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DISSERTATION

The Role of the Neurotransmitter Glutamate for the Development and Maintenance of Alcohol Use Disorder

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Abbreviations

ACC	anterior cingulate cortex	LCModel	linear combination of model spectra
ADS	Alcohol Dependency Scale	meanAb	Mean Abstinence Time
ALDH	aldehyde dehydrogenase	mGluR	Metabotropic Glutamate Receptors
AMPA	α-amino-3-hydroxy-5 methylisox-azole-4-propionic acid receptor	MLP	Mesolimbic Pathway
AUD	Alcohol Use Disorder	MRI	Magnetic Resonance Imaging
AUDIT	Alcohol Use Disorder Identification Test	MRS	Magnetic Resonance Spectroscopy
AvDay	Average alcohol drunk per day	NAcc	Nucleus accumbens
AvOcc	Average alcohol drunk per occasion	NMDA-R	N-Methyl-D-Aspartate Receptor
B0	Magnetic Flux Density	AUD-P	Alcohol Use Disorder Patient
cAMP	Cyclic adenosine monophosphate	P2	Project 2
CNS	central nervous system	P5	Project 5
CT	computer tomography	PET	positron emission tomography
DSM-V	Diagnostic and Statistical Manual of Mental Disorders - V,	PFC	Prefrontal Cortex
EAAT	sodium-dependent excitatory amino acid transporters	PRESS	Point resolved spectroscopy
FTND	Fagerström Test for Nicotine Dependence	PTB	Physikalisch Technische Bundesanstalt
GABA	Gamma-aminobutyric acid	RNA	Ribonucleic acid
GABA-R	GABA-A Receptor	SD	Standard deviation
GluC	Glutamate concentration	STEAM	stimulated echo acquisition mode
GluR	Glutamate receptor	T	Tesla
GM	Grey Matter	TE	Echo times
HC	Hippocampus	TR	Repetition times
HR	High Risk	vGluT	Vesicular Glutamate Transporter
ICD-10	International code of disease	VTA	Ventral tegmental area
iGluR	Ionotropic Glutamate Receptors	WHO	World Health Organisation
KA-R	kainic acid receptor	WM	White Matter
LR	Low Risk	γ	Gyromagnetic ratio
LTD	Long-term depression	ω	Larmor Frequency
LTP	Long-term potential	μ	Magnetic Moment

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ABSTRACT

1.1 English

The effects of alcohol on the neurotransmitter system in the brain are thought to have an acute physiological effect and also appear to be responsible for the development of addiction. Neuroadaptive changes may induce craving, which is among the reasons why therapy for Alcohol Use Disorder (AUD) is often unsuccessful. To explain the development of an AUD, there has been increasing interest in the neurotransmitter glutamate in recent years. Alcohol intake is assumed to inhibit the glutamatergic system, among others. Prolonged alcohol consumption is thought to lead to a compensatory increase of extracellular glutamate as well as the number and composition of NMDA-Rs. If alcohol is no longer consumed, the inhibitory effect of alcohol is eliminated. Due to the high extracellular glutamate concentration (GluC), and the elimination of inhibition, hyper-excitation occurs. Furthermore, a high GluC can be associated with strong withdrawal symptoms and a high relapse risk.

This study examines changes of the total GluC after chronic alcohol consumption and the relationship between GluC and drinking behaviour. For this, three groups with different drinking behaviour were compared. The first group included 27 AUD patients (AUD-P), the second group included 38 high-risk (HR) subjects with a score > 8 in the Alcohol Use Disorder Identification Test (AUDIT), and the third group consisted of 31 subjects with low-risk (LR) alcohol consumption. All participants were scanned with a three Tesla magnetic resonance tomograph, and magnetic resonance spectroscopy (MRS) was used to assess the GluC in the anterior cingulate cortex (ACC) and the left hippocampus (HC). Additionally, all the participants filled out a drinking calendar and underwent a battery of clinical tests to collect detailed information about their drinking behaviour. The group comparison of the GluC in the respective groups did not show any significant differences. The drinking calendar was used to investigate if there is a correlation between the time of abstinence and GluC dysregulationnin one of the subgroups and all of the groups. A final investigation was carried out to determine if there is a correlation between the amount of drinking and the GluC. A correlation could not be found between GluC and abstinence time or drinking quantity.

1.2 German

Alkohol bewirkt akute physiologische Reaktionen im Neurotransmittersystem des Gehirns, ist aber auch für langfristige neurologischen Veränderungen, die die Entstehung und Aufrechterhaltung einer Sucht begünstigen verantwortlich. Vor allem Glutamat gewann in den letzten Jahren in der Suchtforschung zunehmend an Bedeutung. Alkohol beeinflusst das Glutamaterge-Neurotransmitter System, indem es die N-Methyl-D-Aspartat-Rezeptoren (NMDA-R) hemmt. Es wird angenommen, dass ein chronischer Alkoholkonsum zu einem kompensatorischen Anstieg des Glutamats und der Anzahl und Zusammensetzung NMDA-R führt. Wenn kein Alkohol konsumiert wird, fällt die hemmende Wirkung von Alkohol weg. Durch die erhöhte extrazelluläre Glutamat-Konzentration (GluC) und die fehlende Hemmung kommt es zu einer Übererregung. Eine hohe GluC konnte in Tierversuchen bereits mit einer verstärkten Entzugssymptomatik und einem erhöhten Rückfallrisiko korreliert werden. Bisher gibt es nur wenige Studie am Menschen, welche die Zusammenhänge zwischen Glutamat Dysregulation und Alkoholkonsum betrachten. Ziel dieser Studie ist es, Korrelationen zwischen chronischem Alkoholkonsum und einer Glutamat Dysregulation Nachzuweisen.

Hierfür verglichen wir 3 Gruppen mit unterschiedlichem Trinkverhalten. Die erste Gruppe umfasste 27 AUD-Patienten (AUD-P), die zweite Gruppe 38 Personen mit riskantem Alkohol-trinkverhalten (HR), welche eine Punktzahl > 8 im Alcohol Use Disorder Identification Test erreichten und die dritte Gruppe bestand aus 31 Personen mit niedrigem Alkoholkonsum (LR). Alle Teilnehmer wurden in einem 3-Tesla-Magnetresonanztomograph gescannt und die GluC im im Anterioren Cingularen Kortex (ACC) und Hippocampus (HC) mittels Magnetresonanzspektroskopie gemessen. Darüber hinaus füllten alle Teilnehmer einen Trinkkalender und umfangreichen klinischen Tests aus, um detaillierte Informationen über ihr Trinkverhalten zu sammeln. Die Untersuchung auf Gruppenunterschiede der GluC konnte keine signifikanten Unterschiede zeigen. Mit Hilfe des Trinkkalender wurde untersucht, ob eine Korrelation zwischen der Abstinenszeit und der Glutamat Dysregulation, der einzelnen Untergruppen, sowie allen Gruppen, besteht. Zuletzt wurde die Korrelation zwischen den Trinkmengen und der GluC untersucht. Es konnte keine Korrelation zwischen der Abstinenzzeit oder Trinkmenge und der GluC gefunden werden.

2 INTRODUCTION

The following chapter introduces the key terms and puts them into context. Furthermore, it is important for comprehension to note that ethanol is referred to as alcohol.

2.1 Alcohol Use Disorder (AUD)

2.1.1 Definition of AUD

Alcohol addiction is the most common substance use disorder (SUD) in the world (WHO, 2014). To classify drinking behavior, varied approaches are used; they range from quantitative approaches to those which gauge how alcohol affects and changes people's lives, emotions, and physical health. In this work, we apply the classification from the fourth Diagnostic and Statistical Manual of Mental Disorders (DSM-IV).

The DSM-IV, published in 2003, distinguishes between "alcohol abuse" and "alcohol dependency". Also, the tenth International Classification of Diseases (ICD-10), published by the World Health Organization (WHO), uses a similar approach and distinguishes between acute alcohol intoxication (F10.0), harmful alcohol use (F10.1), and alcohol dependency (F10.2). A popular quantitative approach was released by the National Institute on Alcohol Abuse and Addiction. It categorizes the groups according to drinking quantity. In 2013, the DSM-V established the term Alcohol Use Disorder (AUD) and replaced the two distinct disorders described in the DSM-IV (see Table 1).

Table 1: ***DSM V Criteria for Alcohol Use Disorder Diagnosis***

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

Meeting 2-3 criteria = mild; 4-5 criteria = moderate; ≥ 6 criteria = severe disorder.
Retrieved from the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition; American Psychiatric Association (2013)

If at least two of the eleven criteria are met during a 12-month period, the patient is diagnosed with an AUD. With the unification of the two terms into the one term "AUD", the DSM-V illustrates that AUD is not a fixed condition but rather a continuum. It proposes that there are fluent transitions between normal, mild, moderate or severe disorder. Also, it should be kept in mind that different publications use different methods to classify alcohol consumption.

2.1.2 Epidemiology and Alcohol-related Problems

In Germany, more than 70% percent of the population between 18-59 years drink alcohol on a regular basis. According to a publication by Lange and colleagues, about 18.5% of Germany's male population and 13.1% of the female population consume alcohol in a health-endangering way (Lange et al., 2016) and 3.4% of the German population can be diagnosed with an AUD according to the DSM-V criteria (Pabst et al., 2013). Nevertheless, the harmful effects of alcohol are often underrated. Every year, alcohol is responsible for the death of 3.3 million people worldwide. Alcohol can be associated with over 200 secondary diseases. The best-known diseases associated with AUD are liver cirrhosis, liver cancer and pancreatitis. In Germany alone, 74,000 deaths per year can be associated with the abuse of alcohol.

Furthermore, alcohol burdens the economy. If people do not adhere to culturally accepted drinking patterns, there are also social and financial consequences (Bennett L. et al., n.d.). Subjects are more likely to lose their jobs, friends, and even their family. By losing their workplace, they become dependent on welfare and thus burden social service budgets. The economic costs add up to an estimated 27 billion EUR per year in Germany (Adams M., Effertz T., 2011). Therefore, the WHO has argued that the harmful effects of alcoholism are not discussed sufficiently in our society and preventive measures are insufficient (WHO, 2014).

To avoid the development of AUD in the first place, and also to develop better methods of withdrawal and diminish the risk of relapse, it is essential not only to stop trivializing alcohol abuse but also to understand the aetiology and mechanisms of maintaining an AUD.

2.1.3 Aetiology

The neurobiological development of an AUD is a complex multifactorial process, which is not yet fully understood. Alcoholism is the result of the interplay of A) genetic, B) neurobiological, and C) psychological factors, as well as D) the circumstances/environment that affect the patient over their lifetime (Spanagel et al., 2013).

A) Adoption studies with twins (Goldman et al., 2005; McGue et al., 1992; Risch et al., 2000) suggest a genetic component of alcohol dependency. They reveal that children of AUD parents

have a 3-4 times higher risk of developing an AUD, even when they grow up in an adoptive family (Goodwin et al., 1973). However, it is assumed that there is no “alcohol gene” which follows a Mendelian hereditary pattern. The “inheritance of alcoholism” is presumably manifested in complex patterns, wherein small gene regions contribute to the formation of the phenotype (Li et al., 2008).

B) During the last 20 years, imaging techniques have created new possibilities for investigating neurobiological processes that can affect or be caused by alcohol consumption. In this time, changes in neurotransmitter concentrations in the brain have become the focus of addiction research. It is assumed that by better understanding the neurobiological mechanisms, new drugs can be developed. The detailed neurological effects of alcohol are explained in Chapter 2.1.6.

C) The quest for the characterization of a 'typical addiction personality' remains unsuccessful. However, personality characteristics that are common in AUD patients include impulsivity, sensation seeking, and neuronal and behavioural disinhibition (Mitchell & Potenza, 2014).

D) It was shown that the development of an addiction is often connected to the patient’s environment, their situation at work, the stability of their living conditions, the number of losses or traumas in the past and the social support of others. (Möller et al., 2009; Wilson et al., 1997). These factors can prevent or facilitate the development of an AUD.

A model that tries to combine all these factors is the diathesis-stress model.

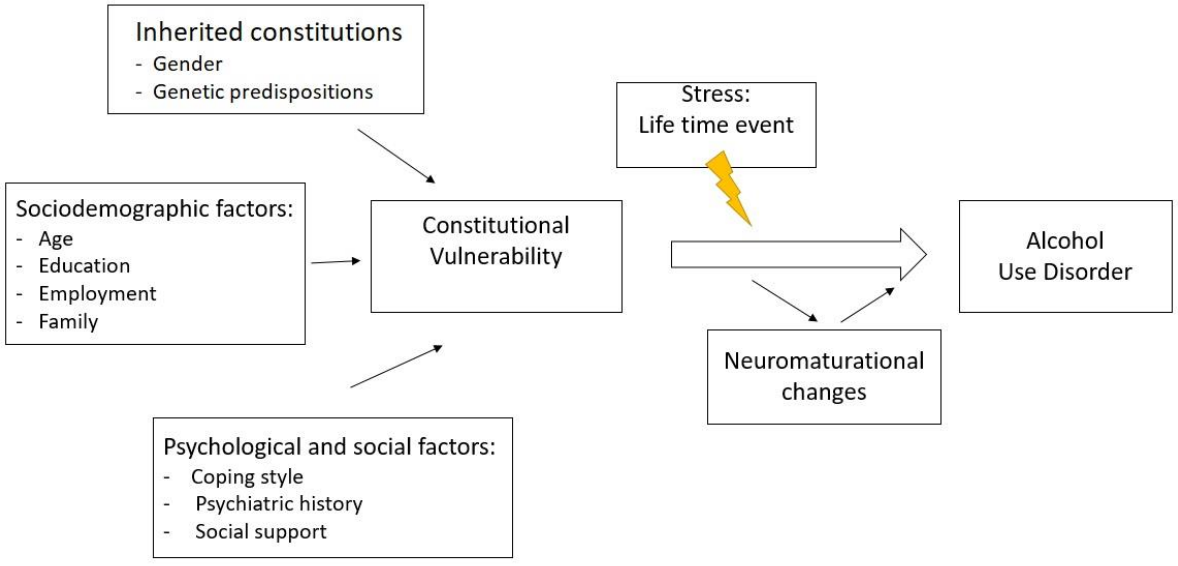

Figure 1: Diathesis-Stress Model, adapted from Schotte et al., 2006.

The model states that every individual has their own constitutional vulnerability, which is created by multiple factors (inherited, sociodemographic, psychological and social, as seen in Figure 1). The vulnerability, or diathesis, helps the individual to cope with stress or elevates their susceptibility. External stressors can affect people in positive and negative ways. Stress leads to neurochemical changes, which may increase the vulnerability of these people to psychological diseases such as AUD. Depending on the diathesis and the amount of stress throughout their lifetimes, some people are more likely to be affected than others.

2.1.4 Withdrawal, Craving and Relapse

In order to successful treat an AUD for a patient, it is essential to become aware of the alcohol-related problems. The first step of treatment is withdrawal, namely the physical and mental effects that occur upon the cessation or reduction of the intake of a substance. If the regular high dose of alcohol is stopped, the blood alcohol level drops significantly. In up to 50% of patients, this sudden absence of alcohol induces a symptomatology called alcohol withdrawal syndrome (AWS) (Schuckit, 2012).

An often used approach is the division of AWS into three phases (Hall & Zador, 1997). However, not all three phases of AWS must be completed. The first phase begins hours after the last drink and lasts about 24 hours. The characteristics of this first phase consist of autonomic hyperactivity including trembling, sweating, nausea, vomiting, anxiety, and agitation. During the second phase, 24 to 48 hours after the last drink, patients may present neuronal excitation, comprising global confusion or epileptic seizures. The third phase is a complication of the withdrawal and is also called alcohol withdrawal delirium (AWD) or delirium tremens, developing in 5-10% of patients (Kosten & O'Connor, 2003). An AWD is characterized by visual and auditory hallucination, disorientation and confusion (Ferguson et al., 1996). If left untreated, it can lead to death by respiratory or cardiovascular collapse (Cushman, 1987).

The symptoms are likely to be evoked by changes in the neurotransmitter system (Guochuan Tsai & Joseph T. Coyle, 1998). In particular, changes in the central inhibitory GABAergic and excitatory glutamatergic systems seem to be related to the severity of the withdrawal. The up-regulation of glutamatergic neurotransmission and the down-regulation of the inhibitory effect of GABA during alcohol misuse leads to insufficient central inhibition and hyperexcitation (Abdelkader Dahchour & De Witte, 2003).

After the acute withdrawal, the maintenance phase begins. In this phase, the patients have to succeed in remaining abstinent. However, craving and relapse often perpetuate an AUD (Fox et

al., 2007). In the DSM-V craving is described as follows: *"an intense desire or urge for the drug that may occur at any time, but is more likely to occur in an environment where the drug previously was obtained or used"* (American Psychiatric Association, 2013). Craving is not a phenomenon that only exists during abstinence, but is also essential for the development and maintenance of an AUD (Drobes & Thomas, 1999; Soyka et al., 2008). Furthermore, it was shown that the extent of craving is associated with the relapse probability (Schneekloth et al., 2012).

The development of craving can be partly explained as classical conditioning, which can be seen as an associative learning process. For the associative learning, changes of the neurotransmitter dopamine are essential (Di Chiara et al., 1999). The place where associative learning takes place is the mesolimbic pathway (MLP), otherwise known as the reward system. The role of dopamine is discussed in Chapter 2.1.6.1.

The development and maintenance of AUD, however, is not influenced only by the neurotransmitter dopamine. Other neurotransmitters like glutamate and gamma-aminobutyric acid (GABA) may facilitate or impede the manifestation of an AUD, so that a better understanding of neurotransmitter changes is essential for the treatment of AUD.

2.1.5 Effects of Alcohol on the Neurotransmitter System

Neurotransmitter changes affect the emotional state of humans (Masserman et al., 1941; Papez 1937). Alcohol can affect the neurotransmitter system of the brain, and also existing neurotransmitter concentrations can facilitate or perpetuate the development of an AUD. The neurobiological changes that lead to the development of an AUD are complex and do not follow a uniform, chronically progressive pattern and are therefore not yet fully understood (Phillips et al., 1989). Thus far, it has been shown that alcohol has an effect on the quantity and constitution (and hence the performance) of the receptors and ion channels in cell membranes. These proteins are important for the regulation of - and are also regulated by – the neurotransmitter system of the central nervous system (CNS). Important structures of the brain and their location, which will be referred to in the following, are delineated in Figure 2. Considering different neurotransmitters, it is assumed that glutamate, dopamine, GABA, opioid, and serotonin play key roles in the development of an AUD (Koob & Le Moal, 2008). In the following chapter, some additional background information concerning these neurotransmitters is given.

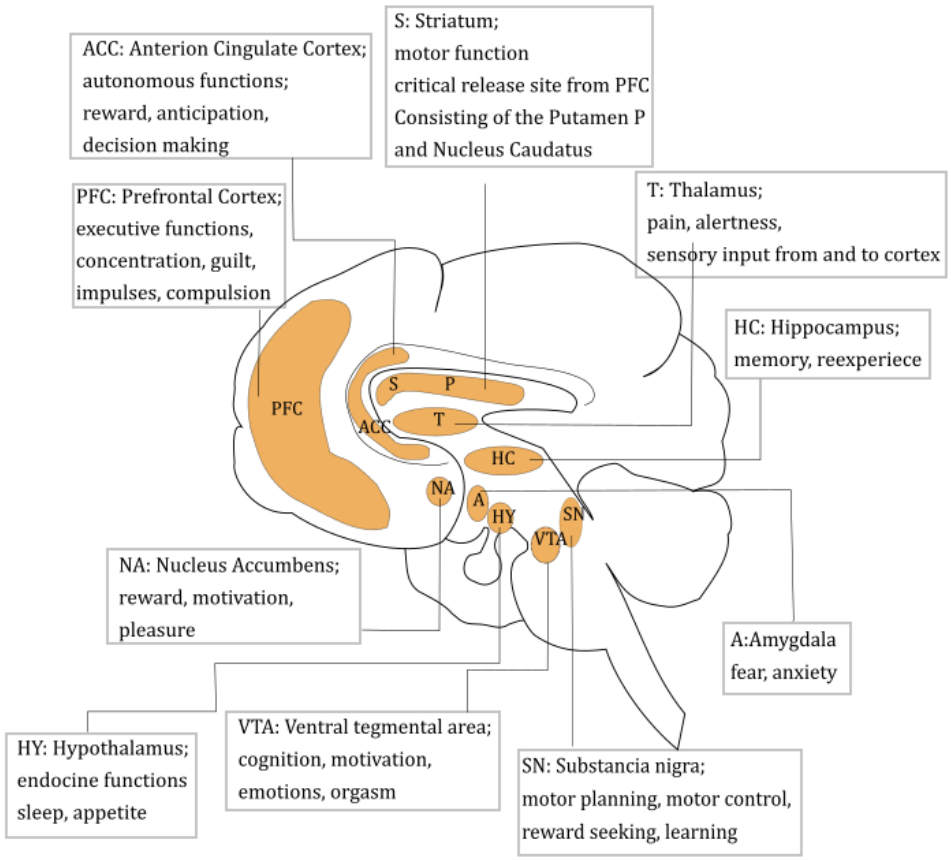

Figure 2: Central nerval system: relevant structures of the brain and their assignment, adapted from Duale Reihe Neurologie (German) Paperback, 23 Oct. 2013.

2.1.5.1 Dopamine

There are two main dopaminergic pathways (see Figure 3) known in the human brain: the nigrostriatal pathway and the MLP. The nigrostriatal pathway connects the substancia nigra with the dorsal striatum and is involved in the production of movement. The MLP is referred to as the reward pathway and connects the afferent dopamine neurons from the ventral tegmental area (VTA) with the nucleus accumbens (NAcc), HC, amygdala and the prefrontal cortex (PFC). There are other dopaminergic neurotransmissions, however (Björklund et al., 2007).

In this chapter, the focus is on the reward and reinforcement system of the brain, i.e., the MLP. Additionally, the MLP is seen as responsible for the reward learning processes (Robinson et al., 1993). Rewards in this neurological context are stimuli that motivate the performance of a certain task. Often, these are tasks which are essential for survival like eating or drinking. However, drinking alcohol can also be rewarded by the brain with a dopamine release. Dysfunction of the dopaminergic reward system has been repeatedly linked to severe craving (Charlet et al., 2013; Heinz et al., 2004, 2005; Sebold et al., 2019).

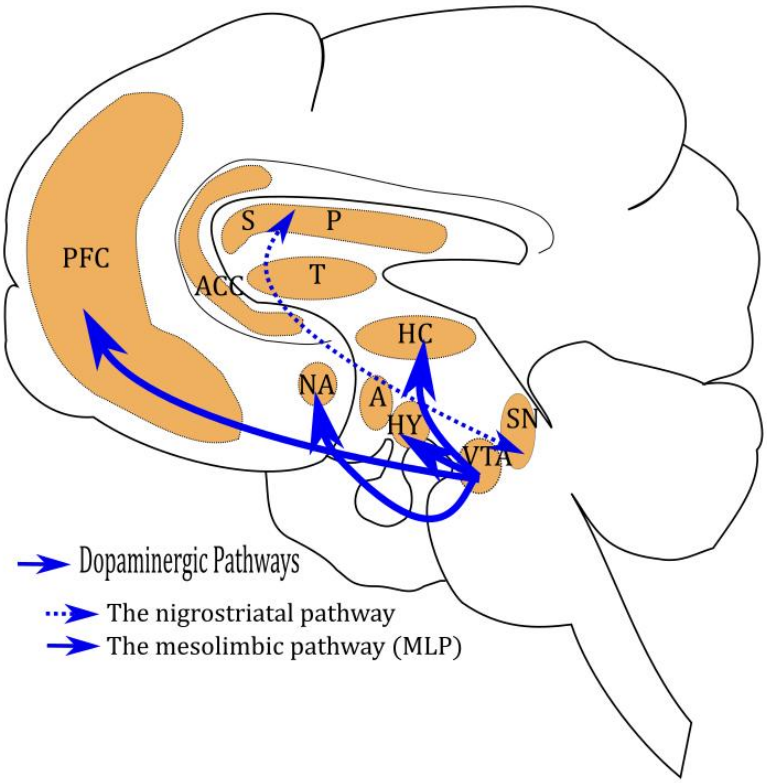

Figure 3: Dopaminergic neurotransmission: dopaminergic pathways. The nigrostriatal pathway and the MLP, adapted from Tammimäki, 2008.

The neurotransmitter dopamine can lead to both excitation (via D1-like receptors, known as D1/5R) and inhibition (via D2-like receptors, including D2/3/4R) of cells, and can thus be seen as a neuromodulator (Seamans et al., 2004). Acute alcohol intoxication leads to increased synthesis and release of presynaptic dopamine in the NAcc (Boileau et al., 2003). Detoxified alcoholics experience a stronger dopamine activation in the MLP after alcohol consumption than healthy controls (Heinz et al., 2004).

Chronic alcohol consumption, on the other hand, leads to a compensatory down-regulation of the dopamine-receptors (Heinz et al., 2009) and to a hypodopaminergic state (Koob & Volkow, 2010). Therefore, AUD patients show dopamine receptor dysfunction and delayed dopamine receptor recovery even after withdrawal. The extent of down-regulation is also correlated with increased craving and relapse risk (Heinz et al., 2004, 2005). However, after prolonged abstinence, D2/3R availability can recover (Rominger et al., 2012; Schultz, 1998).

2.1.5.2 GABA

GABA is the most important inhibitory neurotransmitter in the human brain (Olsen & DeLorey, 1999). There are two types of GABA receptors: ionotropic GABA-A receptors and metabotropic GABA-B receptors. GABA-A receptors are inhibitory chloride and hydrogen carbonate ion channels.

The activation of GABA-A receptors was shown to lead an anxiolytic, anticonvulsant, sedative-hypnotic, cognitive-impairing, and motor incoordination effect on the human behaviour (Kumar et al., 2009). Ethanol, along with other substrates like barbiturates or benzodiazepines, binds to allosteric binding sites of GABA-A receptors, multiplying the activity (Sieghart, 1999; Sieghart & Sperk, 2002).

Furthermore, during acute intoxication, the amount of GABA-A neurotransmission is increased. However, in AUD-P a reduced activity of GABA-A receptors was found (Krystal et al., 2006). Additionally, during detoxification a decreased GABA-A activity was detected, which was associated with a higher relapse probability (Heinz et al., 2009).

The GABA-B receptor is a g-protein-coupled receptor and leads to post-synaptic activation of ligand-directed potassium channels. GABA-B receptors are located in the pre- and postsynaptic membrane. Depending on the distribution of pre- and post-synaptic GABA-B receptors, they can either increase or decrease general excitability in a neuronal network. It is assumed that the stimulation of post-synaptic GABA-B receptors can inhibit long-term potential (LTP) (Olpe et al. 1993; Davies and Collingridge 1996), whereas the activation of presynaptic receptors via the control of GABA secretion from interneurons leads to an amplification of LTP (Davies et al. 1991; Mott et al. 1991).

Considering these results, it is not surprising that GABA receptors may be involved in the development or severity of diseases in which synaptic reorganization and plasticity is altered.

2.1.5.3 Opioid

Alcohol leads to an increased release of endogenous opioids also known as beta-endorphins. The importance of endorphins for the development of an AUD can be explained by the observation that opioids and also ethanol are responsible for the euphoric, well-being effect of alcohol and they are also part of the intrinsic reward system (Economidou et al., 2006). Furthermore, children of AUD patients have a higher alcohol dependent endorphin release than children of healthy controls, which might be one of the reasons why they are more vulnerable to addiction (Froehlich et al., 2000). Andreas Heinz (2005) showed an up-regulation of the postsynaptic opioid receptor concentration in the ventral striatum of AUD-P, and thus the number of opioid receptors could be

directly associated with the craving for alcohol. Additionally, activated opioid receptors in the NAcc provoke an elevated dopamine release in this area and thereby influence the MLP. Therefore, the elevated opioid concentration not only directly influences the emotional state of a person but also the dopaminergic reward and learning system, and therefore it indirectly influences the drinking behavior of a person (Spanagel et al., 1992).

2.2 Glutamate and Alcohol Use Disorder

For several decades the focus of research in drug addiction concentrated on changes of dopamine in the MLP (Björklund & Dunnett, 2007). However, in the past 20 years other neurotransmitters projections and their impacts on the MLP have become the focus of science. The increasing number of studies referring to the role of the neurotransmitter glutamate illustrates the importance of this neurotransmitter for the development and maintenance of addictions.

2.2.1 Glutamatergic Neurotransmission and Glutamate-Receptors

Glutamate is the most important excitatory neurotransmitter in the mammalian brain and is responsible for about 70% of synaptic transmission in the CNS. It is important for fast synaptic neurotransmission, as well as for the process of learning, the development of memory and for neuronal cell death (Ozawa et al., 1998).

The functioning of a glutamatergic neurotransmission is illustrated in Figure 4. In glutamatergic presynaptic terminals, glutamate is stored in vesicles. Once the presynaptic neuron is activated, the vesicles are moved by vesicular glutamate transporters (vGluT), fused into the synaptic membrane, released, and the glutamate is distributed in the synaptic cleft (Dingledine et al., 1999). On the postsynaptic membrane, there is a range of glutamate binding receptors.

To date, two main groups of glutamate receptors have been identified: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). The iGluRs are ligand-gated ion channels and mediate fast excitatory neurotransmission. They can be further divided into NMDA-R, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA) and the kainic acid receptor (KA-R) (Shigeri et al., 2004).

The NMDA ligand-gated ion channels are heterotrimeric protein complexes. They are composed of several subunits, including one NR1 unit plus various combinations of NR2A-D, NR3a or 3B units. For the NR1 subunit alone, there are at least 8 known splice variants and each subunit contains binding sites for a specific neurotransmitter. In their resting position, the NMDA receptors are blocked by magnesium ions. The NMDA receptors can be stimulated by glutamate and glycine. D-serine and other polyamines can become co-agonists. Once activated, the NMDA

receptors are permeable primarily for sodium, though potassium and zinc ions can also pass. The NMDA receptors are mainly located on neurons, but also found on glia cells (Dingledine et al., 1999; Paoletti & Neyton, 2007; Stephenson, 2006).

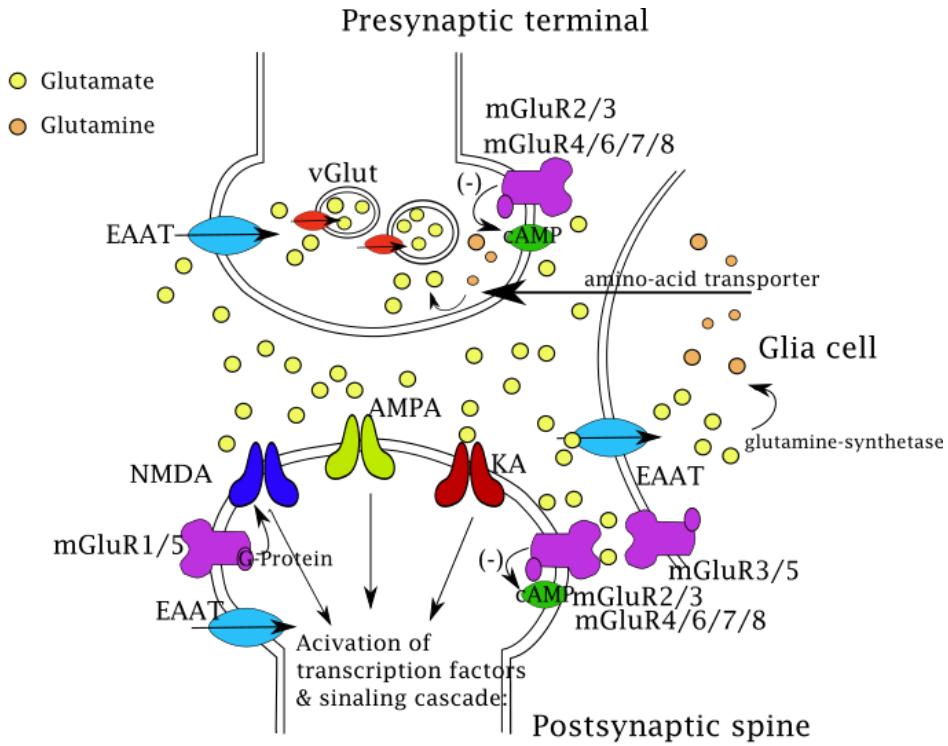

Figure 4: The glutamatergic synapse, vesicular glutamate transporters (vGluT), metabotropic glutamate receptors (mGLUR), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA), kainic acid receptor (KA-R), blocking N-Methyl-D-Aspartate Receptor (NMDA) , cyclic adenosine monophosphate (cAMP) and excitatory amino acid transporter (EAAT), adapted from Noebels et al., 2012.

AMPA receptors, similarly to NMDA receptors, are also heterotrimeric protein complexes. Their subunits are called glutamate receptors (GluR1-4). Each GluR contains binding domains for glutamate and can also be modulated by other polyamides. Once activated, they are permeable for an influx of calcium, sodium and potassium. Research showed that the NMDA and AMPA receptors are important for synaptic plasticity, as they influence the long-term depression (LTD). and the excitatory postsynaptic potential (Bliss & Collingridge, 1993).

The KA-R is also an ion channel. It is formed by a tetrameric protein complex whose subunits are termed GluR5-7 and KA 1 and 2. They are permeable to sodium and potassium ions, responsible for the postsynaptic excitation and also play a role in synaptic plasticity. The KA-R are not only located in the post-synaptic terminal, but also the pre-synaptic terminal, where they influence the release of neurotransmitter (Dingledine et al., 1999).

For the other group of glutamate receptors, the mGluR, eight subunits of mGluR (mGluR 1-8) are known to date. The mGluRs can be located pre- and peri-synaptically and are responsible for a slow, modulatory glutamatergic transmission. The mGluRs can be further subdivided into 3 groups according to their constitution and signal transduction.

1) Group I - consisting of mGluR1 and mGluR5 - stimulates G-proteins, which are connected to several phospholipases and activating intracellular messengers. In particular, mGluR5 can thereby positively influence the function and production of the NMDA receptor.
2) Group II – mGluR2 and mGluR3 – and 3) Group III - mGluR4/6/7/8 - In the case of both of these groups, their activation is negatively associated with the levels of cyclic adenosine monophosphate (cAMP) and can inhibit the release of glutamate in the synaptic cleft (Hermans & Challiss, 2001).

In summary, the activation of mGluR and iGluR is responsible for numerous intracellular signalling cascades which influence neural plasticity, such as the regulation of dendritic messenger ribonucleic acid (mRNA) translation, protein synthesis, changes of gene expression and cytoskeletal remodelling. This neuronal remodelling is thought to be one of the explanations for the impairment of changes in the glutamatergic neurotransmitter system and psychological illnesses.

After evoking a specific reaction, glutamate is removed from the synaptic cleft by sodium-dependent excitatory amino acid transporters (EAATs). To date, five different types of EAATs have been identified, located in the glia cells (EAATs 1 and 2), the presynaptic terminal (EAATs 2 and 5) or the post-synaptic terminal (EAATs 3 and 4) (Shigeri et al., 2004). EAATs prevent an extracellular accumulation of glutamate above the excitotoxic level and are widely spread on the glia cells. Glia cells react to the excitatory neurotransmitter glutamate with an activation of EAATs, which transport the glutamate at high speed into the glia cell.

The absorbed glutamate is then converted into glutamine with the aid of the enzyme glutamine synthetase and is subsequently returned to the neurons via amino acid transporters. There, it is transformed back into glutamate by the glutaminase and stored into vesicles. This is called the glutamate-glutamine cycle (see Figure 5). The neurotransmitter GABA is structurally related to glutamate and can be converted to glutamate or derived from glutamate.

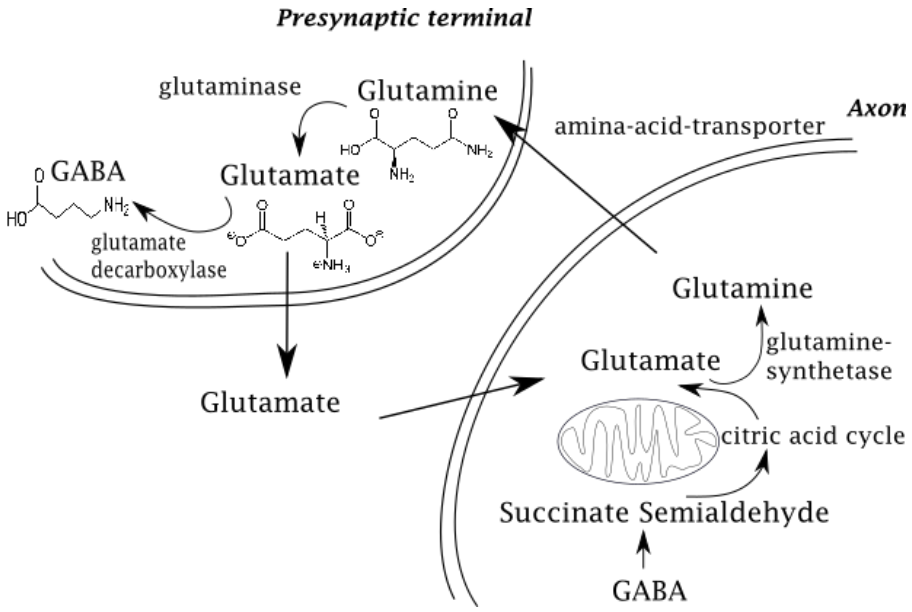

Figure 5: The glutamate-glutamine cycle, author's diagram.

2.2.2 Effects of Acute Alcohol Intoxication on the Glutamatergic System

Alcohol can affect neurotransmitters in the brain and influences glutamate homeostasis, among other effects. To describe the effect of alcohol on the GluC in the brain, it is important to discriminate between the impact of acute and chronic drinking. Furthermore, it should be noted that though various animal studies have proven a correlation between alcohol and changes in GluC, there have been few studies on humans.

The NMDA-R is seen as one of the key targets of alcohol in the glutamatergic system. It is inhibited by ethanol in a non-competitive way (Wirkner et al., 2000). This indicates that a high concentration of ethanol decreases the mean opening time of NMDA-R (Lima-Landman & Albuquerque, 1989) and thus decreases the NMDA-R mediated intracellular increase of Ca 2+ (Dildy & Leslie, 1989). It has also been demonstrated that alcohol can alter the production and composition of subunits of NMDA-R through its effect on mGluR (Suvarna et al., 2005). Furthermore, receptors containing a NR2B subunit were shown to be more sensitive to ethanol than those containing a NR2C or NR3 subunit (Kuner et al., 1993; Smothers et al., 2001; Wirkner et al., 2000). Additionally, Moghaddam & Bolinao (1994) demonstrated that low concentrations of ethanol (0.5g/kg) cause an increase of the extracellular glutamate level in the HC and NAcc in rats. Furthermore, high levels of ethanol (2.0g/kg) decrease the amount of extracellular glutamate. Carboni (1993) supported these results by providing evidence that the alcohol suppresses the release of glutamate by inhibiting the NMDA-R.

The activation of VTA neurons and also the extracellular dopamine levels is regulated by excitatory projections from the PFC to the VTA. The glutamatergic neurons of the VTA interact with dopaminergic synapses and influence them, and thereby they also regulate the extracellular levels of dopamine as part of the reward system (Carr & Sesack, 2000).

Further microdialysis studies showed an ethanol-induced increase of extracellular glutamate in the HC (Moghaddam & Bolinao, 1994), amygdala (Quertemont et al., 1998), and NAcc (Selim & Bradberry, 1996; Szumlinski et al., 2007a). The effect of the glutamate increase was particularly strong at low doses, whereas higher doses of ethanol actually reduced the glutamate overflow (Moghaddam & Bolinao, 1994; Yan et al., 1998).

Despite there being evidence that low doses of alcohol elevate the extracellular GluC, there have been several studies that contradict this statement by demonstrating a suppressed glutamate release after acute alcohol intoxication (Dahchour et al., 1996; De Witte et al., 1994; Quertemont et al., 2002).

2.2.3 Effects of Chronic Alcohol Consumption on the Glutamatergic System

A large number of studies indicate that chronic alcohol consumption leads to an increase in extracellular GluC (Melendez et al., 2005; Roberto et al., 2004). In some studies, there is evidence that the result depends on the brain regions examined or on the cell type, along with the abstinence time between the last alcoholic drink and measurement as well as the amount of alcohol (Cheng et al., 2018; Dahchour et al., 2000; Ding et al., 2012a; Zahr et al., 2016).

Many studies agree that glutamate levels are increased immediately after detoxification, in that glutamate levels are increased immediately after withdrawal onset, e.g., in the ACC (Hermann et al., 2012; Yeo et al., 2013), HC (Chefer et al., 2011; Abdelkader et al., 2003; Frischknecht et al., 2017; Hermens et al., 2015), dorsal striatum (Rossetti et al., 1999; Rossetti & Carboni, 1995), NAcc (Dahchour et al., 2000; Das et al., 2015; Melendez et al., 2005; Szumlinski et al., 2007b) or PFC (Hwa et al., 2015), and VTA (Ding et al., 2012a). This effect is further enhanced by repeated detoxification (Abdelkader Dahchour & De Witte, 2003), which in turn may explain a higher likelihood of epileptic seizures after repeated detoxification (Becker, 1999).

However, there are also studies that could not establish a connection between glutamate dysregulation and alcohol consumption (Lee et al., 2007; Mason et al., 2006). Some studies have even obtained the opposite results and assumed that long alcohol consumption leads to a compromise of the glutamate metabolism (Ende et al., 2013a; Prisciandaro et al., 2016, Mon et al., 2012).

Even though the mechanisms through which ethanol affects the glutamate levels remain partly uncertain, there is evidence that glial cell-mediated glutamate uptake can be affected by ethanol (Melendez et al., 2005; Othman et al., 2002; Smith, 1997).

The effect of alcohol on glutamatergic receptors has often been investigated: chronic alcohol abuse leads to a change in the composition of the glutamatergic receptors which influences the development of LTP (Wills et al., 2012). As early as 1978, Michaelis showed that synaptosomal glutamate binding activity increased in rats exposed to alcohol for 16 days (Michaelis et al., 1978). On the cellular level, changes in the NMDA receptor are assessed as decisive. As a result of the lasting blockade of NMDA receptors by ethanol during permanent alcohol consumption, there is a compensatory upregulation and sensitisation of NMDA receptors in the cerebral cortex and HC (Hardy et al., 1999; Hu et al., 1996; Snell et al., 1996; Trevisan et al., 1994).

This increases the prevalence and functionality of the NMDA receptors by affecting their influx capacity and conductance. In this context, the NR1, NR2A and NR2B subunits are thought to play a relevant role. For example, both Ortiz and Floyd were able to show that prolonged ethanol consumption can increase NR1 immunoreactivity (Floyd et al., 2003; Ortiz et al., 1995). However, an AUD does not just affect the NMDA receptor; increased levels of GluR subunits have also been observed in rats. For example, an increase of the GluR1 subunit in the VTA was shown by Ortiz et al. (1995), and in 1997 Brückner et al. demonstrated that GluR3 mRNA was increased by 15-30% in the ACC and HC during withdrawal. Furthermore, the author's own study found an increase in GluR1 or GluR2 in the ACC and HC.

It was also shown in vitro that cells that were permanently treated with ethanol showed an increased AMPA-induced Ca2+ influx (Netzeband et al., 1999). The AMPA receptors in the amygdala are evoked by reinforcing effects and evoke processes like stimulus-reward learning (Zhu et al., 2007).

Unfortunately, the above-mentioned studies are to a large extent animal experiment based. Studies on humans are still rare. Furthermore, the glutamate concentrations were determined with different methods, and thus also in different cellular spaces, which makes comparability difficult.

2.2.4 Areas of Glutamate Measurement: The Hippocampus and Anterior Cingulate Cortex

To examine the influence of alcohol on glutamate, areas in the brain in which changes of the neurotransmitter have a substantial influence on the neurological activity were defined. For the development of AUD learning, more specific reward learning is crucial. Therefore, for the present study, two regions in the brain will be investigated: the HC and the ACC.

The part of the brain which is most important for the creation of long-term memory and thus for the process of learning is the HC. The HC can differentiate between old and new stimuli. To detect new stimuli and differentiate new from already known stimuli, a permanent comparison between the incoming information and already existing memory is necessary. New stimuli can be consolidated to the long-term memory through LTP (Ranganath & Rainer, 2003), and stimuli that are rated as reward-connected stimuli are more likely to be stored in the long-term memory.

The HC is closely linked to other brain structures for the evaluation of stimuli, such as the ACC (Brooks et al., 2011).

The ACC is the other region of measurement. The ACC is surrounded by the frontal part of the corpus callosum and is well connected to many other brain regions via a large number of synapses. Its ventral projections lead to the amygdala, the NAcc and the hypothalamus. The dorsal part is connected to the PFC and parietal cortex. The ACC plays a role in autonomic (e.g., heart rate, blood pressure) as well as higher-level functions (e.g., reward, decision-making). Furthermore, there is proof that the ACC regulates cognitive as well as emotional processing (Bush et al., 2002).

In this way, it controls impulses. The dorsal part of the ACC is influenced by reward-associated decision making and learning, whereas the rostral part concerns affective responses to mistakes, rewards, and losses. The ACC has been attributed to such functions as error detection and reward-based learning. As mentioned above, the predominant neurotransmitter in the MLP is dopamine. However, the striatal dopamine turnover may be regulated by glutamatergic projections from the PFC (Carlsson et al., 1999).

The interactions between glutamatergic neurons of the PFC are important for learning because they project into the midbrain, where they interact with tegmental and hippocampal dopamine (Lisman & Grace, 2005). In animal studies, the inhibition of GluR (NMDA and AMPA) in the NAcc led to the obstruction of operant conditioning and reversed learning (Harris et al., 2004; Hernandez et al., 2005).

2.2.5 Therapeutic Significance

Nevertheless, there are already some drugs that have been developed which successfully support somatic withdrawal. To date, the influence of the drugs supporting long-term abstinence has been under debate, leaving motivation and therefore psychoeducation as the essential component of treatment. It would be important to develop drugs which support AUD-P to maintain long-term abstinence. For this aim, a better knowledge of the neurobiological impairments of addiction is required. However, most of the effects of the drugs were only assessed in rodents and

there are only a few studies to validate the effects these drugs have on humans. As described above, alcohol consumption alters the glutamatergic transmitter system, which is thought to be responsible for some of the effects of ethanol intoxication as well as withdrawal and modulates the MLP.

Evidence could show that drugs that alter the glutamatergic neurotransmitter system also alter alcohol-related behaviour, hence influence drinking behaviour as well as the withdrawal and alcohol seeking behaviour (R. Spanagel, 2003). Therefore, these drugs may offer new approaches of efficiently treating AUD patients. At present, there are two approved drugs that are thought to have an influence on glutamate: acamprosate and topiramate (Blodgett et al., 2014; Maisel et al., 2013).

The mechanisms of acamprosate are not fully understood. But there are indications that it especially influences the formation of NMDA-R subunits in the HC and cortex, while its influence on NMDA-R as an antagonist is weak (Rammes et al., 2001). It can thus help to restore normal glutamatergic neurotransmitter activity in AUD Patients. Patients that were treated with acamprosate reported suffering less under the withdrawal symptoms, successfully completed the withdrawal more often, and showed prolonged abstinence rates (Mason et al., 2001). Unfortunately, other studies couldn't confirm a positive long-term effect of acamprosate on the AUD treatment (Anton et al., 2006; Mason et al., 2006).

The anticonvulsant topiramate appears to affect the NMDA-R and mGluR5. It inhibits the release of glutamate and can block the AMPA-R receptor. It was shown that in rodents that were treated with topiramate the amount of alcohol drunk was reduced, and further craving and withdrawal symptoms decreased (Baltieri et al., 2008; Breslin et al., 2010).

These two examples point out the potential of drugs which alter the glutamatergic neurotransmitter system; however, the possibilities have not yet been completely explored. There are numerous ways in which the GluC in the CNS can be modified. Few of these are understood and used for therapy (Tsai and Coyle 1998) and glutamate alteration may offer a variety of new options for the treatment of AUD.

2.3 Hypothesis

Chronic alcohol consumption leads to an increase of GluC during withdrawal and early abstinence. This increase is due to neuroadaptive changes and can cause withdrawal symptoms. To date, studies have mainly been carried out on animals (Hernandez et al., 2005; Harris et al., 2004; Michaelis et al., 1978; Chefer et al., 2011; Dahchour & De Witte, 2003; Dildy & Leslie, 1989; Ding et al., 2012; Frischknecht et al., 2017) and only few studies on humans (Ende et al., 2013; Hermann et al., 2012; Lee et al., 2007; Mon et al., 2012; Yeo et al., 2013) exist. In this work, the influence of alcohol consumption on the GluC in the ACC and HC will be examined. It is assumed that:

Hypothesis 1 (H.1.)

Abstinent AUD patients have a higher GluC than their healthy controls.

More precisely, the HR Controls also show glutamate dysregulation, represented by a higher GluC in the ACC and HC than their healthy controls.

Hypothesis 2 (H.2.)

The GluC of the HR group lies between those of the AUD-P and LR groups.

Additionally, it can be assumed that the glutamate dysregulation is associated with time-dependent alterations. Participants from the AUD-P and HR group who were tested earlier after the last alcoholic drink exhibit higher GluC than those tested later after the withdrawal.

Hypothesis 3 (H.3.)

Participants tested earlier after withdrawal show higher GluC, whereas longer phases of abstinence lead to lower GluC.

Furthermore, there are many factors, which influence the neuroadaptive changes in the brain. It is suggested, that more alcohol leads to a higher GluC.

Hypothesis 4 (H.4.)

A larger quantity of alcohol consumption is associated with an increased GluC dysregulation.

3 METHODS

3.1 Study Background

The present monograph is written in the context of a cooperative project between the Charité Universitätsmedizin Berlin and Technische Universität Dresden on the topic of "Learning & habituation as predictors of the development & maintenance of alcoholism" – the LeAD Study. The LeAD Study consists of seven research projects that work closely together and share a common data and method pool. The present research is part of Project 5 (P5) “The role of dopaminergic and glutamatergic neurotransmission for learning dysfunction in AUD”. The P5 project shared a data pool with project 2 (P2), "Learning mechanisms as predictors of treatment outcome in alcohol-dependent patients". The exact data recruitment is explained in Chapter 3.1.1.

The data for P5 and P2 was collected in Berlin in collaboration with the Charité Universitätsmedizin, St. Hedwig’s hospital, and the Physikalisch-Technische Bundesanstalt. It was founded by the Deutsche Forschungsgemeinschaft. P5 was supervised by Prof. Dr. Gallinat and was approved by the local Ethics committee.

3.1.1 Recruitment and Description of the Groups

The study was constituted as a cohort study in which three groups were examined: one group of detoxified AUD patients (n=31), one HR control group (n=38), and one LR group (n=32). The AUD patients were recruited in four hospitals in Berlin (St. Hedwig Krankenhaus, Bundeswehr Krankenhaus, Jüdisches Krankenhaus, and Charité Universitätsmedizin Campus Mitte). The patients had to be diagnosed with an AUD by an independent psychiatrist who was not part of the study. They received information about the study and were recruited directly in hospital while undergoing acute detoxification. The conduct of the study is illustrated in Figure 6.

Most AUD-P were recruited and assessed from P2. To verify their diagnosis, a structured clinical interview was conducted during the first assessment session by trained researchers of P2. After being given information about P5, the AUD-P were transferred to P5 for the MRS and PET-CT testing.

All HR and LR controls were recruited via advertisements in supermarkets, local newspapers, and online on eBay Kleinanzeigen (eBay.kleinanzeugen.de). The first contact with LR and HR participants was carried out by telephone. The participants received further information about the study and underwent a questionnaire. During this first phone call, the screening tests were carried out as part of the questionnaire. This allowed us to exclude participants who did not match the

inclusion criteria, as well as allocate subjects into the HR and LR control groups. Most LR controls were recruited from P2, whereas the HR controls were recruited from P5.

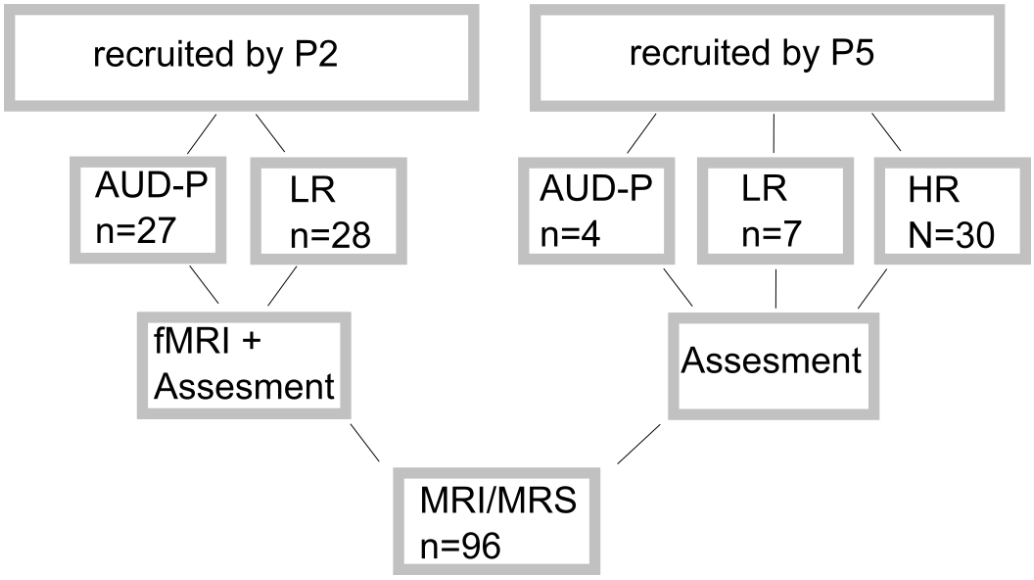

Figure 6: Recruitment of Patients and Conduct of the Study.

For the allocation of the controls to the two groups, we used the alcohol use disorder identification test (AUDIT). Developed by the WHO in 1982, it is a test that enables the simple identification of alcohol-associated problems and risky drinking patterns (Allen et al., 1997). Its reliability and validity were tested by Saunders et al., in 1993. For the screening, a German translation was used.

The AUDIT consists of 10 questions and each answer is scored with a minimum of 0 and a maximum of 4 points. For the interpretation, the points are added to a total score of a minimum of 0 and a maximum of 40 points. The first three questions of AUDIT are core questions concerning the quantity of alcohol consumption. Questions 4 to 6 rate the characteristics of the alcohol abuse, while questions 7 to 10 assess the extent of the hazardousness of the drinking behaviour. Sanders et al. defined a positive case as either: dangerous daily alcohol consumption (i.e. an average intake of 60 g of pure ethanol for men and 40 g for women); repeated intoxication (meaning an intake of 60 g of ethanol daily or 120 g weekly); excessive drinking patterns and therefore meeting at least one of the criteria for AUD but not enough to be diagnosed with an AUD; at least one alcohol-related injury in the past year; an alcohol-related illness; or: a concern mentioned by family, health professionals or friends regarding the alcohol consumption.

To achieve the highest possible sensitivity and specificity, the cut-off value for the assignment of the controls to the HR and LR was set to 8. This provides an overall sensitivity of 92% for the

detection of dangerous and harmful alcohol consumption and a specificity of 94% (Neumann et al., 2004).

The HR and LR controls were matched with the AUD patients regarding age, gender, and education. For all participants the same exclusion criteria were used:

- Lifetime prevalence of DSM-IV bipolar or psychotic disorder
- Current diagnosis of one of the following DSM IV diagnoses: depression, borderline personality disorder, generalized anxiety disorder, post-traumatic stress disorder, or obsessive-compulsive disorder
- Major non-communicable diseases such as untreated diabetes, high blood pressure, thyroid dysfunctions, and infectious diseases like HIV or hepatitis
- Substance dependence, excluding alcohol and nicotine
- Disease history, severe headache, or other neurological diseases
- History of dramatic brain injury, brain operations
- Alcohol consumption in the last 24h
- Medications that interact with the CNS during the last 10 days
- Left-handedness
- Insufficient knowledge of the German language
- Major visual or cognitive impairments
- Claustrophobia
- Pregnant or nursing females
- Metallic implants like pacemakers, dental prostheses, or metal clips

AUD patients that entered this study had to have had the diagnosis of AUD for at least 3 years. Furthermore, we assessed them 72 hours to 90 days after their last drink and only if they only had mild withdrawal symptoms. The withdrawal symptoms were tested using the Clinical Institute Withdrawal Assessment of Alcohol Scale and a cut of value of 8 was used as the defining mark.

3.1.2 Description of Procedures

After the telephone screening, all subjects who met the criteria underwent the same procedures. For simplification, only the tests relevant for this work are described:

1. All participants were invited to either the Charité Universitätsmedizin or St. Hedwig's hospital for further clinical testing. Before the testing was started, the participants were screened for alcohol and other substance consumption (benzodiazepines, cocaine, heroin, cannabis) in the urine and breath. They were only allowed to take part in the study if all tests were negative.

2. The participants had to complete a set of tests, which took around 140 minutes. All tests were explained and supervised by one or two study researchers. For further information visit https://cegg.psych.tu-dresden.de/lead/.
3. Within one to two weeks after testing, the MRS data was collected by the Physikalische Bundesanstalt. A description of the MRS investigation can be found in Chapter 3.3.
4. Also, within two weeks after the initial testing, a PET-CT was to be carried out to measure the extrastriatal dopamine receptor availability, using (18F)-Fallypride as a tracer. The testing was carried out at the Charité Campus Virchow.
5. 6 months after the first testing, the telephone follow-up (FU) was carried out and 12 months later the participants were invited to the Charité to complete questionnaires similar to the ones in the first testing. The testing period lasted from 2013 to 2017.

3.2 Clinical Methods

3.2.1 Amount of Consumed Alcohol and Time of Abstinence

To evaluate the amount of drinking, on the day of the initial testing all participants had to fill out a drinking calendar for the last 4 months. The calendar was used to calculate the average amount of pure gram alcohol consumed at every drinking occasion (AvOcc) and the average amount of pure gram alcohol consumed every day (AvDay) during the past half-year. As the patients were not allowed to drink alcohol after their withdrawal, the last drinking day mentioned in the calendar could be used to calculate the abstinence time between the last alcoholic beverage and the MRS. As the HR and LR groups were not asked to stop drinking between the initial testing and the MRS, a mean abstinence time (meanAb) was determined by calculating the mean abstinence time between two drinking episodes. The meanAb was calculated by dividing the AvOcc by the AvDay.

3.2.2 FTND - Fagerström Test for Nicotine Dependence

To quantify the severity of nicotine dependence, the Fagerström Test for Nicotine Dependence (FTND) was used. The FTND is a 6-item long questionnaire. Two items score on a four-point scale (0, 1, 2, and 3 points), along with four items on a two-point scale (0 or 1 point). This leads to a sum score of 10 points. This sum score is matched to a verbal score, which again decodes to a 0 to 5 point score. High total values equal a heavier dependence.

For example, a sum score from 0 to 2 points equals a verbal score of "no dependence or a very light dependence", which is decoded as a score of 1; a sum score of 3-4 points is seen as a "light dependence" and a score of 2; a sum score of 5 is a "medium dependence" and a score of 3; a sum

score of 6-7 points is designated as a “heavy dependence” and a score of 4; a sum score of 8-10 points equals a “very heavy dependence” and a score of 5 (see Table 2).

Table 2: Encoding of the Fagerström Test for Nicotine Dependence

Total score from test	Verbal score	Final score
	Not smoking	0
0-2	no dependence	1
3-4	light dependence	2
5	medium dependence	3
6-7	heavy dependence	4
8-10	Very heavy dependence	5

Participants that do not smoke received a score of 0 (Kunze et al., 1998). The retest-reliability of the questionnaire is r_{tt} = .88, and the internal consistency $\alpha = 0.61$ (Bleich et al., 2002).

3.3 Magnetic Resonance Spectroscopy (MRS)

To determine the neurotransmitter concentration in the ACC and HC, MRS was used, which can be seen as an advancement of magnetic resonance imaging (MRI). Therefore, firstly, the principles of MRI will be explained.

3.3.1 Basic Principles of Magnetic Resonance Imaging (MRI)

MRI is a non-invasive medical imaging method which does not use x-rays. It allows us to create sectional images of the body in any axial orientation (Katti et al., 2011).
MRI was first used in the 1970s and by now is one of the most important tools of medical imaging. Its functioning principles are based on the ability of nuclei with odd nucleons to absorb and emit radiofrequency energy (Katti et al., 2011).

Nuclei with an odd number of nucleons (i.e., protons and neutrons) have a magnetic moment (μ) and they can therefore spin. The spin is comparable to the angular moment and its speed is specific for every nucleus. The spin and the μ are connected over a proportionality quotient, the gyromagnetic ratio (γ). It is a matter constant, which defines the detection sensitivity of a nucleus. Nuclei with a high γ are easier to detect than those with a low γ (Pekar, 2006).

Single proton hydrogen has a high γ and a high prevalence in fat and water. Therefore, it is frequently used for the creation of MRI images, and because of this, one can say that MRI scans map the location and quantity of hydrogen in water and fat in the body. Hydrogen atoms behave

like magnets. Under normal conditions, the protons and electrons spin freely. In a neutral position the hydrogen atoms all form dipoles which are randomly aligned in the human body; therefore, they neutralize each other and do not generate detectable radiofrequency (Pekar, 2006).

An MRI scanner creates a strong magnetic field, which is measured in Tesla (T). In the human clinical imaging field, a strength of 1.5 to 3 T is frequently used. If we place the sample we want to map in a strong magnetic field, the magnetic flux density (B0) and the μ of the nucleus line up and create a magnetic vector. This change of orientation of the rotational axis is also called precession. The precession of a μ in an external magnetic field is also called the Larmor frequency ω0. This precession or Larmor frequency is measured in Megahertz (MHz). It is proportional to B0, therefore $\omega = \gamma * B0$.

The vector, created by the alignment of nuclei, is oriented along the axis of the MRI scanner, which can also be defined as the field lines or Z-axis. The spins in the nucleus align parallel or antiparallel to Z. The μ rotate around the field lines. As the parallel orientation of the spins is more effective than the antiparallel, there are more parallel-oriented spins than antiparallel spins. Therefore, the magnetisation of the spins equals the spins of the surrounding magnetic field.

With additional energy, such as radio waves, the alignment of the spins can be changed. The magnetic vector is deflected. The surrounding magnetic field is overlaid with magnetic fields of less intensity in three dimensions. If these other electromagnetic coils deflect a high-frequency impulse, an energy transfer to the protons is applied; this is also called resonance. This resonance is limited to a located area. It introduces a change in the magnetization of the protons in this area and their alignment is moved to one equal angle for all protons. The vector is changed from the previous longitudinal plane to a transversal one. Now, the protons start to process around this transverse magnetization, which creates tension. This tension can be detected by detection coils and is used to create images (Weishaupt et al. 2009).

After cutting off the radiofrequency source, the spins return to their resting position. The transverse magnetization vector drops, and the magnetisation goes back to that of the longitudinal magnetisations. Next, electromagnetic radiation with the substance-specific Larmor frequency is emitted. Receiver coils in the MRI are used to detect free induction decay. The free induction decay defines the measurable signal of the nuclear spin resonance. The nuclear spin resonance is measured after the deflection of the equilibrium magnetisation of the nuclear spins in the external magnetic field by a resonant high-frequency pulse. Furthermore, the time it takes for the magnetic vector to return to its resting state is variable, but specific for a substance. These principles are used to create MRI pictures. In a T1-weighted image, the longitudinal relaxation time, also called

the “spin-lattice” relaxation of a tissue, is measured. This means the time needed for spins to align in a magnetic field B0 after a transverse deflection through a radiofrequency. As the tension emitted by only one deflection through radiofrequency is not significant, there are several radiofrequencies in rapid succession, and the results are averaged to improve the signal-to-noise ratio. T2-weighted images use the transverse relaxation time or “spin-spin” relaxation. At the same time as the spins relax from the transverse plane towards B0, spins decay from their precession in the transverse plane. The differences in the decay are detectable and captured in T2 (Katti et al., 2011). Repetition time (TR) describes the frequency in which excitation pulses are applied and thereby determines how long it takes for the longitudinal magnetization to recover. Echo time (TE) is the time between the application of a signal and its detection (both measured in milliseconds). T1-weighted images use short repetition times and echo times (TE<30ms or rather TR<600ms), whereas T2-weighted images use long repetition times and echo times (TE>60ms or rather TR>1500ms) (Weishaupt et al. 2009).

The received signals are plotted according to their signal intensity in greyscale and cross-sectional images can be built. The signal intensity depends on the number of spins in the tissue and how quickly a spin relaxes after deflection, which is measured by T1, as well as on how long is needed for the tension to neutralize after the radiofrequency pulses, which is measured by T2. T1 and T2 identify tissue parameters which help us to distinguish between different tissues (Katti et al., 2011).

3.3.2 Functioning of Magnetic Resonance Spectroscopy (MRS)

MRS is based on the same physical principles as MRI. It enables the depiction of neurotransmitters and metabolites in the brain in vivo. Instead of producing pictures like an MRI, an MRS determines a spectrum which plots the number of metabolites in a certain area. With an MRS it is possible to trace several metabolites at one time.

MRS is frequently used in science to depict the biochemical constitution of tissue in vivo and often the metabolism of the brain is in focus. Therefore, MRS can be used to investigate the influence of alcohol on brain metabolism. As MRI and MRS both use strong magnetic fields, the same magnetic resonance tomographs can be used. However, for the MRS the data is processed in a modified manner, which is why a separate processing software must be provided.

To choose the exact anatomic region in which the MRS data is going to be collected, before the MRS investigation, T1-weighted pictures with high resolution are taken. Afterwards, the exact location in the MRI picture is determined and the MRS can be carried out in this area. The MRS shows an intensity spectrum and not a picture of the brain like an MRI. The spectrum reflects the

concentration of different metabolites in this area. Additionally, it can detect a great variety of concentrations and both very high and low values. To do so, the much stronger signal of hydrogen protons has to be suppressed.

As described above, every magnetic resonance sensitive nucleus has a specific ω. The ω is defined through γ and B0. In an ideal setup, the external magnetic field would be homogenous and it would therefore have the same effect on each nucleus. In reality, nuclei in chemical compounds are shielded by other atoms in the same compound. This effect is called J-coupling or scalar coupling. It can also be described as an interaction between different nuclei and electron clouds of one compound. This process causes changes in the local magnetic field, and therefore the ω of this compound is not homogenised but rather in a permanent changing process. This is called a chemical shift. This minimal change of the ω in different chemical compounds is the basis for the identification and quantification of neurotransmitter and metabolites in the brain.

When MRS is carried out, the change in the ω induces a signal in a detection coil surrounding the body. The difference between the detected ω and the ω of the expected "pure" nucleus is measured in parts per million (ppm).

This time-domain signal can be digitised, and with the aid of the Fourier transform, a frequency spectrum can be calculated. The Fourier transform is a logarithmic function that helps to transform a continuous, aperiodic signal into a continuous spectrum. This resulting spectrum is an integral function. In this function, the full width half maximum describes the rate at which the time signal is decreasing. This rate, also known as T2, depends heavily on the compound and the homogeneity of the magnetic field. The metabolites are identifiable through characteristic lines with resonance and peaks. The surface under the spectrum lines demonstrates the concentration of the compound we are looking at. There are different methods for calculating an MRS:
Single voxel spectroscopy detects one to four volumes of interest in one cuboid voxel of 4-16cm³. It is used to explore localised pathologies and has a relatively high signal-noise ratio. When the local distribution at multiple lesions is investigated, chemical shift imaging is used. Also, in the MRI several admissions have to be carried through to achieve a good signal-to-noise ratio. For single-voxel spectroscopy, there are two commonly used sequences: *point resolved spectroscopy (PRESS),* which has a better signal-to-noise ratio and was used in this project, and *stimulated echo acquisition (STEAM)* (Frahm et al., 1987).

3.3.3 MRS based glutamate analysis in this study

In this study, a 3-Tesla scanner (Siemens TRIO) with a circularly polarized head coil was used. In the first step, T1-weighted pictures were acquired to localize the site for MRS. In the second

step, the MRS data was required in two brain regions: the 2x3x2 cm^3 voxel in the ACC, and a 2x2x2 cm^3 voxel including the HC (see Figure 7). For the quantification, spectra from metabolite phantoms of equal voxels were taken. The spectra were acquired with PRESS. It was used to receive spectral data with a multi-echo single-shot technique. During PRESS, there are three slice selective pulses in a 90°-180°-180° sequence. The 90° radiofrequency pulse causes the spins to rotate in the yx-plane. The first 180° pulse rotates the spins in the xz-plane, while the second 180° pulse spins in the xy-plane, which produces the signal. The signal is then transformed using the Fourier transform to produce a spectrum (see Figure 8).

Glutamate quantification was calculated, based on a publication by Schubert et al., 2004. To achieve a high selectivity for the glutamate C4 resonance, an 80ms echo time was selected, since at this time the resonance of other disturbing macromolecules has largely decayed. Before additional analysis, with the help from water-unsuppressed spectra, the 8 resulting subspectra-metabolites are adjusted for turbulent currents. Also, frequency shifts due to subject movements and system instabilities are corrected automatically. The quantification of the spectra was carried out using a time-domain/frequency-domain based program. Additionally, remaining, minor contributions caused by macromolecules are taken into account in the baseline by the fitting procedure.

Extensive previous testing revealed uncertainties for glutamate and NAA. In ACCl voxels, the mean uncertainty was adjusted to 10.1% for glutamate and 2.1% for NAA, and in HC voxels to 13.0% for glutamate and 2.6% for NAA (Elster et al., 2005). Additionally, the adjusted metabolite amplitudes are also modified, taking different coil loading into account based on the phantoms, the participants' individual heads, and relaxation effects (Schubert et al., 2004b).

An MRS, however, can only measure the total amount of glutamate in one area of the brain at a time (Rothman et al., 2011). There is no possibility to differentiate between different brain tissues. Therefore, the unified segmentation approach was used to decide between white matter (WM), grey matter (GM) and cerebrospinal fluid (CSF) within the investigated MRS voxels (Ashburner & Friston, 2005). Hence, the absolute GluC in the voxels was determined for GM and WM by using the following formula: glutamate adjusted divided by glutamate absolute.

To calculate the concentration of the metabolites, T1-weighted images were divided into grey and white matter using a superimposition model. Next, the linear combination of model spectra (LCModel) was used to calculate the spectrum. This calculates an in vivo spectrum as a linear combination from in vitro model spectra, which consist of individual metabolite solutions. To utilise a lot of preliminary information in the calculations, complete model spectra are used rather

than individual resonances. Due to the almost model-free, restricted regularisation method, the baseline as well as the in vivo line course is automatically taken into account. The LCModel works automatically without subjective input, but not interactively.

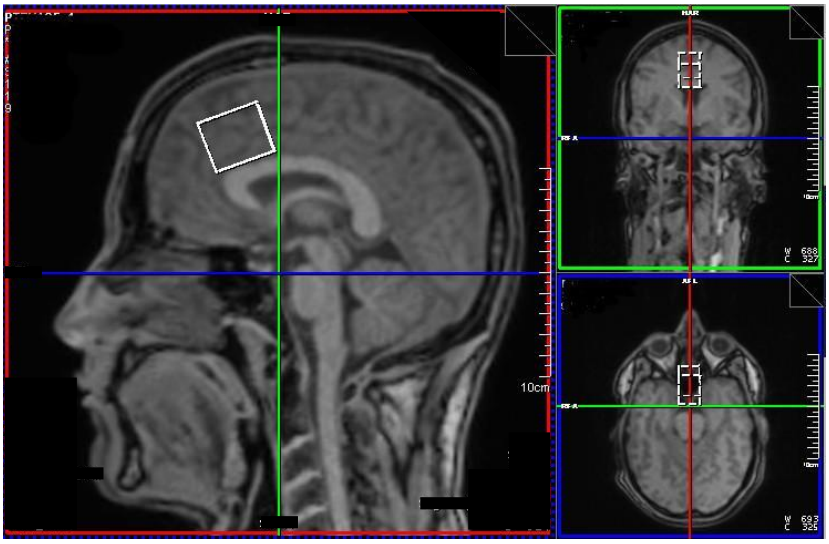

Figure 7: Placement of a voxel (2x2x2 cm3) in the area of the FMPFC/ACC with a 3-Tesla scanner, Siemens-TRIO.

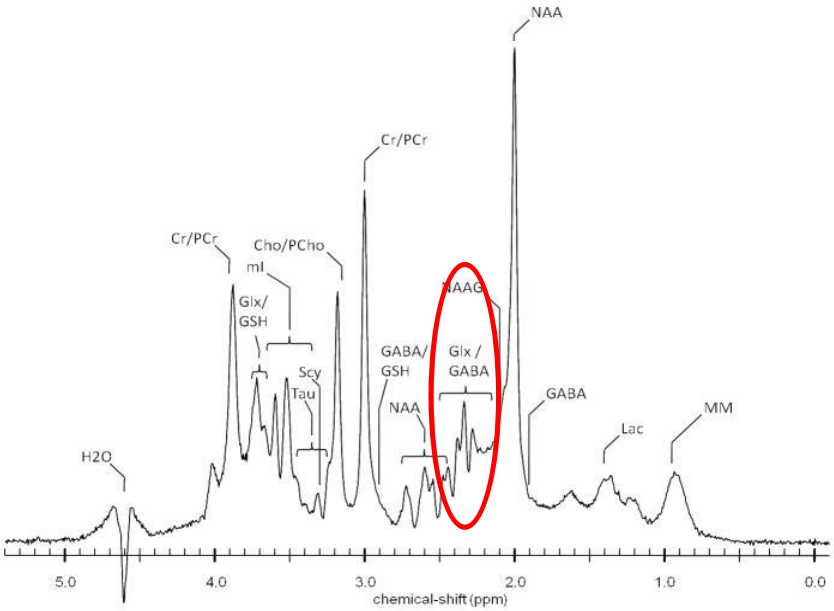

Figure 8: Proton spectrum of the parieto-occipital cortex of a 26-year-old healthy subject (Licata SC & Renshaw PF, 2011).

In this study, GluC in the brain was assessed with MRS. However, there are other methods of measuring GluC, such as PET-CT or microdialysis, which will not be discussed here (Frahm et al., 1987; O'Gorman Tuura et al., 2019; Zieminska et al., 2018). It is important to note, however, that the different measurement methods cannot be compared because the GluC is assessed in

different sites (total glutamate in a region, glutamate intracellularly or in the synaptic cleft), therefore the concentrations are not comparable.

3.4 Statistics

The statistical data analysis was completed with the 26th Version of IBM SPSS Statistics. First, the data was examined for completeness and statistical outliers, which were excluded. The sample was described calculating mean values and standard deviations. To select a valid test procedure, the data was examined for normal distribution and variance homogeneity. The dichotomous data was analysed using the Chi-square test. The Kolmogorov-Smirnov test was used for testing the normal distribution of the nominal variables. Variance homogeneity was calculated using Levene's test.

As GluC in the HC and ACC were not normally distributed, the Kruskal-Wallis test was used for the calculation of group differences. To control for the possible influence of covariates, at first group differences of the demographical data were also calculated using the Kruskal-Wallis score for nominal scaled data, along with Chi-square tests for dichotomous data. In a second step, for the variables that showed significant group differences, a Spearman's rank-order correlation was calculated. The Mann-Whitney U test was used for calculating possible differences between the groups. The differences were visualized in graphs. A two-tailed p-value $< .05$ was set as the threshold for significance.

Spearman's rank-order correlation was applied to calculate the correlations between GluC, meanAb and drinking quantity. The threshold of value for significance was set at $p = 0.05$

Significant results should be corrected for multiple comparisons with post-hoc tests (Mann-Whitney tests, Bonferroni correction). p-values less than 0.05 were considered as statistically significant. The p-values of the statistical tests are thus to be understood as exploratory ones with no confirmatory generalization of the results (Hart, 2001; Hochberg, 1988; Holm, 1979; Mann & Whitney, 1947).

4 RESULTS

To present the results of the study, firstly, there is a short description of the examined groups and the demographic characteristics of the participants. Secondly, for testing the first and second hypothesis, the GluC of the different groups was examined for significant differences. Finally, the third and fourth hypothesis were tested calculating a correlation between the abstinence time/amount of drinking and alcohol.

4.1 Sample Description

In this study, 96 participants were admitted to the clinical and MRS testing.

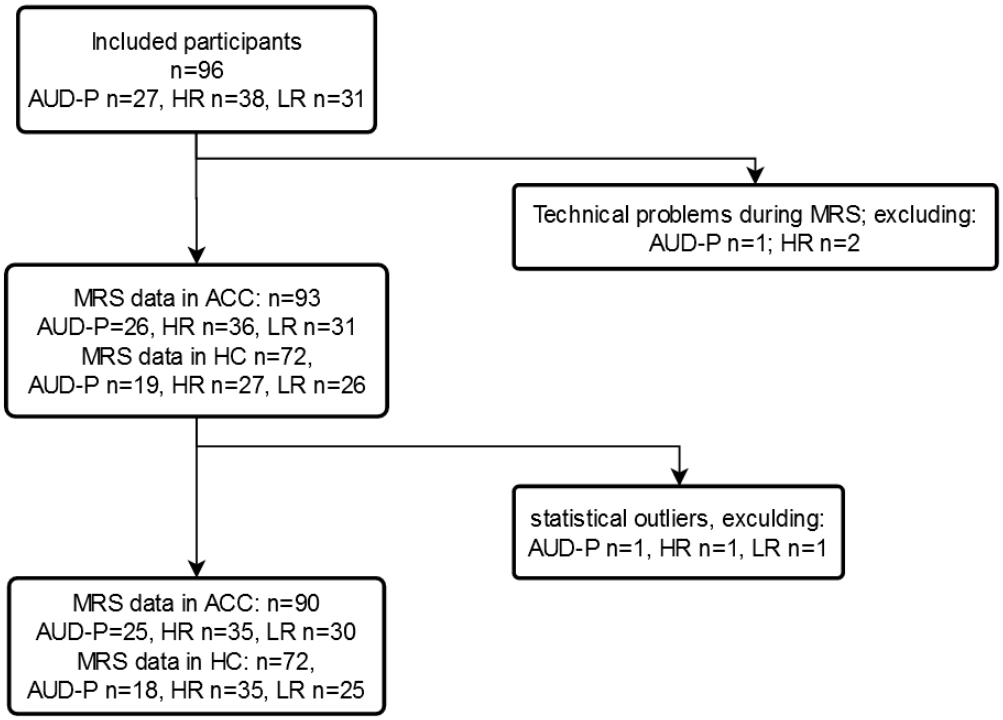

Figure 9: Study Process and Participant Numbers.

For 3 participants (AUD-P n=1, HR n=2), MRS data had not been collected in the ACC or in the HC. These participants were therefore excluded. The GluC measurement in the HC was unsuccessful for several other participants; for these participants, however, the results of the GluC of the ACC were included in the data analysis. Figure 9 visualizes this process.

A statistical outlier was defined as more than 1.5 times the interquartile distance outside the quartile distance, $Q_{75} - Q_{25}$ of the GluC in the ACC and HC. In this dataset, 3 outliers (AUD-P n=1, HR n=1, LR n=1) were identified and excluded, leaving a total number of n=90 participants.

The participants can be subdivided into the three subgroups regarding the GluC in the ACC, with AUD-P n=25, HR n=35 and LR n=30. Data collection in the HC area proved to be more difficult, which is why less data is available here, with AUD-P n=18, HR n=35, LR n=25.
The demographic composition of the groups is described in Table 3.

Table 3: Overview of the sociodemographic factors

Variable	Total		LR		HR		AUD-P	
	Mean (n)	SD	Mean (n)	SD	Mean (n)	SD	Mean (n)	SD
Age in Years	44.41 (90)	9.681	46.240 (30)	9.973	42.784 (35)	8.449	44.490 (25)	10.870
Gender (m/f)	75/15 (90)		24/6 (30)		31/4 (35)		20/5 (25)	
FTND	1.31 (61)	1.478	1.23 (30)	1.591	1.14 (7)	1.464	1.46 (24)	1.382
Smoking (yes/no)	61/27 (88)		15/15 (30)		3/31 (34)		9/15 (24)	
Education in Years	14.978 (89)	3.676	14.533 (30)	2.834	15.264 (34)	4.351	15.120 (25)	3,664

Low Risk = LR, High Risk = HR, alcohol use disorder patient = AUD-P, LR+HR+AUD-P = total, Standard deviation = SD, Fagerström Test for Nicotine Dependence = FTND.

In all groups, there were more male participants than female participants, but all groups were structured similarly (ratio male/female - LR: 24/6, HR: 31/4, AUD-P 20/5). The statistics are illustrated in Table 6. Regarding the smoking habits/routines, the groups differed from each other in terms of composition (Table 6). Nevertheless, the results of the FTND suggested a similar smoking behaviour in all three groups. This could be due to the fact that only 7 HR participants completed the FTND.

At the beginning of the assessment, the groups were matched in terms of age and gender. Yet, despite the deliberate parallelisation, there were some differences found regarding the means. Those will be referred to later in this chapter. To determine a test procedure for the group comparison of the demographic distribution data, a normal distribution was also calculated using the Kolmogorov-Smirnov test (see Table 4). The testing showed no normal distribution of the demographical data except for the age.

Furthermore, the drinking calendar was relevant to this analysis. At the first clinical testing, all participants were asked to complete a drinking calendar for the last 4 months.

Table 4: Test for normal distribution of the demographical data

Variables	K-S statistic	Df	p-value
Gender	0.505	90	0.001
Education in Years	0.144	89	0.001
Smoking	0.439	88	0.001
FTND	0.255	61	0.001
Age in Years	0.099	90	0.029

Kolmogorov Smirnov Test, Degrees of freedom (df), $p < .05$;
Fagerström Test for Nicotine Dependence = FTND.

Using the drinking calendar, the average daily quantity of pure alcohol in grams drunk during the last 4 months (AvDay), and the average amount of pure alcohol in grams drunk during each drinking occasion (AvOcc) was determined. Furthermore, we attempted to ascertain the abstinence time of the participants. As the MRS took place a few days after the initial testing and the participants (HR and LR) were not asked to stop drinking in between the testing, there was no reliable data for the time in between the last alcoholic beverage and the MRS testing for LR and HR. Therefore, a mean abstinence time for HR and LR controls was determined by calculating the average time between each drinking occasion, dividing the average quantity of ethanol during each drinking occasion by the average ethanol quantity per day. The detailed results for the drinking behaviour as well as the results of the clinical tests are discussed in Chapter 4.3. The descriptive results of the drinking behaviour are illustrated in Table 5.

Table 5: Examination of the participants' drinking behaviour regarding quantity of drinking and abstinence time over a 4-month period

	Total		LR		HR		AUD-P	
Variable	Mean (n)	SD	Mean (n)	SD	Mean (n)	SD	Mean (n)	SD
AvDay in Gram	67,3 (80)	67,57	6.68 (30)	8.534	47.97 (35)	75.146	140.21 (25)	101.559
AvOcc in Gram	94,46 (90)	67,12	38.10 (30)	33.732	79.26 (35)	72.693	183.36 (25)	73.639
Abstinence time in Days	14,49 (90)	11,29	10.76 (21)	8.988	5.97 (34)	8.723	35.04 (25)	20.703

Standard deviation (SD)
Low Risk = LR, High Risk = HR, alcohol use disorder patient = AUD-P, LR+HR+AUD-P = total, Standard deviation = SD, Average alcohol drunk per day = AvDay, Average alcohol drunk per occasion = AvOcc, Mean Abstinence Time = MeanAb.

To screen for possible group differences regarding the demographical variables and the mean abstinence, a Chi-square test was performed for the dichotomous data (see Table 6). For the continuous data, a Kruskal-Wallis test was used, since the data was not normally distributed (see Table 7).

The variables with a detected significant difference were used as covariates in the following analyses. AvDay and AvOcc are not independent variables and therefore cannot be used as covariates.

Table 6: Group differences regarding gender and smoking

Gender			**Smoking**		
Pearson-Chi2	**df**	**p**	**Pearson-Chi2**	**df**	**p-value**
1.131[a]	2	0.568	13.427a	2	0.001
n=90			n=88		

Test statistics: Pearson's chi-squared test, Grouping variable: groups.
Degrees of freedom = df, $p < .05$.

Table 7: Group differences regarding age, abstinence time, FTND and education

Variables	**H-statistic**	**df**	**p-value**
Age in Years	3.125	2	0.210
Abstinence time in Days	42.236	2	0.000
FTND	0.744	2	0.689
Education in Years	0.359	2	0.836

H- statistics: Kruskal-Wallis Test, Grouping variable: groups.
$p < .05$, degrees of freedom = df, Mean Abstinence Time = MeanAb, Fagerström Test for Nicotine Dependence = FTND.

Table 6 and Table 7 show that significant group differences were identified only for the variables meanAb and smoking. Therefore, these two variables were used as covariates. The other variables were equally distributed in the three groups.

4.2 Glutamate Concentration in Group Comparison

To determine the test for GluC group differences, a Kolmogorov–Smirnov test with a significance level of < 0.05 was calculated to check for normal distribution. Table 8 shows that GluC was not normally distributed in all of the groups.

Table 8: Test for normal distribution of glutamate in the total sample and subgroups

	K-S Statistic	df	p-value
GluC ACC (total) in mmol/l	0.099	90	0.030
GluC ACC (LR) in mmol/l	0.115	30	0.200*
GluC ACC (HR) in mmol/l	0.155	35	0.032
GluC ACC (AUD-P) in mmol/l	0.095	25	0.200*
GluC HC (total) in mmol/l	0.077	69	0.200*
GluC HC (LR) in mmol/l	0.127	25	0.200*
GluC HC (HR) in mmol/l	0.175	26	0.040
GluC HC (AUD-P) in mmol/l	0.127	18	0.200*

K-S Statistic: Kolmogorov-Smirnov test, $p < .05$.
Degrees of freedom = df, corrected glutamate concentration in the ACC = GluC ACC, corrected glutamate concentration in the HC = GluC HC, Low Risk = LR, High Risk = HR, alcohol use disorder patient = AUD-P, LR+HR+AUD-P = total.

As discussed before, the possible covariates were not distributed normally. Therefore, a non-parametric test was used for the calculation of group differences. The non-parametric tests, however, do not allow covariates.

To test for possible influences of the dichotomous covariate *smoking* on the GluC, the GluC of smokers was compared with that of non-smokers. The influence of the other possible covariate "meanAb" is investigated in Chapter 4.5.1.

4.3 Group Differences of the Glutamate in the Anterior Cingulate Cortex

The calculation of the group means of GluC in the ACC indicated that there were differences between the groups: the highest values were found among the AUD-Ps, the lowest values were in the HR group, and the values of the LR were in between (see Table 9).

Table 9: Comparison of means of the GluC in the ACC in mmol/l

Groups	mean	n	SD
LR	11.236	30	1.324
HR	11.188	35	1.355
AUD-P	11.599	25	1.516
Total	11.318	90	1.387

Standard deviation = SD, Low Risk = LR, High Risk = HR, alcohol use disorder patient = AUD-P, LR+HR+AUD-P = total.

The examinations for group differences concerning GluC in the ACC using the Kruskal-Wallis test could not show any significant group differences (compare Table 10).

Table 10: Comparing for group differences of the GluC in the ACC in mmol/l

H-Statistics	df	p-value
1.246	2	0.536

H-Statistic: Kruskal - Wallis test, grouping variable: groups.
Degrees of freedom = df, $p < .05$, corrected glutamate concentration in the ACC = GluC ACC.

As the results of the Kruskal-Wallis test were not significant, no further testing was required. As mentioned above, it was assumed that smoking could have a significant influence on the GluC. To calculate this connection, the GluC of the smoking and non-smoking participants was compared for significant differences with the help of the Mann-Whitney U Test (see Table 11). Additionally, a graphical comparison was carried out (see Figure 10).

Table 11: Comparison of smokers and non-smokers concerning GluC in the ACC in mmol/l

Mann-Whitney-U Test	Z	Asymp. Sig. (2-tailed)
748.00	-0.683	0.495

Asymptotic significance = Asymp. Sig.

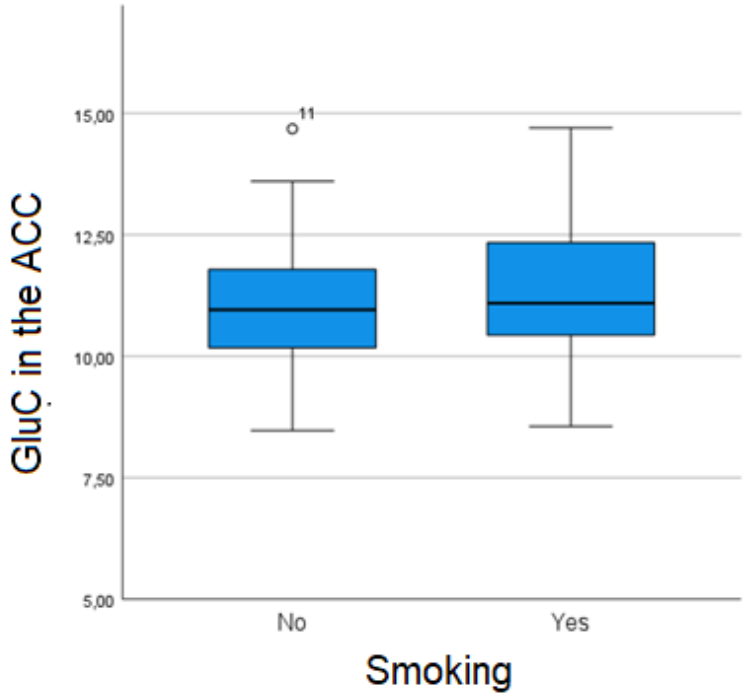

Figure 10: Visual differences concerning the corrected Glutamate concentration in the Anterior Cingular Cortex in mmol/l in non-smoking and smoking subgroup; using boxplot.

The Mann-Whitney-U Test did not show significant differences between smoking and non-smoking participants (p=0.495).

4.4 Group Differences of the Glutamate in the Hippocampus

In the HC, a comparative analysis of the mean values of GluC showed no significant differences between the three groups. The highest values were found in the HR subgroup, followed by the GluC in the AUD-P group. The lowest values were found in the LR group (see Table 12).

Table 12: Comparison of means of the GluC in the HC in mmol/l

Groups	mean	n	SD
LR	7.366	25	1.488
HR	7.894	26	1.417
AUD-P	7.710	18	1.651
Total	7.655	69	1.502

Standard deviation = SD, Low Risk = LR, High Risk = HR, alcohol use disorder patient = AUD-P, LR+HR+AUD-P = total.

Also, when comparing the groups concerning GluC in the HC using Kruskal-Wallis tests, the differences were not significant (compare Table 13).

Table 13: Comparing for group differences in the GluC in the HC in mmol/l

	H-statistic	df	Asymp. Sig.
GluC HC	0.932	2	0.627

H-Statistic: Kruskal-Wallis test, grouping variable: groups.
Degrees of freedom = df, Asymptotic significance = Asymp. Sig, corrected glutamate concentration in the HC = GluC HC.

The group difference between the GluC of smokers and non-smokers was calculated using the Mann-Whitney U test (see Table 14). No differences could be found in either the calculation or the graphical comparison (see Figure 11).

Table 14: Comparison of smokers and non-smokers concerning GluC in the HC in mmol/l

Mann-Whitney-U Test	Z	Asymp. Sig. (2-tailed)
468.000	-0.642	0.521

Asymptotic significance = Asymp. Sig.

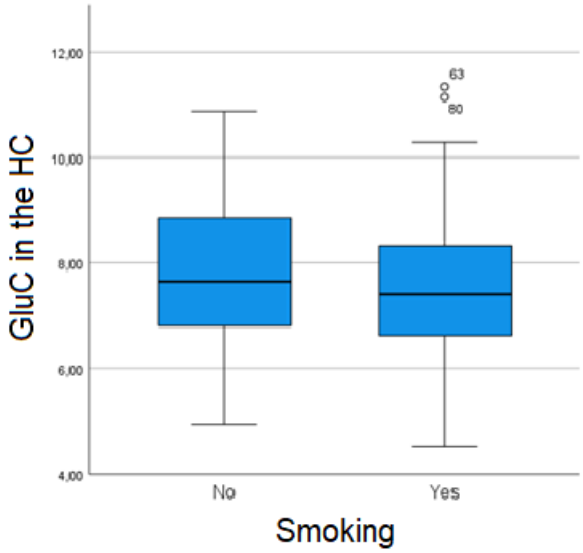

Figure 11: Visual differences concerning corrected Glutamate concentration in the Hippocampus in mmol/l in non-smoking and smoking subgroup; using boxplot.

4.5 Correlations of the Glutamate Concentration with Drinking Behaviour and Clinical Testing

4.5.1 Correlation between Time of Abstinence and Glutamate Concentration

For the determination of a correlation between the time of abstinence and the GluC in the two measured regions, a Spearman's Rho correlation was calculated regarding the two brain areas as well as the 3 subgroups. Significant correlations between the meanAb and GluC could not be found in any of the subgroups, nor the overall group (see Table 15).

Table 15: Correlation between the abstinence time and the corrected glutamate concentration in the ACC and HC

		GluC ACC in mmol/l				**GluC HC in mmol/l**			
		total	**LR**	**HR**	**AUD-P**	**total**	**LR**	**HR**	**AUD-P**
Abstinence time in days	r.	0.117	0.066	0.087	-0.208	-0.089	-0.349	0.113	-0.027
	Sig. (2-tailed)	0.302	0.778	0.625	0.318	0.498	0.169	0.590	0.916
	n	80	21	34	25	60	17	25	18

r.= Spearman's Rho correlation, *Correlation is significant at the 0.05 level (2-tailed).
Significant = Sig., corrected glutamate concentration in the ACC = GluC ACC, corrected glutamate concentration in the HC = GluC HC, Low Risk = LR, High Risk = HR, alcohol use disorder patient = AUD-P, LR+HR+AUD-P = total.

4.5.2 Correlation between Amount of Drinking and Glutamate Concentration

Additionally, a Spearman's Rho correlation was created for the calculation of correlation between the GluC in the HC and ACC with AvOcc (see Table 16) and the AvDay (see Table 17) A significant correlation could not be found in any of the groups.

Table 16: Correlation between average grams of pure alcohol that were ingested during each drinking episode and the corrected glutamate concentration in the ACC and HC

		GluC ACC in mmol/l				**GluC HC in mmol/l**			
		total	**LR**	**HR**	**AUD-P**	**total**	**LR**	**HR**	**AUD-P**
AvOcc in gram	r.	0,172	0,231	0,193	0,052	0,05	-0,026	0,157	-0,198
	Sig. (2-tailed)	0,104	0,219	0,268	0,803	0,682	0,9	0,444	0,431
	n	90	30	35	25	69	25	26	18

r.= Spearman's Rho correlation, *Correlation is significant at the 0.05 level (2-tailed).
Average alcohol drunk per occasion = AvOcc, Significant = Sig., corrected glutamate concentration in the ACC = GluC ACC, corrected glutamate concentration in the HC = GluC HC, Low Risk = LR, High Risk = HR, alcohol use disorder patient = AUD-P, LR+HR+AUD-P = total.

Table 17: Correlation for connections between the average amount of drinking during each day and the corrected glutamate concentration in the ACC and HC

		GluC ACC in mmol/l				**GluC HC in mmol/l**			
		total	**LR**	**HR**	**AUD-P**	**total**	**LR**	**HR**	**AUD-P**
AvDay in gram	r.	-0.014	0.120	-0.001	-0.149	-0.039	0.055	-0.025	-0,198
	Sig. (2-tailed)	0.898	0.528	0.996	0.476	0.751	0.795	0.904	0,431
	n	90	30	35	25	69	25	26	18

r.= Spearman's Rho correlation, *Correlation is significant at the 0.05 level (2-tailed).
Average alcohol drunk per day = AvDay, Significant = Sig., corrected glutamate concentration in the ACC = GluC ACC, corrected glutamate concentration in the HC = GluC HC, Low Risk = LR, High Risk = HR, alcohol use disorder patient = AUD-P, LR+HR+AUD-P = total.

5 DISCUSSION

In the following chapter, the results will be discussed.

5.1 Glutamate Concentration in Group Comparison

Alcohol consumption in large amounts is assumed to cause glutamate dysregulation during withdrawal and early abstinence, especially in the MLP. These changes are thought to be due to neuro-adaptive changes (Airagnes et al., 2019).

In the direct comparison of the GluC in the ACC of the three groups, 93 participants were included (AUD-P N=26, HR N=36, LR N=31). While the mean GluC in the P group was the highest, followed by LR and HR, the difference between the groups was not statistically significant.

Furthermore, it is of interest that the pure sequence of the concentrations in the groups of GluC in the HC differed from its equivalent in the ACC. In the HC, the HR participants showed the highest GluC, followed by P and LR. Again, the differences between the groups could not be shown to be significant. Therefore, the first and second hypotheses must be discarded.

It should be mentioned that the GluC measurements in the HC and ACC showed different results. The results are affected by voxel-size, different infiltration depths, as well as the resulting deflection of the radio-magnetic radiation. While this results in a general concentration difference, it does not imply a different ranking of the groups. As the differences between the groups are marginal, one could argue that these differences are purely objective and therefore have no meaning. However, it could be of interest to review if alcohol-related changes of the GluC differ in distinct locations in the brain.

5.1.1 Group Comparison of Glutamate Concentration in the Anterior Cingulate Cortex

As described above, no significant differences between the three tested groups were found. Nevertheless, a GluC change after alcohol consumption had already been confirmed by other studies. The cross-section study by Lee including 13 P and 18 controls was one of the first human studies dealing with the GluC in the ACC of detoxicated AUD patients (Lee et al., 2007). It could not find any significant differences between the absolute GluC in the ACC of either the P or the controls. As the P group was abstinent for 15.5 days on average, the authors postulated that the GluC had already decreased, compared to a possible higher GluC during withdrawal and early abstinence. In our study, the participants also feature a high variation in the duration of the

abstinence time days and a long mean abstinence time. For the LR and HR, no valid abstinence time exists.

Further limitations of the study by Lee et al. are that the participants took benzodiazepine until 48 h before the MRS and that they did not control for the impacts of smoking on the GluC, though smoking is considered to influence glutamate. In our study, the influence of smoking was assessed and participants taking other drugs or medications that interfere with the neurotransmitter system were excluded. Even though we could not show an association between smoking and the GluC, other publications suggest that there is a correlation, albeit the results of the studies are not concordant. A study by Barbara J. Mason et al. (2006) included 12 AUD patients and 12 controls, showing that smoking increased GluC in AUD patients. This study, however, could not show any significant difference between the GluC of AUD patients and controls, neither at one week nor at one month after the beginning of their withdrawal. Also concerning smoking, a study by Lallemand et al. (2006) showed opposing results in rats. Here, nicotine consumption decreased the GluC in addicted rodents during withdrawal (Lallemand et al., 2006).

A major difference between our work and other related studies is the inclusion of an HR group. In most studies, one group of detoxicated AUD patients and a healthy control group are compared. The HR group included in this study shows the symptoms of risky drinking behaviour but does meet all the criteria for AUD. An HR group can be of use as a reference group to study neurotransmitter concentrations of subjects who are not yet dependent but drink alcohol in the same concerning amounts. Some of the HR participants are at increased risk of developing a dependency, while others are less vulnerable to developing an addiction. The examination of HR subjects can be used to find explanations of neurotransmitter constellations that simplify or prevent the emergence of addiction. Another important difference between HR and P is that the HR as well as the LR were not asked to stop drinking for the time of testing, though they had to be sober for the examinations. Thus, it can be assumed that – within the HR group – the intervals between the last alcoholic drink and the MRS testing are shorter.

Another study in which heavily drinking participants were not asked to stop drinking before the testing - similarly to our HR group - was published by Yeo et al. in 2013. The study included 279 participants aged between 19 and 55 years. 213 of the participants were diagnosed with an AUD and reported at least 5 or more binge drinking sessions in the last month, and 66 participants were used as controls.

Before the 3T MRS, the participants self-reported a mean abstinence time of 2.66 days (SD=2.85) and the blood alcohol was zero. The study demonstrated higher brain metabolite

concentrations, including glutamate and glutamine, for the heavily drinking participants, when compared to the controls. The results of this study - with the short and uniform abstinence times of the participants - support our assumption that the long abstinence times of our subjects affected our results considerably. Unfortunately, this review concentrated especially on the GABA concentrations and only mentioned glutamate incidentally.

Additionally, a publication by Hermann et al. (2012) demonstrated the suspected increase of GluC during early withdrawal. In his study, including 47 AUD patients and 57 healthy controls, all AUD-P were tested on two dates: the first MRS scan took place one day after the last alcoholic drink and a second test was carried out on day 14 after the withdrawal. All the participants were scanned in a 3T MRS. Furthermore, their research tries to compare the results directly to an animal model. Therefore, 8 out of 16 male rats were exposed to ethanol vapour to induce dependency. The rats were also scanned at one day and at fourteen days after withdrawal in a 9.4T MRS. Hermann et al. showed a significantly increased glutamate level at the first point of measuring in both rats and humans. The glutamate – and also the alteration of other neurotransmitters including noradrenaline, choline, and creatine – normalized by the second measurement. Compared to our results, this again highlights the importance of the abstinence time.

A downregulation of GluC was suggested by Ende et al. (2013) and Mon et al (2012). Mon et al. (2012) carried out a longitudinal study with 45 AUD patients. Here, a significant lower concentration of the metabolites (glutamate, noradrenaline, choline) was detected at the first MRS testing and increased significantly between one and five weeks of abstinence.

Ende et al. (2013) supported these results in their publication. The participants were divided into heavy drinkers that experienced a loss of control over their drinking routine and participants who could control their drinking behaviour. The study found significantly lower glutamate levels in drinkers (both heavy and light drinkers) reporting a loss of control than in individuals that kept control over their drinking behaviour. It is of interest – even though the amounts of alcohol consumed in the groups were similar – that there was a difference in the GluC between the heavy-drinking participants who fulfilled the criteria for AUD and those who did not fulfil the DSM-V criteria: AUD patients showed significantly lower glutamate levels in the WM than “non-dependent” heavy drinkers. Therefore, the authors concluded that a decreasing GluC in the frontal WM may indicate the shift from non-dependent heavy drinking to dependence.

Consequently, as in this study, not all the participants fulfilled the criteria for addiction, and it could be assumed that the neurological correlation of losing control is marked by a temporary

down-regulation of glutamate. Furthermore, neurological remodelling (loss of control in drinking behaviour) could be seen as one of the first signs of addiction.

For the application of these results to our HR group, it would be of interest to evaluate the drinking habits of the HR groups more closely for already experienced losses of control. However, all our values were not significant. Our results, as well as the partly inconstant results of the studies described above, illustrate how complex the changes in neurotransmitters in the brain are, and that even small changes in the conditions can lead to decisive changes in the results.

5.1.2 Group Comparison of Glutamate Concentration in the Hippocampus

In the HC, the HR group had the highest GluC, followed by P and LR. The differences between the groups were again not statistically significant in the HC.

The measurements of the metabolites in the HC are prone to error, as due to its location the coupling effect (compare Chapter 5.4) is reinforced. The recordings of the magnetic shifts are more vulnerable as the distance to the recording coils, and therefore the resulting deflection of electromagnetic radiation by the tissue surrounding the HC, is greater. This results in a greater measurement inaccuracy, which leads to greater error susceptibility of the results. These challenging conditions for measurement might be the reason why, to the best of our knowledge, this study is the first to investigate the correlation between alcohol and in vivo GluC changes in the HC in humans.

Since neurotransmitter changes in the HC might reinforce the development of the pathological learning process of an addiction, changes of the excitatory glutamate-mediated neurotransmission in the HC may hold a key role in the mechanisms of the synaptic memory, by affecting the LTP and depressions (Martin et al., 2000).

As there are no comparable human studies, animal-based models, as well as models using different measuring techniques will be used. It has to be taken into account, that the methods can greatly change the results and therefore the results cannot be directly compared.

In an animal and human-based model using MRS, Frischknecht et al. supported our first hypothesis by showing that the neurotransmitter-related dynamics from the ventral HC to the NAcc can be altered by alcohol. During acute in vitro alcohol exposure, neuronal plasticity between the ventral HC and the NAcc was inhibited. However, this study also showed an increased signal transmission after the stopping of acute alcohol consumption (Frischknecht et al., 2017). Another publication supporting a general alcohol-related glutamate increase in mice directly after alcohol intake using microdialysis was published by Chefer et al. in 2011. It should be noted that

a direct comparison of values obtained by microdialysis and MRS is not possible. In contrast to our research, Frischknecht and Chefer et al. examined the ultimate effects of alcohol.

The effects of alcohol are complex, as they depend on other factors like animal species, age, dose and the frequency of alcohol consumption (Zorumski et al., 2014).

5.2 Abstinence Time and Glutamate Concentration

Previous MRS based research indicated a time-dependent change of the GluC (Dahchour & De Witte, 2003; Ding et al., 2012; Zahr et al., 2016). Therefore, the third hypothesis suggested a correlation between the GluC and the meanAb. Based on the Spearman's Rho correlation between the meanAb of the GluC, no significant correlation could be found either in the ACC or the HC, or when considering the subgroups individually. Hence, the third hypothesis has to be rejected.

A major challenge of this study is that the exact time between the last alcoholic drink and the MRS testing was only recorded for P and no exact information can be given on time-dependent glutamate changes in HR and L. For the other groups, the mean time between each drinking session was used as an approximation for the abstinence time. This estimation of the mean abstinence time must be treated with great caution.

Furthermore, the mean abstinence time in AUD-P was very long with an average of 36.92 days and it showed a high SD of 20.11 days. The minimum abstinence time recorded in the AUD-P group amounted to 10 days, whereas the maximum abstinence time amounted to 95 days. Unfortunately, if all the participants with an abstinence period over 21 days were excluded, only six AUD-P participants would remain. Thus, the initial target of a maximum abstinence period of 21 days was generously exceeded. Since the abstinence period in the AUD-P varied greatly (between 10 and 95 days) and the AUD-P had a total of 25 participants, the meanAb can only be used very imperfectly due to the large deviations. Therefore, it is possible that – regarding the particular measurement values – the results were not traceable because of the small sample sizes.

For the comparison to other publications focusing on time-dependent GluC during withdrawal, the publications will be sorted chronologically by the time between the last alcoholic drink and the MRS investigations.

In three publications, the MRS screening was carried out *24 h after* the last alcoholic drink. Ende et al. found decreased GluC in the WM of the frontal cortex in 2013. Contradicting these results, in 2012 Hermann et al. found elevated glutamate levels in the ACC of 47 AUD-P, when compared to the 57 healthy controls. Another MRS study by Yeo et al. with 213 participants presented higher glutamate and glutamine levels in the ACC of AUD patients compared to the control group.

The main difference between these publications was the location where the spectrum was taken. Whereas Ende et al. (2013) assessed the frontal WM of the left hemisphere, Hermann et al. (2012) and Yeo et al. (2013) tested the ACC. The results in these studies differ, which strengthens the hypothesis that glutamate levels respond differently to alcohol in certain areas of the brain.

For the period of *two to five days* after drinking the last alcoholic drink, a study by Prisciandaro et al. (2016) could not find a significant difference between non-treatment seeking participants with an AUD and the control group. It even found lower GluC in the ACC of AUD patients compared to the control group, though the differences were again not significant in this study. For the 2–5-day period, no other investigations were found, presumably because the study participants suffer the strongest withdrawal symptoms at this stage. Regrettably, this is also the period of major dysregulation. Again, no studies were found for a period of *one week* after the last alcoholic drink, probably for the same reasons.

It can be suspected that the formerly increased GluC reached a normal level compared to the control group *in week two* after withdrawal. A GluC reduction to normal levels was also shown in an MRS study including 20 AUD-P, measured 19.6 days after starting the withdrawal (Zahr et al., 2016).

Mon et al. (2012) found no difference between the healthy control group and the AUD-P in the ACC (n = 34, +/-4 days after withdrawal). Notably, the studies led to different results. It can be assumed that the large time difference in the abstinence time between the two studies also influenced the results. In all the referenced studies, the period between the withdrawal and the MRS measurement is shorter than in our research.

Most of the referenced studies as well as our investigation included two or fewer MRS measurements. Therefore, it is difficult to reconstruct the time-dependent progression of the GluC after the withdrawal. There was also no follow-up MRS carried out in P5.

5.3 Amount of Alcohol Drunk and Glutamate Concentration

The fourth hypothesis presumed that participants drinking more alcohol show a higher GluC during withdrawal. The non-parametric data analysis using Spearman's Rho tests could not find any significant correlation between the drinking behaviour of the three groups, whether for AvOcc or for AvDay. Therefore, the fourth hypothesis can also be discarded.

Nevertheless, several studies indicate a dose-dependent change of the glutamate level. In one publication featuring rats injected with different doses of ethanol, the authors showed decreased levels of glutamate after high doses of alcohol (2.0 g/kg), whereas lower doses (0.5 g/kg) led to an increase of the GluC in the posterior VTA (Ding et al., 2012b). However, in contrast to our

examination, Ding et al. mostly investigated the short-time effects of alcohol as the measurements were taken directly after a singular alcohol dose. Furthermore, they used an animal model and microdialysis as a means for GluC determination.

In the publication by Prisciandaro et al. (2016), non-severe and non-treatment seeking AUD participants were tested one to five days after their last drink. Similarly to our research, the assessment was carried out with an H-MRS scan and in the ACC. Prisciandaro et al. found a negative correlation between the number of heavy drinking days within the last 2 weeks and the GluC. Likewise, negative associations between the basal glutamate level in the ACC and the number of drinking days in the past two weeks were found by Cheng et al. (2018). They did not find significant differences between the baseline GluC of AUD and the control group, which is explained by the short time their AUD patients suffered from their dependence (onset of AUD 3 years prior to the testing). Considering these other studies, a relation between the amount of alcohol consumed and the concentration of glutamate is likely.

Regarding the research by Cheng et al. (2018), one could conclude that only the quantity of alcohol leads to a change in the GluC. However, Ende et al. (2013) refuted this theory suggesting that a connection exists between the change of GluC and the control over alcohol consumption, rather than between the GluC and the amount of drinking. They further assumed that a change in the glutamate level is an indicator of the beginning of an addiction. Taking these findings into consideration, it is worth mentioning that a loss of control over one's drinking behaviour – associated with a change in GluC according to Ende et al. – can frequently lead to an increase in alcohol consumption.

The studies could not produce consistent results, thus illustrating the complexity of neurotransmitter changes. Changes in GluC react – besides their reaction to alcohol consumption – to other variables like smoking, age, and abstinence time. Although we found no evidence in our study that these variables influence GluC, it is possible that important influencing factors have been overlooked.

5.4 Involved Factors and Limitations

When considering the conduct of the study, difficulties must be acknowledged. Firstly, this is a cohort study; it is possible the groups are not differentiated enough, and consequently, possible differences are not clearly discernible. In particular, the HR drinkers could be examined again to rule out uncertainties concerning the selection criteria and therefore overlaps with P or LR drinkers. Furthermore, our groups are rather small. This leads to less test strength and therefore differences or correlations could be overlooked.

Secondly, GluC determination using MRS continues to show weaknesses. One is the coupling effect: each neurotransmitter - such as glutamate, glutamine or GABA - in the brain evokes its own resonances on the spectrum. As depicted in Figure 5, Glutamate is closely linked to glutamine and GABA via the glutamate/GABA glutamine cycle (Bak et al., 2006). In the MRS, the peaks of similar composited molecules like glutamate, glutamine and GABA lay close together, which makes the demarcation of the peaks difficult. This is why the corresponding spectrum of the peaks is challenging and summarised as a single value in many publications (referred to as Glx). Furthermore, other macromolecules can also cause resonances (Hillmer et al., 2015). The Glx metabolites fall into a spectrum of the MRS which has many interfering molecules. This strongly increases the measurement uncertainty, due to an even perturbation of the spectrum (Thoma et al., 2011). This implies that the determination of neurotransmitter concentrations in deeper brain regions such as the HC is more prone to errors.

Third, the MRS does not provide insights into the cellular mechanisms, as intracellular and extracellular glutamate cannot be distinguished. The MRS detects the concentration of a metabolite in a single region at a given instant of time. The values for intra- and extracellular GluC are calculated based on comparative values.

Forth, regarding the drinking calendar (on the day of the first clinical test, the participants were asked to fill out a drinking calendar for the last half year), it can be assumed that the subject's statements in the drinking calendar are inaccurate since the calendar is retrospective and covers a long period. Fifth, data interpretation is hampered by the lack of information about meanAB for HR and LR as well as the violation of the length of the abstinence period for AUD-P (meanAb mean: 36.96 days), as previously discussed.

Fifth, regarding the statistical evaluation, our variables were not normally distributed. Non-parametric procedures were therefore used for the calculation. The disadvantage of non-parametric methods is that they can only cover simple analyses (mostly univariate and single factorial). In addition, the power of the tests (or test strength) is lower compared to parametric methods.

Finally, the criticism can be made that there are significantly lower numbers of women in all groups (LR n = 3, HR n = 2, AUD n = 3). Also, no gender-typical differences were applied in the division of the groups into LR and HR. However, the proportion of women in all groups is low and there are no significant differences in group composition, which is why we decided to use the same criteria for men and women in this study.

5.5 Summary and Perspectives

There are considerable indications that alcohol and glutamate dysregulation are related. Furthermore, it can be assumed that glutamate dysregulation is time dependent. The evaluation of this study could however, neither verify significant differences between the GluC of the groups, nor any relationships between the time-dependent or quantitative drinking behaviour and GluC. One of the reasons could be the lack of a valid abstinence time for HR and LR. Other research suggests that alcohol-induced glutamate changes are highly dependent on time. It could be concluded that in this study, the long periods of abstinence for AUD-P as well as the lack of an abstinence time for HR and LR contributed to the absence of significant results. Thus, the lack of significant results can also be seen as an indication of the impact of the meanAb. Therefore, in future projects, it would be of interest to more closely examine the role of time-dependent changes of neurotransmitters, especially GluC, and to therefore to define the meanAb limits more strictly.

One strength of this study was the large number of subjects and large amount of data collected; thus, other less time-dependent neurotransmitters could be assessed.

Furthermore, in particular, the HR participants contributed important insights to a target group that to date has rarely been examined. The HR group could be of particular interest as its members exhibit an elevated risk of developing an AUD. In future studies, possible vulnerability factors could be recognised by including the examination of HR groups. It might even be possible to draw conclusions about the influence of GluC on this vulnerability.

Additionally, the different sequence of GluC in the HR and ACC could indicate different reactions of different brain regions to alcohol. It could be of interest to identify brain regions that are particularly vulnerable to neurotransmitter dysregulations. Therefore, by comparing the extent of neurotransmitter dysregulation, regions that play a key role for the development of craving and relapse may be identified. For the investigation of distinct neurotransmitter changes at different sites, the examination of different loci of the brain would be of interest.

Furthermore, to illustrate a time-dependent GluC change in AUD patients after alcohol intake, a longitudinal study with AUD patients, as well as a control group with multiple measurements over a period of 1-2 weeks after the withdrawal could be of interest. If, in addition, the tests were carried out at different locations in the brain, this would provide more reliable information on time-dependent changes of the GluC after the start of withdrawal as well as information on regional specific GluC changes.

It remains of great importance to better understand the neuronal changes that promote alcoholism. So far, it has been shown that alcohol alters the synaptic transmission of glutamate

and thus provokes a permanent synaptic change. For alcoholism, some of the mechanisms of neuronal dysfunction are already understood, yet further research and understanding of these illnesses might also offer possibilities for the prevention and treatment of an AUD.

For the development of effective pharmacological drugs that improve and complement the existing treatment, further investigations are needed. on the glutamatergic receptors that offer a wide range for modulation.

The goal of the research should always be to better understand a dysfunctional system. This allows the identifying of solutions for the reduction and prevention of existing dysfunctions, improving existing treatments and therefore reducing the impact of the disease on public health.

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7 APPENDIX

7.1 Eidesstattliche Versicherung

„Ich, Samira Klein , versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Die Bedeutung des Neurotransmitters Glutamat für die Entwicklung und Aufrechterhaltung der Alkoholkonsumstörung (The Role of the Neurotransmitter Glutamate for the Development and Maintenance of Alcohol Use Disorder)“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

7.2 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht

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