicide by hanging</td>
</tr>
</tbody>
</table>

4.4 Post-mortem analysis for drugs and alcohol

One of the control subjects had a small amount of alcohol in the blood (0.036‰) at the time of death. Nine of ten Cloninger type 1 alcoholics had alcohol in their blood at the time of death and the tenth subject had had a 10-hours’ period of incarceration previous to his death. Six of the eight Cloninger type 2 alcoholic subjects had alcohol in their blood at the time of death. One of the sober Cloninger type 2 alcoholics had an abstinence period of five days while the abstinence period of the other subject had lasted from three to seven days. Two type 1 alcoholics and three type 2 alcoholics had traces of diazepam in their blood and
one type 2 alcoholic subject tested positive for cannabinoids. This unconfirmed finding might have been a false positive for ibuprofen with the diagnostic test that was used.

### 4.5 Autoradiography

The imaging technique used in the experimental part of this thesis is post-mortem whole-hemisphere autoradiography of the human brain tissue. This autoradiography technique produces images of tissues that are exposed to a radiosensitive plate or film. These images provide information of the distribution and intensity of radioactive signals in the studied tissue (Manuel et al. 2015). By using a radioactively labeled ligand that selectively binds to specific receptors in the tissue, the binding density of this receptor can be measured from the images.

#### 4.5.1 Post-mortem whole hemisphere human autoradiography

In whole-hemisphere autoradiography technique, the selected brain slices are first preincubated in a buffer to remove any endogenous ligands, which could interfere with the test ligand. After preincubation, brain slices are incubated in a buffer-solution containing the radioligand of interest. An adjacent brain slice from each of the study subjects is incubated in a buffer-solution containing the radioligand with an excess of displacer. The displacer is a compound, which has high affinity for the same binding site as the radioligand. Due to concentration difference, the displacer is able to displace or inhibit the binding of the radioligand to the specific receptor. As a result, the images of the brain slices incubated in a displacer-solution display only the nonspecific binding of the radioligand to brain tissues other than the specific receptor of interest. The result of the subtraction of the binding values of the brain images of the adjacent brain slices of the same subject reveals the intensity of specific binding. After incubation of the adjacent slices in two types of solution (one containing the ligand, the other containing the ligand and the displacer), the brain slices are rinsed in a buffer and finally, in deionized water to remove the excess of the radioligand and salts. The temperature, pH and presence of ions need to be set in advance and carefully controlled during the laboratory work, since these factors affect the receptor binding. The aim is to optimize the specific binding in order to obtain images where the radioactive signal is detectable and clear, so that possible differences in binding density can be reliably detected. After drying, the brain slices are exposed to a phosphor-imager plate (BAS IP-TR 2040, Fuji Photo Film, Japan) for a certain time (typically around 14 days), and then scanned (Storm 860 PhosphorImager scanner, Amersham). The autoradiograms were analyzed in a phosphor imager analysis (Image J, National Institutes of Health, USA), and the resulting values for the binding intensity of the data have been transformed into tissue properties (fmol/mg or pmol/mg) by the use of [3H]-calibrating scales (cat. no. RPA 507, Amersham). The details of the four autoradiographies of the experimental part of the thesis can be seen in Table 3.
Table 3. Details of the four autoradiographies.

<table>
<thead>
<tr>
<th>Targeted receptor</th>
<th>GABA&lt;sub&gt;A&lt;/sub&gt; (study 1)</th>
<th>mGluR2/3 (study 2)</th>
<th>MOR (study 3)</th>
<th>MOR (study 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand</td>
<td>[³H]flunitrazepam, 4 nM</td>
<td>[³H]LY341495, 5 nM</td>
<td>[³H]naloxone, 3 nM</td>
<td>[³H]DAMGO, 1.49 nM</td>
</tr>
<tr>
<td>Displacer</td>
<td>Flumazepam, 17 µM</td>
<td>L-glutamate, 1 mM</td>
<td>Naltrexone, 10 µM</td>
<td>Naltrexone, 6 µM</td>
</tr>
<tr>
<td>Buffer</td>
<td>Tris-HCl, 50 mM</td>
<td>10mM potassium phosphate + 100mM potassium bromide</td>
<td>50 mM Tris-HCl + 100 mM NaCl</td>
<td>50 mM Tris-HCl + 100 mM NaCl</td>
</tr>
<tr>
<td>Temperature</td>
<td>20°C</td>
<td>20°C</td>
<td>4°C preincubation, 20°C incubation</td>
<td>4°C preincubation, 20°C incubation</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.6</td>
<td>7.4</td>
<td>7.4</td>
</tr>
<tr>
<td>Preincubation</td>
<td>30 min</td>
<td>30 min</td>
<td>15 min</td>
<td>15 min</td>
</tr>
<tr>
<td>Incubation</td>
<td>40 min</td>
<td>90 min</td>
<td>60 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Washing</td>
<td>3 x 2 min buffer, 1 x dip to ice-cold distilled water</td>
<td>3 x 2 min buffer, 1 x dip to ice-cold distilled water</td>
<td>1 x 30 sec buffer, 2 x 2 min 30 sec buffer, 1 x dip to ice-cold distilled water</td>
<td>2 x 5 min buffer, 1 x dip to ice-cold distilled water</td>
</tr>
<tr>
<td>Film exposure</td>
<td>20 days</td>
<td>14 days</td>
<td>14 days</td>
<td>14 days</td>
</tr>
</tbody>
</table>

4.6 Statistical analyses

The statistical tests used in the studies 1-3 were selected considering the special characteristics of this study sample. Above all, the challenges were the small sample size, relatively great variation in binding density among the study subjects and the fact that it was the goal to study simultaneously several brain areas from the same study subjects. These cannot be answered through analysis of variance, but required resampling statistics such as bootstrapping and permutation-based testing. Pearson’s method was used to reveal possible correlations between receptor binding density and age, PMI and BAC. The p-value of 0.05 or less was considered as statistically significant in all analyses. Effect sizes of the significant findings were calculated with Cohen’s method (Cohen 1988). An effect size of 0.5 was considered as a medium, and effect size of 0.8 as a large effect. STATA (releases 11.2 and 13.1, StataCorp LP, College Station, TX) was used for all statistical analyses.

In study 1, a bootstrap-type generalized estimation equation was used to analyze differences in [³H]flunitrazepam binding density between three study groups. Generalized estimating equations (GEE) were developed as an extension of the general linear model (e.g., OLS regression analysis) to analyze correlated data. GEE models observe the correlation between measurements of the same study subject. Bonferroni adjustment was
applied to correct for the levels of significance for multiple testing. In study 2, the measured [$^3$H]LY341495 binding densities were normalized by standard deviation to the same mean and distribution within the region and across the study groups. The standardized data of each brain area was then analyzed using a permutation-type ANOVA (analysis of variance). Age was used as a covariate. In study 3, a bootstrap-type ANOVA was used to analyze the [$^3$H]naloxone and [$^3$H]DAMGO binding densities. Based on the anatomical and functional connections, certain brain areas were paired in order to confer added power into the analysis.
5 Results

In the three paragraphs below, I will list the key findings of four autoradiography studies of the experimental part of the thesis. These results are described more thoroughly in the three published articles (studies 1-3), which are to be found at the end of this thesis.

5.1. \([\text{³H}]\text{flunitrazepam binding to GABA}_{\alpha}\) receptors
In study 1, \([\text{³H}]\text{flunitrazepam binding density}\) was studied in eight brain areas: the hippocampus, dentate gyrus (DG), caudate (Cau), putamen (Pu), internal globus pallidus (GPi), external globus pallidus (GPe), frontal cortex (FC) and perigenual anterior cingulate cortex (pACC). The highest binding densities were measured in the hippocampus, DG and pACC. A GEE model revealed a statistically significant difference (p < 0.001) in \([\text{³H}]\text{flunitrazepam binding density}\) between the three study groups (Cloninger type 1 alcoholics, Cloninger type 2 alcoholics and controls). This difference was localized to three brain areas: the hippocampus, (p=0.003, type 1; p=0.04, type 2), DG (p=0.04, type 2) and GPi (p=0.03, type 1; p=0.006, type 2) when compared with the controls (See Figure 2 in Publication 1). No significant differences in \([\text{³H}]\text{flunitrazepam binding density}\) between alcoholic subgroups were revealed. Alcoholics displayed lower \([\text{³H}]\text{flunitrazepam binding}\) density in all studied brain areas. In six areas, Cloninger type 2 alcoholics displayed the lowest mean binding of the group, but in the hippocampus and GPe, the mean binding of type 1 alcoholics was the lowest. Age, PMI or BAC did not correlate significantly with \([\text{³H}]\text{flunitrazepam binding density}\) in any of the study groups.

5.2. \([\text{³H}]\text{LY341495 binding to group II metabotropic glutamate receptors}\)
In study 2, \([\text{³H}]\text{LY341495 binding density}\) was measured in six brain areas: the hippocampus, DG, amygdala, NAc, FC and pACC. It was observed that the binding within the subject was not independent in anatomically and functionally connected brain areas. Therefore, the variables within correlated brain areas were normalized to the same mean and distribution across the study groups according to the standard deviation, and the data was expressed as z-scores. Permutation type ANOVA with age as a covariate was used to analyze the values of standardized data. We found a statistically significant difference in \([\text{³H}]\text{LY341495 binding density}\) between three groups (Cloninger type 1 and type 2 alcoholics and controls) in the functionally connected block of NAc, FC and pACC. The statistically significant finding was further localized to the pACC, where Cloninger type 2 alcoholics displayed increased \([\text{³H}]\text{LY341495 binding}\) compared with the controls (p= 0.046) (See Figure 2 in Publication 2). The effect size of this finding in type 2 alcoholics was large (1.09). No statistically significant differences in \([\text{³H}]\text{LY341495 binding density}\) were detected in the area of hippocampus and DG or amygdala. Type 2 alcoholics displayed the highest binding \([\text{³H}]\text{LY341495}\) in five of the six studied brain areas. In four of six areas, the controls displayed the lowest binding. Pearson’s method did not reveal any significant correlations between the \([\text{³H}]\text{LY341495 binding density}\) and PMI or age in any of the groups. \([\text{³H}]\text{LY341495 binding}\) correlated significantly with BAC in pACC of type 2 alcoholics.

5.3. \([\text{³H}]\text{naloxone and [³H]}\text{DAMGO binding to mu-opioid receptors}\)
Study 3 examined differences in MOR binding between Cloninger type 1 and 2 alcoholics and controls in eight selected areas, i.e. in the anterior prefrontal cortex, mPFC, NAc, pACC, hippocampus, DG, anterior insular cortex (aINS) and posterior insular cortex (pINS) and utilized two separate binding ligands. \([\text{³H}]\text{naloxone, a competitive antagonist of MOR, was used in the first autoradiography. To confirm the findings of the [³H]naloxone study, a}\)
subsequent autoradiography was performed with [³H]DAMGO, a selective MOR agonist. In order to gain power for the analysis of the small sample, hippocampus was paired with DG, as well as anterior and posterior insula and anterior and medial PFC with each other. Cloninger type 1 alcoholics displayed lowest [³H]naloxone and [³H]DAMGO binding in most brain areas (See Table 4 A and B). There was a statistically significant difference in [³H]naloxone binding between the study groups in the paired areas of hippocampus and DG (p= 0.027) (See Figure 4). This finding was further localized to exist between type 1 alcoholics and controls in the DG (p= 0.019). The differences in [³H]DAMGO binding density between study groups were statistically nonsignificant, but the binding trends were similar to [³H]naloxone, suggesting lowest [³H]DAMGO binding in Cloninger type 1 alcoholics. [³H]DAMGO binding correlated strongly with [³H]naloxone binding density in the hippocampus and DG (R= 0.73; 95% CI: 0.4-0.89; Pearson).

Table 4A. [³H]naloxone binding density in control subjects and Cloninger type 1 and 2 alcoholics in eight brain areas. Legends: aINS anterior insula; aPFC anterior prefrontal cortex; DG, dentate gyrus; Hippo, hippocampus; mPFC medial prefrontal cortex; NAc, nucleus accumbens; pACC perigenual anterior cingulate cortex; pINS, posterior insula; SD, standard deviation.

| Brain area | Control (n:10) | | Type 1 (n:10) | | Type 2 (n:8) | |
|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD |
| Hippo | 15.21 | 5.71 | 11.09 | 5.47 | 15.88 | 7.58 |
| DG | 18.84 | 8.09 | 11.92 | 4.64 | 18.30 | 9.18 |
| NAc | 32.83 | 13.20 | 24.30 | 13.30 | 30.11 | 12.5 |
| aPFC | 35.48 | 12.14 | 29.72 | 18.45 | 28.85 | 19.75 |
| mPFC | 31.88 | 14.11 | 28.56 | 16.71 | 26.14 | 11.73 |
| aINS | 36.55 | 14.63 | 26.15 | 17.79 | 28.65 | 19.17 |
| pINS | 39.01 | 19.86 | 26.68 | 18.48 | 33.66 | 21.85 |

Table 4B. [³H]DAMGO binding density in control subjects and Cloninger type 1 and 2 alcoholics in eight brain areas. Legends: aINS anterior insula; aPFC anterior prefrontal cortex; DG, dentate gyrus; Hippo, hippocampus; mPFC medial prefrontal cortex; NAc, nucleus accumbens; pACC perigenual anterior cingulate cortex; pINS, posterior insula; SD, standard deviation.

| Brain area | Control (n:10) | | Type 1 (n:8) | | Type 2 (n:8) | |
|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD |
| Hippo | 2.20 | 1.58 | 1.39 | 1.77 | 2.53 | 2.38 |
| DG | 3.36 | 2.26 | 1.93 | 1.83 | 3.86 | 3.76 |
| NAc | 6.98 | 4.19 | 6.61 | 7.08 | 7.61 | 4.49 |
| pACC | 11.99 | 8.37 | 10.60 | 12.46 | 11.84 | 8.22 |
| aPFC | 9.36 | 6.05 | 8.12 | 9.32 | 10.71 | 9.08 |
| mPFC | 10.49 | 6.95 | 9.33 | 10.78 | 10.65 | 8.02 |
| aINS | 13.41 | 9.00 | 12.53 | 16.41 | 13.91 | 11.89 |
| pINS | 13.32 | 9.22 | 10.54 | 13.02 | 14.79 | 11.81 |
Figure 4. Distribution of the standardized values of [³H]naloxone binding density in the region of hippocampus and dentate gyrus. P indicates the p-value of difference in binding density between three study groups. Leged: DG, dentate gyrus, Hippo, hippocampus.
6 Discussion

The results of each three studies are discussed in depth in the published articles reprinted at the end of this thesis. In this chapter, I will cover the main findings of each study (studies 1-3) and discuss the possible implications for clinical work with patients and future research. In addition, the research approach, study sample and limitations of the study are discussed. This thesis is a part of a research project, which has been on-going for almost twenty years. The basis of this project was formed by the collection of a unique sample of human post-mortem brains. By using methods like post-mortem whole-hemisphere autoradiography and liquid chromatography-tandem mass spectrometry, several researchers have published study results about altered neurotransmitter or receptor transporter binding densities and neurotransmitter levels in either in Cloninger type 1 or 2 alcoholics, or in both alcoholic subgroups. A summary of the main findings of our study group from this set of post-mortem human brain samples can be found in Tables 5A and 5B and the related brain areas are visualized in Figure 5.
Table 5A. Alterations in the neurotransmitter binding densities of Cloninger type 1 and 2 alcoholics when compared with the control subjects. Legends: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Amy, amygdala; Both, Cloninger type 1 and 2 alcoholics; Cau, caudate; DG, dentate gyrus; D2/D3, dopamine receptor type 2/3; GABA\textsubscript{A}, γ-aminobutyric acid receptor A; GP, globus pallidus; Hipp, hippocampus; mGluR1/5, metabotropic glutamate receptor 1/5; mGluR2/3, metabotropic glutamate receptor 2/3; MOR, μ-opioid receptor; NAc, nucleus accumbens; NMDA NR2B, N-Methyl-D-aspartate receptor with NR2B-unit; pACC perigenual anterior cingulate cortex; Pu, putamen; Rec, receptor; Type 1, Cloninger type 1 alcoholics; Type 2, Cloninger type 2 alcoholics, 5HT\textsubscript{1A}, serotonin receptor type 1A.

<table>
<thead>
<tr>
<th>Rec type</th>
<th>GABA\textsubscript{A}</th>
<th>NMDA NR2B</th>
<th>AMPA</th>
<th>mGluR 1/5</th>
<th>mGluR 2/3</th>
<th>D2/D3</th>
<th>5HT\textsubscript{1A}</th>
<th>MOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cau</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DG</td>
<td>(\downarrow) Both (Study 1 of this thesis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\downarrow) Type 1 and 2 (Tupala et al. 2003a)</td>
<td></td>
</tr>
<tr>
<td>GP</td>
<td>(\downarrow) Type 2 (Study 1 of this thesis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\downarrow) Type 1 and 2 (Tupala et al. 2003a)</td>
<td></td>
</tr>
<tr>
<td>Hipp</td>
<td>(\downarrow) Both (Study 1 of this thesis)</td>
<td></td>
<td></td>
<td>(\uparrow) Type 1 (Kupila et al. 2013)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAc</td>
<td>(\downarrow) Type 2 (Kupila et al. 2015)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\downarrow) Type 1 (Tupala et al. 2001a)</td>
<td></td>
</tr>
<tr>
<td>pACC</td>
<td>(\uparrow) Type 2 (Kärkkäinen et al. 2013a)</td>
<td></td>
<td></td>
<td>(\uparrow) Type 2 (Study 2 of this thesis)</td>
<td></td>
<td></td>
<td>(\downarrow) Both (Storvik et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>Pu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\downarrow) Type 1 and 2 (Tupala et al. 2003a)</td>
<td></td>
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</tbody>
</table>
Table 5B. Alterations in the transporter densities and neurotransmitter concentrations of Cloninger type 1 and 2 alcoholics when compared with the control subjects. Legends: AEA, anadamide; Amy, amygdala; Both, Cloninger type 1 and 2 alcoholics; Cau, caudate; FC, frontal cortex; DAT, dopamine transporter; DHEA, dehydroepiandrosterone; Hipp, hippocampus; Ins, insula; NAc, nucleus accumbens; pACC perigenual anterior cingulate cortex; PCC, posterior cingulate cortex; PHG, parahippocampal gyrus; PREG, pregnenolone; Pu, putamen; SERT, serotonin transporter; TC, temporal cortex; Type 1, Cloninger type 1 alcoholics; Type 2, Cloninger type 2 alcoholics.

<table>
<thead>
<tr>
<th>Transporter/Transmitter</th>
<th>DAT</th>
<th>SERT</th>
<th>Endocannabinoids</th>
<th>Keto-steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain area</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amy</td>
<td>↓ Both (Storvik et al. 2007)</td>
<td>↑ Docosahexaenoylethanolamide, Type 1 (Kärkkäinen et al. 2013b)</td>
<td>↑ DHEA and PREG, Both (Kärkkäinen et al. 2016)</td>
<td></td>
</tr>
<tr>
<td>Cau</td>
<td>↓ Both (Tupala et al. 2001b, Tupala et al. 2003a)</td>
<td>↓ Both (Storvik et al. 2006)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FC</td>
<td>↓ AEA, Type 1 (Lehtonen et al. 2010)</td>
<td>↑ DHEA and PREG, Both (Kärkkäinen et al. 2016)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hipp</td>
<td>↓ Both (Kärkkäinen et al. 2015)</td>
<td>↑ DHEA and PREG, Type 1 and 2 (Kärkkäinen et al. 2016)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ins</td>
<td>↓ Type 1 (Tupala et al. 2001a)</td>
<td>↓ AEA, Type 1 (Lehtonen et al. 2010)</td>
<td>↑ DHEA and PREG, Both (Kärkkäinen et al. 2016)</td>
<td></td>
</tr>
<tr>
<td>NAc</td>
<td>↓ Both (Mantere et al. 2002)</td>
<td>↓ AEA, Type 1 (Lehtonen et al. 2010)</td>
<td>↑ DHEA and PREG, Both (Kärkkäinen et al. 2016)</td>
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<tr>
<td>pACC</td>
<td>↓ Both (Kärkkäinen et al. 2015)</td>
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<tr>
<td>PCC</td>
<td>↓ Type 2 (Kärkkäinen et al. 2015)</td>
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<tr>
<td>PHG</td>
<td>↓ Both (Kärkkäinen et al. 2015)</td>
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<tr>
<td>Pu</td>
<td>↓ Both (Tupala et al. 2003a)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>↑ Type 2 (Tupala et al. 2006)</td>
<td></td>
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</tbody>
</table>
Figure 5. Two Nissl-stained canto-meataly oriented brain slices, at the level of dorsal striatum (above) and amygdala (below), visualizing the brain areas with altered receptor binding or transporter binding densities, or altered neurotransmitter concentrations in Cloninger type 1 and 2 alcoholics when compared with the controls. Legends: aINS, anterior insula; Amy, amygdala; Cau, caudate; DG, dentate gyrus; FC, frontal cortex; GPe, external globus pallidus; GPi, internal globus pallidus; Hippo, hippocampus; NAc, nucleus accumbens; pACC, perigenual anterior cingulate cortex; PCC, posterior cingulate cortex; PHG, parahippocampal gyrus; pINS, posterior insula; Pu, putamen; TC, temporal cortex.

6.1 Study subjects

6.1.1 Age at the time of death
The mean age of Cloninger type 1 alcoholics and controls is about the same (51.8 years, SD: 12.0 versus 53.5 years, SD: 10.6), but the mean age of Cloninger type 2 alcoholics is strikingly different, the mean age at the time of death being more than 15 years younger for Cloninger type 2 alcoholics than for type 1 alcoholics or controls (34.6 years, SD: 12.2). Since neurotransmitter binding of the CNS undergoes changes during aging, one has to pose the reasonable question of whether age has an effect on the [³H]flunitrazepam, [³H]LY341495, [³H]naloxone or [³H]DAMGO binding densities, which were studied in the experimental part of the thesis.
Cloninger type 2 alcoholics tend to die at a young age (Repo-Tiihonen et al. 2001, Repo-Tiihonen et al. 2002). This tendency can be viewed as an intrinsic feature of Cloninger type 2 alcoholics, which stems from their character (high in novelty-seeking, low in harm avoidance and dependency of social reward) (Cloninger et al. 1988), as well as from the high occurrence of antisocial personality disorder and criminal record among these subjects (Cloninger et al. 1988, Repo-Tiihonen et al. 2001). The impulsive and non-deliberative lifestyle of this subgroup often leads to premature death. This is also visible here when one examines the causes of death of Cloninger type 2 alcoholics (See Table 2, page 34). In order to take account of the effect of the aging, as mGluR2/3 has been reported to correlate negatively with aging in humans (Frank et al. 2011), age was used as a covariate in the analysis of [³H]LY341495 binding density (study 2). In all studies, 2-tailed Pearson’s correlation coefficient was used to determine possible correlations between age and each ligand binding densities in all studies of the thesis. No significant correlations between [³H]flunitrazepam, [³H]LY341495, [³H]naloxone or [³H]DAMGO binding densities and age were found in any of the three study groups.

6.1.2 Gender
The study sample consists of both genders, as two control subjects and two Cloninger type 1 alcoholics were females. The group of Cloninger type 2 alcoholics, according to its definition, consists only of males (Cloninger et al. 1988) (See Table 2, page 34). There are known to be some gender differences in neurotransmitter binding densities of the CNS, and alcohol-induced alterations in binding density may vary between men and women. For example, MOR binding has been reported to be higher among women in several cortical and subcortical areas (Zubieta et al. 1999). Two human sex hormones, allopregnanolone and pregnanolone, are positive modulators of GABA\(\alpha\) receptor (Andreen et al. 2009). In a rodent study it was found that the alterations in protein levels of several GABA\(\alpha\) receptor subunits after alcohol exposure correlated strongly with sex (Devaud and Alele 2004, Devaud et al. 2003). The possibility of altered binding was considered during exploration of the data from each study, but no statistical outliers were detected and thus, all subjects were included in the analyses. The possible differences in binding density between the sexes in alcoholics remain to be revealed by further studies.

6.1.3 Smoking
About 80% of alcoholics use nicotine products on a regular basis and they report more symptoms of nicotine dependency and more difficulties in cessation of smoking (Weerts et al. 2014). At the time of collection of our study sample (1997-1998), about 28% of Finnish men and 20% of women smoked (Heloma et al. 2012). It is likely that smoking was more common among the alcoholics than in the controls in our study sample. However, the reports of smoking in the medical records of the subjects were defective, and the smoking status of the subjects could not be reliably estimated and included in the analyses. This is unfortunate, since nicotine and smoking may have exerted an effect on the binding density assessments, and the effect of smoking in binding densities will need to be clarified in future studies. In experiments conducted in animals, nicotine enhances GABA\(\alpha\)ergic function (Hernandez-Vazquez et al. 2014, Xu et al. 2015), and in Cosgrove and co-workers’ study of human subjects, smoking impaired the normalization of GABA\(\alpha\) receptor levels in abstinent alcoholics, which correlated with craving intensity (Cosgrove et al. 2014). Smoking is also known to affect mGluR function. In an experiment conducted in rats, exposing young rats to nicotine altered their mGluR2 function (Counotte et al. 2011), and smoking has been suggested to affect mGluR function and thereby alter LTP and LTD processes (Goriounova and Mansvelder 2012). As far as is known, there are no reports if or how smoking influences mGluR function in alcoholics. In addition, MOR binding is affected by nicotine exposure. Weerts and colleagues detected decreased \[^{11}C\]carfentanil binding potential in those alcoholics with nicotine dependence (Weerts et al. 2014).
6.1.4 Blood alcohol content and post-mortem interval

Both acute and chronic alcohol exert robust effects on neurotransmitter receptor binding including the neurotransmitter systems studied in this thesis (GABA, glutamate and opioid systems). Often, acute and chronic effects are opposite, e.g. acute exposure to alcohol enhances GABA neurotransmission and depresses the glutamate-driven system, whereas chronic exposure to alcohol inhibits the GABA system and produces an over-excited glutamatergic state. Furthermore, neurotransmitter receptors react to changes in alcohol exposure with varying time frames, as some alterations in the receptor subunit occur within minutes, while some adaptations take much longer times, from days to weeks. In our study sample, most of the alcoholics had alcohol in their blood at the time of death, although the amounts differed extensively (See Table 2, page 34). One Cloninger type 1 alcoholic had an abstinence of 10 hours and two Cloninger type 2 alcoholics had an abstinence of some days prior to death. One of the control subjects had a small amount of alcohol in his blood at death. The alcohol status at the time of death inevitably had an effect on neurotransmitter binding. However, our study sample is a naturalistic sample of human subjects living their daily life. If we had selected patients at a certain point of alcoholism, e.g., after rehabilitation and certain period of abstinence, this would also have skewed both the sample and the results to a certain direction.

The correlations were calculated between BAC and binding density of each studied ligand ([³H]flunitrazepam, [³H]LY341495, [³H]naloxone and [³H]DAMGO) in three study groups. No significant correlations were detected, the only exception being a statistically significant correlation between [³H]LY341495 binding density in the pACC and BAC of Cloninger type 2 alcoholics (R= 0.863, p= 0.006). Since BAC did not correlate with [³H]LY341495 binding in any other areas of Cloninger type 2 alcoholics, and no correlation existed between BAC and [³H]LY341495 binding in type 1 alcoholics, it is considered that this finding may be a statistical artifact. It is unlikely that BAC would affect the [³H]LY341495 binding in only one specific brain area, exclusively in Cloninger type 2 alcoholics, when no effect is seen in other brain areas, or other group of alcoholics, whose alcoholism also was severe.

The mean PMI of the study subjects is within the same range in each study groups (12.9 hours in type 1 alcoholics, 14.1. hours in type 2 alcoholics and 14.9 hours in controls). However, there was a rather wide variation in the PMI between the subjects in each group, ranging from 4.5 to 33 hours (See Table 2, page 34). As far as is known, there are no reports about the impact of PMI on receptor binding affinity. However, in human post-mortem binding assay and autoradiography studies, PMI did not affect GABA receptor binding (Freund and Ballinger 1988, Freund and Ballinger 1989b, Lewohl et al. 1997). In the present study, no significant correlations between ligand binding densities and PMI were revealed.

6.2 Advantages and limitations of autoradiography technique

Radioligand binding assay is used widely to localize receptors of different neurotransmitters. Radioligand binding assay of a tissue homogenate is often one of the first experiments while studying the pharmacological and pharmacodynamic characteristics of a new compound (Manuel et al. 2015). An in vitro autoradiography, where the tissue is not homogenized but imaged as a whole (for example, tissue samples from brain of whole-brain slices), can give detailed information about the distribution the receptors. This technique has been widely used in the research of different neurotransmitters (Manuel et al. 2015). In the present study, whole-hemisphere human brain slices were used. The autoradiographic images of the brain slices made it possible to observe and measure the binding density of the selected ligand in several different brain structures simultaneously without technical variation between the samples. The resolution of the autoradiography images is better than the resolution of current in vivo –techniques, like PET. The selection of available radioligands for post-mortem imaging is huge
compared to the availability of current in vivo radioligands suitable for human imaging. In comparison with better resolution post-mortem techniques, such as immunohistochemistry, autoradiography is cheaper and this means that it is possible to examine a larger sample set all at the same time, thus preventing technical variation.

The limitations of autoradiography technique are the same as in all post-mortem analyses. The measured value at a given time point cannot provide any information about the history of this value. In the present study, this means that one cannot specify whether a certain change in the neurotransmitter binding properties found in alcoholics proceeded alcoholism or is a result of alcoholism.

6.3 Limitations of the study

The obvious limitation of this study is the small number of study subjects. Therefore, the results of the studies 1-3 should be viewed as preliminary and will need to be confirmed in subsequent studies with different techniques or larger samples. However, although small, this sample of human whole hemispheres is unique in the world. Unlike most human autoradiography samples, which usually consist of small pieces of brains or even small parts of a particular brain area, the whole-hemisphere method meant that it was possible to measure and compare binding densities of several brain areas simultaneously.

Due to technical reasons, which set limitations on the laboratory procedure, such as the size of the incubation containers and the amount of radioligand needed for each autoradiography, we were limited to a single brain level of each study subject in each autoradiography run. Therefore, we were not able to simultaneously examine the ligand binding density of brain structures on different canto-meatally oriented levels of the brain, e.g. amygdala and dorsal striatum. While planning each autoradiography study, we chose the brain level of interest according to the previous reports about the individual receptor binding densities and their roles in the development and maintenance of alcoholism.

During the progression of the experimental part of the thesis, we become more interested in the cortical areas. This explains why we conducted more assays of frontal cortical areas in [³H]naloxone and [³H]DAMGO autoradiographies (study 3) than in [³H]flunitrazepam and [³H]LY341495 autoradiographies (study 1 and 2). For the same reason, the measurements of insular cortex were included in study 3. Therefore, the findings of [³H]naloxone and [³H]DAMGO binding densities in mPFC, aINS and pINS cannot be compared with the binding densities of [³H]flunitrazepam and [³H]LY341495 at the moment, but this is intended to be a topic for future work.

Another problem concerning the study sample is the heterogeneity of the study subjects. The study sample consists of both males and females, whose age range is wide. In addition, some variables, like smoking, the exact length and heaviness of alcohol abuse, or the length of the abstinence/alcohol-intake period prior to death, could not be defined from the available records. These issues concerning the study sample have been discussed above in this chapter.

A post-mortem sample means that one can only measure the studied phenomena, i.e. here the neurotransmitter binding density, at one time point. This method cannot reveal the possible changes in the measured values over the course of time, or if there is any pattern in the changes i.e. one change being the trigger for a secondary effect (Manuel et al. 2015). Thus, the applied research method does not clarify whether the findings in receptor properties preceded alcoholism or are its consequence.

Finally, Cloninger’s typology is a classification which subdivides alcoholics into two groups (Cloninger et al. 1981), but other typologies that are based on different characteristics of the subject, also exist (Leggio et al. 2009). The results of the studies 2 and 3, where statistically significant changes were seen in [³H]LY341495 binding density of Cloninger type 2 alcoholics (study 2) and in [³H]naloxone binding density of Cloninger type 1 alcoholics (study 3) when compared with controls, must be interpreted from this viewpoint.
6.4 GABA<sub>α</sub> receptor binding density

The result of study 1 was that there was decreased [³H]flunitrazepam binding density in the iGP and hippocampus of both alcoholic subgroups and in the DG of Cloninger type 2 alcoholics when compared to the control subjects. This finding is in line with the overall trend of the study, as both alcoholic subgroups displayed lower binding than control subjects in all of the examined brain areas. No difference between Cloninger type 1 and 2 alcoholics was revealed.

The present findings are in line with previous studies, which suggest decreased GABA<sub>α</sub> receptor binding in several cortical areas in human alcoholics without cirrhosis or encephalopathy, both in in vivo PET studies using either [123I]iomazenil or [11C]flumazenil as a binding ligand (Abi-Dargham et al. 1998, Gilman et al. 1996, Lingford-Hughes et al. 1998), and in post-mortem studies of human alcoholics using the same ligand as applied here, [³H]flunitrazepam, i.e. evidence for reduced binding in the hippocampus and frontal cortex (Freund and Ballinger 1988, Freund and Ballinger 1989a). The in vivo -imaging and post-mortem GABA<sub>α</sub> receptor binding studies among alcoholics with cirrhosis, hepatic encephalopathy or Wernicke’s encephalopathy have given mixed results (Dodd et al. 1996, Jalan et al. 2000, Kril et al. 1988). Even though genetic studies have hinted at a link between allelic variation of GABRA2, the gene coding for the α2 subunit of GABA<sub>α</sub> receptor, and externalizing behaviors, extraversion and sensation seeking, as well as increased “high” during alcohol intake and positive family history of alcoholism (Arias et al. 2014, Dick et al. 2009, Dick et al. 2013, Fehr et al. 2006), no difference was detected in [³H]flunitrazepam binding density between Cloninger type 1 and 2 alcoholics.

The globus pallidus is part of the frontal-subcortical circuitry, whose dysfunction associates with addictions, but also with other psychiatric disorders like obsessive-compulsive disorder (OCD) and psychosis (Tekin and Cummings 2002). Interestingly, globus pallidus has been proposed to be part of the neurocircuit mediating GABAergic reinforcement of alcohol (Koob 2004). The present finding of decreased GABA<sub>α</sub> binding in the GPi of both alcoholic subgroups has not been reported before. It may indicate either a predisposing vulnerability of this system, or alcohol’s ability to modulate GABA neurotransmission. The finding of decreased GABA<sub>α</sub> receptor binding in alcoholics in the area of hippocampus is in accordance with previous studies (Freund and Ballinger 1989a).

In conclusion, the findings of the previous studies and the results of the study 1 of the thesis suggest strongly that the observed alterations in GABA<sub>α</sub> receptor binding density are a consequence of chronic exposure to alcohol, but one cannot rule out the possibility that they are a manifestation of a certain phenotype predisposing to alcoholism. In addition, this finding of decreased [³H]flunitrazepam binding density in alcoholics points out the importance of managing the symptoms of GABAergic imbalance when treating alcoholism and especially withdrawal. This is reflected in the treatment of severe alcohol withdrawal, with benzodiazepines being the treatment of choice of this syndrome (Schmidt et al. 2016).

6.5 mGluR2/3 binding density

In study 2, it was observed that there was increased [³H]LY341495 binding density in the pACC of Cloninger type 2 alcoholics when compared with the controls. No statistically significant differences in [³H]LY341495 binding between the study groups were revealed in other studied brain areas. Cloninger type 2 alcoholics displayed the highest binding density in five areas out of six.

The mGluR2/3 binding density in human alcoholics has not been studied before, in fact, there has not been much previous research investigating mGluR2/3 binding in humans. Fueled by the possible future treatment of psychosis and mood disorders by mGluR2/3
modulators, mGluR2/3 binding density has been studied in post-mortem samples of depressive, bipolar and schizophrenia patients. These autoradiography studies found no difference in mGluR2/3 binding density between patient groups and controls (Frank et al. 2011, Matosin et al. 2014).

Meinhardt and his co-workers have studied group II mGluR function in animals chronically exposed to alcohol, and in post-mortem human alcoholics. They reported decreased transcript levels of GRM2, a gene coding for mGluR2, in the ACC of human alcoholics and similar findings of decreased Grm2 levels in rats. This indicates that there may be decreased mGluR2 expression in the ACC in humans, and in the mPFC in rats, as a result of chronic exposure to alcohol (Meinhardt et al. 2013). The findings of Meinhardt and colleagues are opposite to the present findings. These discrepancies may stem from different research methods and grouping of the subjects, as our finding only applies to Cloninger type 2 alcoholics.

Interestingly, our research group has reported Cloninger type 2-specific alterations also in other components of glutamate neurochemistry i.e. increased AMPA receptor binding in the ACC and decreased NR2B-subunit containing NMDA receptor binding density in the NAc of Cloninger type 2 alcoholics (Kärkkäinen et al. 2013a, Kupila et al. 2013). It has been suggested that the mGluR2 signaling between these areas is an essential factor in relapse and in the reinstatement of alcohol seeking in alcoholism (Meinhardt et al. 2013). Interestingly, group II mGluR function, especially the effect of mGluR2, is believed to regulate cognitive flexibility and behavioral inhibition. In the light of the animal studies, it seems that optimal function of mGluR2/3 is crucial for impulse control and maintenance of optimal information processing, as mGluR2 PAM administration in certain studies promotes, but in other circumstances, these types of drugs disturb these actions (Aultman and Moghaddam 2001, Nikiforuk et al. 2010). In addition, reversal of the alcohol-induced alterations of mGluR2 function in the mPFC of rats, a brain region that is anatomically and functionally related to the ACC in humans, has been shown to terminate elevated alcohol seeking (Meinhardt et al. 2013, Uylings et al. 2003). The role of the ACC is to choose and sustain optimal rewarding actions on the basis of previous errors and reinforcements (Kennerley et al. 2006). Considering the role of the ACC, the altered [³H]LY341495 binding density in the pACC of Cloninger type 2 alcoholics could be linked with the impulsivity and low deliberation encountered in this alcoholic subgroup.

In conclusion, our finding of increased mGluR2/3 binding in the pACC of Cloninger type 2 alcoholics, together with the findings in our previous studies of altered AMPA receptor binding in the same area and NMDA receptor binding in functionally connected area of NAc, may suggest that impulsive and low-deliberative Cloninger type 2 alcoholics display a type-specific alteration of glutamatergic neurotransmission. However, the findings of the study 2 will need further confirmation in a larger sample or by other research techniques.

### 6.6 MOR binding density

One goal of study 3 was to study possible differences in MOR binding density between Cloninger type 1 and type 2 alcoholics and controls. Therefore, a [³H]naloxone binding density study was performed followed by a subsequent evaluation of the [³H]DAMGO binding density. There was a trend of decreased [³H]naloxone binding in all studied brain areas and a statistically significant decrease in [³H]naloxone binding in the DG in Cloninger type 1 alcoholics when compared with the control group. Although the differences in [³H]DAMGO binding density between the study groups were not statistically significant, the binding trends were similar to those found in the [³H]naloxone study i.e. decreased binding density in Cloninger type 1 alcoholics compared to controls in all studied brain areas. [³H]naloxone and [³H]DAMGO binding correlated strongly in the area of hippocampus and DG. The accordance of the results of the two autoradiographies with two separate ligands adds to the confidence on the reliability of the findings of the study.
The idea of exploring possible differences in MOR binding between study groups, stems from the results of both the animal and human studies. Several animal studies have revealed innate differences in MOR composition between alcohol preferring and non-preferring animal lines, and these differences possibly affect the vulnerability to alcoholism (de Waele et al. 1995, Learn et al. 2001, McBride et al. 1998). In addition, the treatment response to opioid antagonists, naltrexone and nalmefene, has been speculated to be modulated by the onset age of alcoholism and the MOR binding density of the patients, linking young onset of alcoholism and high MOR binding with a more beneficial treatment outcome (Heinz et al. 2005, Kiefer et al. 2008). It has been postulated that Cloninger type 2 alcoholics would benefit more of opioid antagonist treatment (Spanagel and Kiefer 2008). Our finding of a trend of decreased MOR binding in Cloninger type 1 alcoholics is in line with the suggestions of the aforementioned studies and further indicate impaired MOR function in Cloninger type 1 anxiety-prone and harm-avoidant alcoholics. Thus it would be predicted that these individuals would experience a poorer response to treatments inhibiting MOR function than would be obtained in Cloninger type 2 alcoholics, whose MOR system was similar to the controls. Therefore, MOR function modulating agents should be targeted to the treatment of Cloninger type 2 alcoholism.

6.7 Future directions and possible implications for clinical work

Study 2 found evidence of increased [³H]LY341495 binding density implying that there may be elevated mGluR2/3 binding in the pACC of impulsive Cloninger type 2 alcoholics. It has been suggested that disrupted group II mGluR function in the ACC underlies the relapse propensity in alcoholism (Meinhardt et al. 2013). There is ongoing development of drugs capable of modulating group II mGluR functions, and some compounds are already in the clinical testing phase, for example in the treatment of schizophrenia (Li et al. 2015). Possibly, we will see group II mGluR modulators being evaluated in the treatment of alcoholism in the future. It would be interesting to explore the effects of these compounds on different subgroups of alcoholics, divided according to either the onset age of alcoholism or personal characteristics of the subjects, such as impulsivity.

While waiting for the novel glutamatergic compounds to reach the market, one should not forget the fact that a hyperglutamateric state is one of the core phenomena underlying the symptoms of alcoholism, like withdrawal, craving and compulsive use. This has been clearly demonstrated in several studies, and more importantly by the fact of the popularity of topiramate in the off-label clinical treatment of alcoholism (Del Re et al. 2013). It is proposed that there should be well-designed clinical trials of currently available compounds affecting glutamate neurotransmission, like topiramate and memantine in different subgroups of alcoholics. Interestingly, topiramate is used to treat aggression, which is a trait with close links to impulsivity. In addition, clinical trials testing topiramate in the treatment of binge eating, pathological gambling and OCD have found evidence for the drug’s efficacy in these disorders of impulsivity and compulsivity (Dannon et al. 2005, McElroy et al. 2003, Rubio et al. 2006). A clinical trial of topiramate among alcoholics suggested that the topiramate-induced reduction in alcohol intake correlated with improved inhibitory control and attention. Considering the finding of study 2 of the thesis and the results of the aforementioned studies, topiramate could be especially beneficial in Cloninger type 2 impulsive alcoholics. This possibility will need to be clarified in future studies.

The study 3 of the thesis found a tendency of decreased MOR binding in Cloninger type 1 alcoholics, and this difference achieved statistical significance in the DG. Our finding may be a reflection of impaired MOR function in this subgroup of anxiety-prone, harm-avoidant and deliberative alcoholics. MOR function is involved in the regulation of arousal and anxiety. It affects the expression of brain-derived neurotrophic factor in the hippocampus as a response to stress, and thereby one could speculate that if the MOR is dysfunctional it could increase vulnerability to stress-related psychiatric disorders like anxiety and
depression (Komatsu et al. 2011, Thorsell 2013). In the light of these findings, it seems reasonable to postulate that an important treatment focus among Cloninger type 1 alcoholics should be their mood; the present study has found evidence for a disturbance in MOR function and this could well be related to vulnerability to mood disorders. Instead, when selecting which type of alcoholic patient would benefit most from opioid antagonist treatment, it would seem that the characteristics of Cloninger type 2 alcoholics make them better candidates to respond to this class of drugs.
7 Conclusions

Based on the results of the studies 1-3 in this thesis, one can draw the following conclusions:

1. Cloninger type 1 alcoholics display decreased [³H]flunitrazepam binding density indicating decreased GABA\(_A\) receptor binding in the internal globus pallidus and hippocampus, and Cloninger type 2 alcoholics in the internal globus pallidus, hippocampus and dentate gyrus, when compared with control subjects.

2. [³H]LY341495 binding density, which reflects the mGluR2/3 binding density, is increased in the perigenual anterior cingulate cortex of impulsive Cloninger type 2 alcoholics when compared with the controls.

3. [³H]naloxone binding density, indicating mainly MOR binding density, is decreased in the dentate gyrus of anxiety-prone Cloninger type 1 alcoholics.

These findings suggest that GABA\(_A\) receptor binding undergoes similar alterations in Cloninger type 1 and 2 alcoholics, which may indicate that these changes are a consequence of intensive alcohol use. The type-specific alterations found in studies 2 and 3 of this thesis suggest that within alcoholics, there are subgroup-specific differences in neurotransmission; these may predispose to alcoholism or affect the treatment response. These specific alterations need to be clarified in order to improve the efficacy of and adherence to the treatment of alcoholism. The increased mGluR2/3 binding in the pACC of Cloninger type 2 alcoholics might be a reflection of altered group II mGluR neurochemistry in this subgroup of subjects, especially in brain areas implicated in relapse propensity and action selection. The decreased MOR binding density in the dentate gyrus of Cloninger type 1 alcoholics, together with the trend of decreased MOR binding in type 1 alcoholics suggest that this group may not be able to respond optimally to the current treatments of alcoholism like naltrexone and nalmefene which have their mode of action as to inhibit MOR function. Therefore, these compounds may benefit those early-onset alcoholics with characteristics more typical of Cloninger type 2 alcoholics. Finally, above all, these findings emphasize the importance of viewing alcoholism as a psychiatric disorder with profound changes in CNS’s neurotransmission. This approach will help researchers and clinicians in the search for better understanding and more efficacious treatment of alcoholism.
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The heterogeneity of alcoholics complicates the selection of suitable treatment for alcoholism. The composition and function of neurotransmitter systems affects the course of alcoholism. This thesis conducted a post-mortem investigation of brain specimens of anxious Cloninger type 1 and impulsive Cloninger type 2 alcoholics and controls. GABA<sub>A</sub> receptor binding was decreased in all alcoholics. mGluR2/3 binding was increased in type 2 alcoholics; μ-opioid receptor binding was reduced in type 1 alcoholics.