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OLLI KÄRKKÄINEN

POST-MORTEM BRAINS OF ALCOHOLICS

Changes in the Glutamatergic, Serotonergic, Endocannabinoid and Neuroactive Steroid Systems

KÄRKKÄINEN OLLI

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ABSTRACT

Alcohol (ethanol) consumption is one of the leading risk factors for many serious diseases. The pharmacology of ethanol is extremely complex, affecting many of the signaling systems not only in the brain but widely throughout the body. Alcoholics are a heterogeneous group of subjects suffering a wide spectrum of problems. Cloninger's typology divides the spectrum of alcoholics into two subgroups: anxiety-prone late onset type 1 alcoholics and early onset, impulsive and antisocial type 2 alcoholics. Here the post-mortem brain samples of Cloninger type 1 (N=9) and type 2 (N=8) alcoholics have been studied and compared to non-alcoholic controls (N=10).

The first sub-study evaluated [^{3}H]AMPA binding to AMPA receptors. Increased binding was observed in the anterior cingulate cortex of type 2 alcoholics in comparison with controls. This elevated [^{3}H]AMPA binding could be associated with increased impulsivity in these individuals.

The second study investigated the endocannabinoid levels in the post-mortem samples of hippocampus and amygdala. Increased docosahexaenoylethanolamide levels were observed in late-onset type 1 alcoholics in the amygdala. Furthermore, a negative correlation was observed between anandamide levels and previously published metabotropic glutamate receptor 1/5 levels in the hippocampus in type 1 alcoholics, but not in controls or type 2 alcoholics. These observations could be associated with the transient receptor potential vanilloid type 1 mediated synaptic plasticity which is dependent on metabotropic glutamate receptor 5 and anandamide function.

In the third study, [^{3}H]citalopram binding to serotonin transporters was measured in brain regions associated with social cognition. Decreased [^{3}H]citalopram binding was observed in the posterior cingulate cortex and posterior insula in all alcoholics when compared to non-alcoholic controls. Furthermore, decreased [^{3}H]citalopram binding in the parahippocampal gyrus was seen only in the antisocial type 2 alcoholics. The decreased serotonin transporter binding in alcoholics could be associated with altered social cognitive processes.

The fourth study examined levels of neuroactive steroids in the post-mortem brain samples. Increased dehydroepiandrosterone levels were seen in all alcoholics compared to controls. There were also negative correlations detected between pregnenolone levels and the previously published [^{3}H]naloxone binding to μ-opioid receptors and similarly, increased pregnenolone levels were observed only in a sub-group of alcoholics with decreased [^{3}H]naloxone binding in comparison with the controls.

Overall, the findings of the present thesis improve our understanding of the differences between the brains of alcoholics and controls. Furthermore, they highlight the need to recognize the spectrum of alcoholics in research which hopefully will be translated into improvements in the treatment of alcoholism.

National Library of Medicine Classification: QV 84, QZ 59, WL 104, WL 300, WL 348, WM 274
Medical Subject Headings: Alcoholism/pathology; Alcoholics; Brain/pathology; Amygdala; Hippocampus; Receptors, AMPA; Endocannabinoids; Serotonin; Cognition; Steroids; Dehydroepiandrosterone; Pregnenolone

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TIIVISTELMÄ

Alkoholin (etanoli) käyttö on riskitekijä moniin vakaviin sairauksiin. Alkoholin farmakologia on monimutkaista ja etanoli vaikuttaakin moniin aivojen viestinvälitysjärjestelmiin. Alkoholistit ovat heterogeeninen joukko, joilla on paljon ongelmia. Cloningerin typologiassa alkoholistit jaetaan kahteen alaluokkaan. Cloningerin tyypin 1 alkoholisteilla alkoholismi alkaa myöhäisellä iällä ja he ovat taipuvaisia ahdistuneisuuteen Epäsosiaalisilla ja impulsiivisilla tyypin 2 alkoholisteilla alkoholismi puhkeaa jo nuorella iällä. Tässä väitöskirjassa tutkittiin tyypin 1 (N=9) ja tyypin 2 (N=8) alkoholistien post-mortem aivokudosnäytteitä verrattuna kontrollien näytteisiin (N=10).

Ensimmäisessä osatutkimuksessa mitattiin [^{3}H]AMPA:n sitoutumista AMPA-reseptoreihin. Tyypin 2 alkoholisteilla havaittiin korkeampi [^{3}H]AMPA sitoutuminen anteriorisessa pihtipoimussa kontrolleihin verrattuna. Tämä muutos saattaa liittyä heidän impulsiiviseen luonteeseensa.

Toisessa osatutkimuksessa mitattiin endokannabinoiditasoja amygdala ja hippokampus alueiden aivonäytteistä. Tyypin 1 alkoholisteilla havaittiin kohonneet docosahexaoneylamide-tasot verrattuna kontrolleihin. Tyypin 1 alkoholistien hippokampuksessa havaittiin myös negatiivinen korrelaatio anandamidikonsentraatioiden ja metabotropisten glutamaattireseptorien 1/5 – tasojen välillä. Nämä tulokset voivat liittyä muuntuneeseen endocannabinoidijärjestelmän toimintaan tyypin 1 alkoholisteilla.

Kolmannessa osajulkaisussa tutkittiin [^{3}H]sitalopraamin sitoutumista serotoniinitransporttereihin. Kontrolleihin verrattuna alhaista [^{3}H]sitalopraami sitoutumista havaittiin posteriorisessa pihtipoimussa ja posteriorisessa insulassa kaikkilla alkoholisteilla, sekä parahippokampaalisessa poimussa tyypin 2 alkoholisteilla. Alhainen serotoniinitransportteriin sitoutuminen posteriorisilla aivoalueilla voi liittyä epänormaalisti toimiviin sosiaalisiin prosesseihin alkoholisteilla.

Neljännessä osatutkimuksessa mitattiin neuroaktiivisten steroidien määrää aivonäytteissä. Dehydroepiandrosteronitasot olivat kohonneet alkoholistien näytteissä verrattuna kontrolleihin. Tutkimuksessa havaittiin myös negatiivinen korrelaatio pregnenolonitasojen ja aikaisemmin julkaistujen μ-opioidireseptoriin -sitoutumistulosten välillä. Kontrolleihin verrattuna pregnenolonitasot olivat koholla vain alkoholisteilla, joilla μ-opioidisitoutuminen oli laskenut.

Kokonaisuudessaan tutkimuksen tulokset lisäävät ymmärrystämme muutoksista alkoholistien aivoissa. Tulokset myös korostavat tarvetta huomioda alkoholistien heterogeenisyys niin tutkimuksessa kuin hoidossakin.

Luokitus: QV 84, QZ 59, WL 104, WL 300, WL 348, WM 274
Yleinen suomalainen asiasanasto: alkoholi; alkoholinkäyttö; alkoholistit; aivot; patologia; hippokampus; endokannabinoidit; serotoniini; kognitio; steroidit

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Olli Kärkkäinen

List of the original publications

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II Kärkkäinen O, Lehtonen M, Laukkanen V, Tupala E, Hyytiä P, Kautiainen H, Tiihonen J, Callaway J C, Storvik M. Endogenous cannabinoids in amygdala and hippocampus in post-mortem brains of Cloninger type 1 and 2 alcoholics. *Alcohol 47 (5):399-403, 2013.*

III Kärkkäinen O, Laukkanen V, Haukijärvi T, Kautiainen H, Tiihonen J, Storvik M. Lower [^{3}H]citalopram binding in brain areas related to social cognition in alcoholics. *Alcohol and Alcoholism, 50 (1):46-50, 2015.*

IV Kärkkäinen O, Häkkinen M, Auriola S, Tiihonen J, Storvik M. Increased steroid hormone dehydroepiandrosterone and pregnenolone levels in post-mortem brain samples of alcoholics. *Submitted.*

Contents

Abbreviations

2-AG	2-arachidonoyl glycerol
3α/β-HSD	3α/β-hydroxysteroid dehydrogenase
5-HT	5-hydroxytryptamine, serotonin
5-HTTLPR	5-HT transporter-linked promoter region
AAS	anabolic-androgenic steroids
ACC	anterior cingulate cortex
ACTH	adrenocorticotropic hormone
ADH	alcohol dehydrogenase
ADHD	attention deficit hyperactivity disorder
AEA	N-arachidonoylethanolamine, anandamide
ALDH	aldehyde dehydrogenase
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMY	amygdala
AR	androgen receptor
BAC	blood alcohol concentration
CA	cornus ammonis
cAMP	cyclic adenosine monophosphate
CB	cannabinoid
CBD	cannabidiol
CeA	central nucleus of amygdala
CI	confidence interval
CNS	central nervous system
CPP	conditioned place preference
CREB	cAMP response element-binding protein
CRH	corticotropin-releasing hormone
D	dopamine
DAGL	diacylglycerol lipase
DAT	dopamine transporter
DHA	docosahexaenoic acid
DOR	δ-opioid receptor
E2	estradiol
EAAT1	astrocyte glutamate transporter
EGF	epidermal growth factor
ER	estrogen receptors
ERK	extracellular signal-regulated kinase
ESI	electrospray ionization
FAAAs	fatty acid amides of amino acids
FAAH	fatty acid amide hydrolase
FC	frontal cortex
FGF	fibroblast growth factor
fMRI	functional magnetic resonance imaging
FSH	follicle-stimulating hormone
GABA	gamma-aminobutyric acid

GHB	sodium oxybate, sodium salt of γ-hydroxybutyric acid
GluA	AMPA receptor subunit
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
Gq-mER	membrane estrogen receptor
GR	glucocorticoid steroid receptor
HPA	hypothalamic-pituitary-adrenal
KOR	κ-opioid recept
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LH	luteinizing hormone
LTD	long-term depression
LTP	long-term potentiation
MAGL	monoacylglycerol lipase
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
mGluR	metabotropic glutamate receptor
MOR	μ-opioid receptor
mRNA	messenger ribonucleic acid
NAC	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NRD	N-arginine dibasic convertase
P4	progesterone
PCC	posterior cingulate cortex
PEA	n-palmitoyl ethanolamine
PET	position emission tomography
PFC	prefrontal cortex
PHG	parahippocampal gyrus
PINS	posterior insular cortex
PMI	post-mortem interval
PR	progesterone receptor
SERINC2	serine incorporator 2
SERT	serotonin transporter
SN	substantia nigra
SPECT	single-photon emission computed tomography
SSRI	selective serotonin transporter inhibitor
StAR	steroidogenic acute regulatory protein
T	testosterone
TFAP2B	transcription factor AP2
TH	tyrosine hydroxylase
THC	Δ9-tetrahydrocannabinol
TRVP1	vanilloid receptor 1
WHO	World Health Organization
VTA	ventral tegmental area

1 Introduction

Alcohol (ethanol) is one of the oldest psychoactive substances used by humans to alter consciousness. In addition to the desired properties, alcohol also exerts many negative effects and the consumption of alcohol has been claimed to account for 5.1% of the total global disease burden (Lim et al., 2012). Compared to other substances of abuse, alcohol causes approximately a similar disease burden, including morbidity and mortality, than all illicit drugs combined (Degenhardt et al., 2013a; Degenhardt et al., 2013b). On a global scale, only tobacco smoking is a more important cause of the disease burden attributable to substances of abuse (Lim et al., 2012). Alcohol consumption is an especially important cause of impaired health in the working age population and has been associated with 5.9% of all deaths, a greater number than for example who dies from AIDS or violence. (World Health Organization, 2014).

Alcoholism is a commonly used term, which can be defined in different ways. In the present thesis, the term alcoholism is used as an overall term to refer to alcohol dependence and alcohol use disorder which are the diagnoses used in ICD-10 and DSM-5, respectively. The diagnostic criteria for alcohol dependence and alcohol use disorder include medical, social and psychological factors (APA, 2013; WHO, 2005). Furthermore, there is a need to establish limits for heavy use if one wishes to investigate the relationship between alcoholism and damage to human health (Rehm et al., 2013). At the present moment, limits for heavy use vary from country to country. For example, in Finland, one unit equals 12g of ethanol and over 24 alcohol units per week for men and 16 units for women are considered high risk heavy use (Käypä hoito -suositus, 2015). It was estimated that in 2014 almost every twentieth member (4.9%) of world's population (240 million people) suffered from alcohol use disorder (Gowing et al., 2015). Furthermore, alcohol use disorder is more common in males than females (7.8% versus 1.5% of population in past 12 months, respectively). However, these values are larger in European and northern American countries, like Finland and United States of America. In northern Europe, the prevalence of alcohol use disorder in the past 12 months is 9.3% of the whole population but much higher, 14.3%, in males.

The current treatment of alcoholism utilizes several approaches, namely fear of negative consequences and reduction of reinforcement. The now classical drug for treatment of alcoholism is disulfiram. When ethanol is consumed in a patient who has received disulfiram he/she suffers an intense aversive flushing and other symptoms (antabus reaction) and thus the basis of the treatment lies in the fear of experiencing this reaction. The efficacy of disulfiram treatment is highly dependent on the supervision of adherence to the treatment, for this reason non-supervised treatment seem to be of low value (Hughes and Cook, 1997). Other current treatments include naltrexone and nalmefene. These are μ-opioid receptor (MOR) antagonists and their therapeutic value lies in their ability to reduce the reinforcing effects of ethanol (Nutt, 2014). Because the psychological component is essential for the action of disulfiram, the efficacy of disulfiram has been studied in open randomized clinical trials, in which disulfiram has been shown to be more effective than naltrexone in the treatment of alcoholism (Laaksonen et al., 2008; Skinner et al., 2014; Yoshimura et al., 2014). Even though MOR antagonists can reduce alcohol cue induced relapses of heavy drinking, they are not effective in reducing relapses due to stress (Litten et al., 2012). This could in part explain their lack of efficacy for many alcoholics (Kiefer et al., 2005; Kiefer et al., 2008; Rubio et al., 2005). Acamprosate (N-acetyl homotaurine) has also been used in the treatment of alcohol use disorder, however its pharmacological mechanism of action has remained unclear (Holmes et al., 2013; Johnson et al., 2003b; Popp and Lovinger, 2000). Cochrane reviews on naltrexone and acamprosate indicate that approximately only one alcoholic out of nine is helped by these medications (Rosner et al., 2010a; Rosner et al., 2010b). Since there is a clear room for

improvement, novel medications are being developed to treat alcoholism for example, compounds which influence the glutamatergic, serotonergic, endocannabinoid and steroid systems (Litten et al., 2012).

However, alcoholics are not a homogenous group of subjects. Ethanol has a very complex neuropharmacological profile (Vengeliene et al., 2008) and therefore individuals with different genomic and environmental backgrounds are differently affected by chronic consumption of ethanol; they could experience different pathologies and may well have individual reasons to consume alcohol. One of the unmet needs for both treatment and drug development is the ability to recognize the patient groups which could benefit most from different types of pharmacological interventions (Litten et al., 2014). Several distinctive typologies have been devised to subdivide alcoholics into clinically relevant subgroups. One of the simplest and most widely investigated is Cloninger's typology, where alcoholics are divided into type 1 and 2 alcoholics. Type 1 alcoholics have a late-onset of alcoholism and are anxiety prone, whereas type 2 alcoholics are impulsive and antisocial and have an early-onset of alcohol abuse (Cloninger, 1995). In relation to prevalence, it has been estimated that approximately 80% of alcoholics can be considered to belong to the type 1 category. These subgroups of alcoholics seem to exhibit different changes in the CNS compared to non-alcoholic population (Leggio and Addolorato, 2008; Tupala and Tiihonen, 2004).

Our current understanding of the causes of alcoholism relies on a biopsychosocial model of addiction, where all three elements, biology, psychology and social environment, interact to produce addiction type behavior (Engel, 1977). The focus of the present thesis will be on biomedical aspects. However, it is acknowledged that there is a complex interaction between these elements, where the biology affects the psyche and the individual interacts with the social environment which then influences both psyche and biology.

However, in the case of substance use disorders, there is always the exposure to the compound, in this case ethanol. Both behavior and bodily functions are changed by chronic and repeated exposure to ethanol (Koob, 2013). As with any external exposure, how and what type of alterations occur are related to the genetic background and personal history of the exposed individual, and interpretations of the effects of the exposure depend on the psychosocial frame work of the exposure (Ott, 1996). Many theoretical models have been devised to explain the causes behind the development of alcoholism (Hyman et al., 2006; Koob, 2013; Paulus and Stewart, 2014). However, many of the current theoretical models rely heavily on results obtained from animal models which do not fully represent the biological complexity of human addiction, let alone its psychosocial aspects (Koob et al., 2009). Therefore, there is a need to advance our knowledge of biomedical changes in the human alcoholics, especially in the brain which is the organ where the interaction of human biology and psychosocial environment takes place. Understanding this human pathology might enable the development of more personalized treatment of alcoholics and might also improve research models for finding novel treatment options for alcoholism and other addictions (Litten et al., 2014). In order to further this goal, the present study has determined differences in the post-mortem brain samples of Cloninger type 1 and type 2 alcoholics and non-alcoholic controls in levels of AMPA receptors, endocannabinoids, serotonin transporters and ketosteroids.

2 Review of the Literature

2.1 SPECTRUM OF ALCOHOLICS

2.1.1 Theoretical framework for development of alcoholism

Many theories have been proposed to explain the pathology of alcoholism and addiction. I will briefly review some aspects of these theories to provide an overview of the current theoretical framework which is used to explain the pathology from drug exposure to addiction (Everitt and Robbins, 2013; Koob, 2013; Paulus and Stewart, 2014; Robinson and Berridge, 1993). Some of the details will also be discussed in subsequent chapters. The theoretical framework is that the development of addiction occurs via maladaptive changes in the positive and negative reinforcement systems (Hyman et al., 2006; Koob, 2013).

The positive reinforcement learning (reward system) has been long considered to be at the centre of the development of addiction. The key structures in the reinforcement system are the dopaminergic projections from ventral tegmental area (VTA) and substantia nigra (SN) to ventral and dorsal striatum. Recent meta-analyses have identified that the function of many brain regions which are connected to VTA and SN is altered in addiction (Tomasi and Volkow, 2013). In particular, substance use disorders were associated with changes in the properties of prefrontal cortical brain regions, e.g. anterior cingulate cortex (ACC), which exert inhibitory control over behaviour and are involved in decision making. This has been considered to be associated with the loss of control over drug intake. However, also more temporal regions connected to striatum, such as the hippocampus and posterior insula have been linked with addictions (Tomasi and Volkow, 2013). These regions are important for interoception, context and conditioning. However, although significantly associated with drug use, in the meta-analysis, these regions were considered as being more important for obesity and eating disorders, suggesting that they are more involved in eating behaviour or intake, rather than drug use (Tomasi and Volkow, 2013). However, in the case of alcohol, these regions are obviously important factors since alcohol is usually consumed orally. The molecular mechanisms of the positive reinforcement system will be discussed in more detail in chapters 2.3 and 2.4.

Furthermore, also the stress system has been linked with the development of alcoholism and it has been shown that stress can lead to a relapse of alcohol addiction (Koob, 2013; Uhart and Wand, 2009). The function of the stress system is altered by both genetic and environmental factors, and alterations in the function of the stress system may help to explain the individual diversity to the vulnerability to alcoholism. It has been proposed that stress, activation of hypothalamic-pituitary-adrenal (HPA) axis and the consequent release of steroid hormones alters the function of both the brain reinforcement circuits and the CNS stress system (Koob, 2013). There are several definitions for stress, and therefore stress must always be defined to avoid misunderstandings (Chrousos, 2009). In the present thesis, stress is defined as a stimulus, i.e. a stressor, which threatens the homeostasis of the individual. A stressor can be either an internal or external threat or both; it can also be both emotional and physical. When the homeostasis of individual is threatened, for example by alcohol, the effect of stress of the response is an attempt to adapt to the stressor and to return to homeostasis (Uhart and Wand, 2009). The endocannabinoid system is an important modulator of stress responses (see chapter 2.5).

Three adaptive mechanisms which are related to addiction can be recognised: 1) HPA axis is activated by increased release of CRF in the hypothalamus, which leads to release of ACTH from the pituitary, which further leads to release of steroid hormones, 2) noradrenaline and adrenaline are released by the sympathetic nervous system, 3) anxiety and other stress related symptoms are induced by CRF release in brain regions like amygdala and

hippocampus. In addition, stress responses also influence functions that are not directly associated but might be important in the development of alcoholism, for example catabolism, immunosuppression, inhibition of vegetative functions (Chrousos, 2009). Chronic or repeated stress may lead to allostasis of the stress system (McEwen, 1998). Allostasis is defined as a chronically altered state of homeostasis, which is formed when organism cannot return to the normal homeostatic range and the allostatic state is formed so that the organism remains functional if not entirely healthy. In relation to the stress response, this means that the body gradually adapts to the constantly activated HPA axis and the increased levels of stress mediators. Allostasis of the stress system can lead to the development of mood and anxiety disorders which are common comorbidities with the alcoholism.

Therefore, it has been proposed that the development of alcoholism is a process that involves the brain reinforcement and stress systems (Koob, 2013). At first, alcohol is enjoyed for its rewarding properties and the use is characterised by positive reinforcement learning and the impulsive use of alcohol. In the second state, alcohol usage changes from being impulsive to compulsive because of the negative reinforcement (relief from aversion). The basic cycle of alcohol use (anticipation, consumption and withdrawal/abstinence) remains the same but the motivation of use is considered to shift from positive reinforcement to the avoidance of the aversive state caused by alcohol abstinence (negative reinforcement). The negative emotional states are considered to be the main reason for relapse after a period of abstinence and stress is one of the key risk factors triggering a relapse because stress induces both craving and anxiety (Annis et al., 1998; Chrousos, 2009; Fox et al., 2008; Noone et al., 1999).

However, this simple theoretical framework does not clearly address some of the major components of human addiction. The environment, especially the social environment, has a large influence on substance use in humans and animals (Alexander et al., 1978; Alexander et al., 1981; Robins, 1974; Samson and Falk, 1974). The brain aspects of these influences in addiction pathology have been less extensively studied than the role of reinforcement and stress (Paulus and Stewart, 2014; Volkow et al., 2012). It has been argued that for the development of addiction, the pharmacological properties of ethanol are meaningful only in those individuals who are prone to excessive behaviour and only when meaningful behavioural alternatives are limited (Falk, 1983). Therefore, understanding the neural correlates of these predispositions is one approach to elucidating in more detail the pathology of alcoholism.

Furthermore, from the theoretical models, mostly derived from work done in animal models, one could easily come up with idea for a uniform group of alcoholics. However, alcoholism is a complex disease and alcoholics are recognised as being a heterogenic group; this can be seen for example in diagnosis, where alcohol use disorder has many different criteria i.e. medical, psychological and social criteria, and only part of those need to be fulfilled for the diagnosis to be made (APA, 2013). Each patient is considered to develop alcoholism as a result of a complex interaction of underlying genetic and environmental mechanisms influenced by the person's neurobiological makeup and lifetime experiences (Dick and Kendler, 2012). Heterogeneity among alcoholics causes differences for example in age of onset of heavy use of alcohol, rate of alcohol metabolism, drinking patterns (binge vs. continuous) and comorbid illnesses. This heterogenic group has been divided into subgroups using different typology methods for treatment and research (Leggio et al., 2009b). The primary reason for the sub-classification of alcoholics is to help clinical work in targeting the mechanisms behind the alcoholism in these subgroups; in other words to help diagnosis and provide targeted therapy according to these intermediate phenotypes.

2.1.2 Typologies of alcoholism

E.M. Jellinek was one of the most influential investigators in the alcoholism research field. Jellinek influenced the WHO Declaration of 1954 that alcoholism is a disease and a public

health problem. Jellinek divided alcohol use into five categories (Jellinek, 1960). Of these, the gamma and delta subtypes can be considered to resemble the current view of alcohol dependence. Gamma type alcohol users were able to have abstinence periods between periods of alcohol use whereas delta alcohol users would drink alcohol more or less constantly.

This dichotomy in alcoholics is also seen in the Cloninger's typology of alcoholism which is used in the present thesis. Cloninger and colleagues divided alcoholics into two groups according to their different attributes (Cloninger, 1987; Cloninger, 1995). The two main differences are in the time of onset of alcoholism (<25 years in type 2 alcoholics) and behavioural traits. Type 1 alcoholics are anxiety prone and social-conforming whereas type 2 alcoholics are antisocial and impulsive. Cloninger's type 1 alcoholics can be both male and female, whereas type 2 alcoholics are thought to be primarly male. The metric used for division is the tridimensional personality questionnaire. Type 1 alcoholics have high harm-avoidance (cautious, apprehensive, pessimistic, inhibited, shy, and susceptible to fatigue), low novelty-seeking (rigid, reflective, loyal, orderly and attentive to details) and high reward dependency (eager to help others, emotionally dependent, warmly sympathetic, sentimental, sensitive to social cues, and persistent) (Cloninger, 1987; Cloninger, 1995). Opposite characteristics are seen in type 2 alcoholics. However, not all studies have been able to observe such sharp differences in these personality features in subgroups of alcoholics. For example, harm avoidance scores were similar in type 1 and type 2 alcoholics in a Japanese cohort (Yoshino et al., 1994).

Cloninger and colleagues reasoned that the observed psychological features, anxiety, low novelty seeking and social-conformity, in type 1 alcoholics resemble those seen in Parkinson patients (Cloninger, 1987). In line, Cloninger's type 1 alcoholics seem to have diminished dopaminergic neurotransmission in the striatum (Tupala et al., 2000; Tupala et al., 2001a; Tupala and Tiihonen, 2004). Furthermore, the initial hypothesis was that type 2 alcoholics would have a deficiency in serotonergic function (Cloninger, 1987). However, current evidence suggests that both types 1 and 2 alcoholics experience problems in the serotonergic system although there might be more subtle differences between the subgroups (Mantere et al., 2002; Sari et al., 2011; Storvik et al., 2006a; Storvik et al., 2007; Storvik et al., 2009).

In addition to Cloninger's typology, there are several other two cluster models for alcoholism. Babor and colleagues divided alcoholics into two clusters by evaluating the characteristics of alcoholics in 17 different domains e.g. personality, co-morbidity and family history (Babor et al., 1992). These two clusters were named type A and B which resemble Cloninger's types 1 and 2 respectively (Table 1). Schuckit and colleagues showed that five of these domains (consumed amount of alcohol per day, relief from negative affect, social and physical consequences and medical conditions) exhibited the greatest variance between these two clusters and that these attributes could be used in a clinical setting to differentiate between the two groups (Schuckit et al., 1995). The main factor distinguishing between these two models is that Cloninger's typology derives from personality theory whereas Babor's typology is based on a cluster analysis of 17 domains (Leggio et al., 2009b). Furthermore, Babor type B alcoholics can also be women, whereas Cloninger type 2 alcoholics are considered to be primarly men.

Moreover, an even more simplified two group characterisation can be used. Alcoholics can be divided only by time of onset of alcoholism into early-onset alcoholism and late-onset alcoholism (Table 1). It has been suggested that the Cloninger's and Babor's typologies are over-complicated compared to the onset of alcoholism model, since this is the aspect shared by both typologies (Epstein et al., 2002). However, it has also been considered that the two subgroup models are not complex enough to capture the diversity in all alcoholics (Epstein et al., 2002; Leggio et al., 2009b). Therefore more complex typologies have also been proposed. Of these, I will review here the Lesch typology, which is one of the more extensively studied sub-categorizations.

Table 1. Comparison of different two cluster models for typology of alcoholics

Typology	Subtypes	
Cloninger	**Type 1**	**Type2**
	late-onset (>25 years)	early-onset (<25 years)
	childhood environment influences	inherited, no influences from childhood
	both male and female	primarily male
	periods of abstinence	constant drinking
	high harm avoidance	low harm avoidance, antisocial
	alcohol used for self-medication	euphoria seeking
	generally responds to treatment	poor response to treatment
Babor	**Type A**	**Type B**
	late-onset	early-onset
	few childhood risk factors	many childhood risk factors, inherited
	less psychopathology	more psychopathology
	less life stress	more life stress
	usually no prior treatment	history of treatment periods
Onset	**Late-onset alcoholism**	**Early-onset alcoholism**
	late-onset (>25 years)	early-onset (<25 years)

Reference: Leggio et al. 2009b.

The classification proposed by Lesch and colleagues has little in common with the two cluster models. In Lesch's typology, alcoholics are divided into four different subtypes (Lesch et al., 1988; Lesch and Walter, 1996). Lesch's type 1 alcoholics, "Model of Allergy", have severe withdrawal symptoms, frequent treatment periods, family history of alcoholism and alcoholism develops from occasional drinking into alcoholism when a person consumes alcohol to prevent withdrawal symptoms. Lesch's type 2 alcoholics, "Model of Anxiety or Conflict", are characterized by the use of alcohol for self-medication, often with other sedative drugs, and they undergo extensive changes in behaviour while drinking, but no somatic disorders or severe withdrawal symptoms. In Lesch's type 3, "Model of Depression", alcohol is used as an anti-depressant; affective disorder and family history of addiction are in the background of the alcohol problem and behaviour is characterized by self-destruction and periods of abstinence. Finally Lesch's type 4 alcoholics, "Model of Adaptation", have behavioural disorders, high social burden at early age, enuresis nocturnal (bed wetting) and pre-morbid cerebral defects.

Since the typologies of alcoholism are meant to help in clinical work, the obvious way to estimate their usefulness is via clinical studies. The two cluster models have been shown to have some predictive value in clinical treatment. For example, ondansetron and sertraline seem to be more effective in late-onset alcoholic patients (Johnson et al., 2000; Kranzler et al., 2011). Naltrexone on the other hand, has been shown to be more effective in Babor A alcoholics in US, but more effective in Cloninger type 2 in European alcoholics (Bogenschutz et al., 2009; Kiefer et al., 2008). More recently, in a small pilot study with non-treatment seeking alcoholics, late onset alcoholism was seen as a moderator of increased sedative effects of ethanol during self-administration after administration of the $GABA_B$ receptor agonist baclofen (Leggio et al., 2013). However, onset of alcoholism was not a significant moderator of reduction of alcohol consumption by baclofen in that study. Moreover, when sub-divided according to Lesch typology, type 3 and type 4 alcoholics seem to benefit most of naltrexone treatment (Kiefer et al., 2005) whereas Lesch type 1 alcoholics seem to respond well to acamprosate (Kiefer et al., 2005; Lesch et al., 2001). In contrast, the reduction of alcohol consumption by sodium oxybate (sodium salt of γ-hydroxybutyric acid, GHB) treatment did

not differ between Lesch subtypes (Caputo et al., 2014). Overall, at present, there is no clear indication or clinical evidence that any one of these typologies would be superior over the others.

2.1.3 Associations between typologies of alcoholism and theory of development of alcoholism

The theoretical framework of influence of positive and negative reinforcement learning in the development of addiction shows some associations with the Cloninger's typology of alcoholics (Cloninger, 1988; Koob, 2013). In Cloninger's typology, the type 2 alcoholics show euphoria seeking impulsive behaviour (Cloninger, 1988; Koob, 2013). It could be proposed that the driving factor for alcohol use in type 2 alcoholics is positive reinforcement combined with lack of impulse control. Furthermore, this would lead to the hypothesis that the neurobiological mechanisms associated with impulse control and reinforcement learning could also explain the formation of alcohol dependence in type 2 alcoholics. However, this simplification does not take into account the antisocial behaviour in type 2 alcoholics as a predisposing factor.

In contrast, the positive reinforcement system seems to be dysfunctional in type 1 alcoholics (Tupala and Tiihonen, 2004). The dysfunctional reward system of type 1 alcoholics might not induce as robust positive reinforcement learning as the functioning reward system of type 2 alcoholics which could explain why alcoholism develops over a longer period of time in the type 1 alcoholics. Moreover, Cloninger's type 1 alcoholics are anxiety-prone and could therefore be predisposed to the negative reinforcement caused by chronic alcohol intake (Cloninger, 1988; Koob, 2013). This indicates that the neurobiological mechanisms associated with stress modulation could be altered in type 1 alcoholics.

Moreover, recently it has been proposed that reinforcement based subgroups could be used as a basis of personalized treatment (Litten et al., 2015; Mann and Kiefer, 2015). Similar to the speculation presented above, in this model alcoholic patients are assigned to subgroups by assessing the importance of positive and negative reinforcement in their alcohol dependence. Simplified, this means that some of the patients crave the rewarding (positive reinforcement) aspect of alcohol consumption, whereas there is a subgroup of patients that tend to drink to obtain the aversion relieving effects produced by alcohol consumption e.g. relief from anxiety, (Glockner-Rist et al., 2013). Psychological measures like affect-modulated startle responses as well as brain imaging methods have been used to assign patients into these subgroups (Lemenager et al., 2014; Mann et al., 2009; Mann et al., 2014). The division of alcoholics into positive and negative reinforcement subgroups seems to predict treatment responses to anticraving medications such as naltrexone and acamprosate, supporting the use of this typology (Mann et al., 2009; Mann et al., 2013b; Mann et al., 2014).

2.1.4 Subtype models in the age of genetics

At the present time, the use of genetic information to aid personalized medicine is becoming a reality. It is important to remember that phenotypes are usually caused by interactions between the genotype and the environment and this is also the case in alcoholism. Therefore, the importance of genetic variation needs always to be considered in a wider context.

Genome wide association studies have identified several possible genomic modifications associated with alcoholism (Zuo et al., 2014). Only the association between the alcohol dehydrogenase (ADH) gene cluster and alcoholism can however be considered to be robust. Based on a functional analysis, at the moment other valid candidates to be associated with alcoholism include the genes for serine incorporator 2 (SERINC2), uncharacterized protein KIAA0040, nardilysin (N-arginine dibasic convertase, NRD1), and 5-hydroxytryptamine (serotonin) receptor 7 (HTR7) (Wang et al., 2011; Zlojutro et al., 2011; Zuo et al., 2014).

Furthermore, age of onset is a key criteria in most of the above mentioned typologies of alcoholism. Three SNPs have been associated with age of onset of alcoholism: rs2168784 on

chromosome 3, the ADP-ribosylation factor like 15 (ARL15) gene and UTP20 small subunit (UTP20) gene (Kapoor et al., 2014). However, the biological significance of these SNPs is still largely unknown. Typology specific studies have also been conducted. For example, a SNP at position -602 in the 5′ region of the neuropeptide Y (NPY) gene has been associated with Cloninger type 1 alcoholism (Mottagui-Tabar et al., 2005) and TH Val 81 - Met polymorphism in tyrosine hydroxylase gene has been associated with early-onset alcoholism (Dahmen et al., 2005).

In the case of treatment of alcoholism, there are also several candidate gene polymorphisms which could affect the treatment outcome, e.g. polymorphism in the gene 5-HTTLPR which codes for serotonin transporters (SERT). However, even in the case of 5-HTTLPR polymorphism, there still seems to be a benefit in dividing alcoholics into late and early-onset subgroups (Dundon et al., 2004; Kranzler et al., 2012; Pettinati et al., 2000). Late-onset alcoholics with the LL 5-HTTLPR –genotype seem to benefit from the sertraline treatment (Kranzler et al., 2011). In contrast, sertraline treatment actually seems to increase alcohol consumption in the early-onset alcoholics (Dundon et al., 2004; Kranzler et al., 2012; Pettinati et al., 2000). This example shows that testing for simple polymorphism will not be enough to determine the benefits of drug treatment and other factors need to be considered.

2.1.5 Summary about the spectrum of alcoholism

In conclusion, good predictive subtypes could help in personalized treatment of alcoholism. Moreover, the advantages of understanding the pathology of alcoholism can be used to modify or create new typologies which could be beneficial in guiding treatment (Litten et al., 2015). However, sole reliance on genetic variance within alcoholics will miss the important role of environmental factors, e.g. epigenetic alterations and metabolic activity, which clearly influence the treatment outcome. Therefore, even in this era of genomic information, there is still a need to guide personalized medicine by meaningful typologies of alcoholics. There are currently over 30 molecular targets which are being studied as ways to improve the treatment of alcoholics (Litten et al., 2012). By applying a combination of genetic information and subgroup division according to other traits such as the role of positive/negative reinforcement in the individual's alcohol use have been claimed to be the best treatment option for individual alcoholics (Litten et al., 2015).

However, alcoholics are individuals and no typology or genetic test can divide all alcoholics into a cluster such that no unclear cases would be left. Therefore, these typologies as well as the theoretical models should be seen as an abstraction of complex factors, and the subgroups are best viewed as stereotypes of alcoholics rather than a true characterization of these subjects. Nonetheless, typologies do provide tools for better understanding the alcoholics as a heterogeneous group and can therefore be helpful when designing more valid research protocols to study the pathology of alcoholism. The main reason for using typologies of alcoholism in research is to recognise the heterogeneity of the studied population and to take this into account in the study design with the ultimate aim being to assist in the clinical work. Furthermore, it is also necessary to bear in mind the benefits and limitations of designating alcoholics into different categories in the following chapters on neuropharmacology of ethanol. Many of the results originate from animal studies and even many human studies consider alcoholics as a single group.

2.2 EFFECTS OF ETHANOL IN THE BODY

Acute ethanol consumption has different behavioral stages: 1) disinhibition, 2) sedation and 3) withdrawal. Over a longer time perspective, ethanol consumption also has different stages with distinctive contributions from many neural inputs: 1) initiation and 2) maintenance of consumption as well as 3) craving and reinstatement of ethanol consumption (Vengeliene et al., 2008). Ethanol exerts these effects via its complex pharmacological properties and some

of it will be reviewed in the following chapters. In contrast, the pharmacokinetics of ethanol are relatively simple but also crucial for the overall effect.

In the fasted state, ethanol is rapidly absorbed from the gastrointestinal tract. However, during fed state there is some metabolism of ethanol in the stomach by ADH. After absobtion, ethanol undergoes substantial first pass metabolism in the liver (Holford, 1987). Once it has passed through the liver, ethanol is quickly distributed throughout the body, with tissue delivery dependent on the blood rate to the individual organs. Most of ethanol is metabolized and only a small percent is excreted unchanged in urine or breath.

The liver is the main ethanol metabolizing organ and metabolism of ethanol in the liver is the subject of saturating kinetics (figure 1). This is important for ethanol consumption, because the genes best associated with high ethanol consumption code for enzymes involved in ethanol metabolism, e.g. ADH and aldehyde dehydrogenase (ALDH) (Treutlein et al., 2009; Zuo et al., 2014). Slow acting variants can protect from further ethanol consumption, because of an aversive flushing reaction attributable to the accumulation of acetaldehyde after ethanol consumption in these individuals. The rate of ethanol metabolism to acetaldehyde seems to be higher in women compared to men, but the difference disappears after correction for liver weight (Dettling et al., 2007).

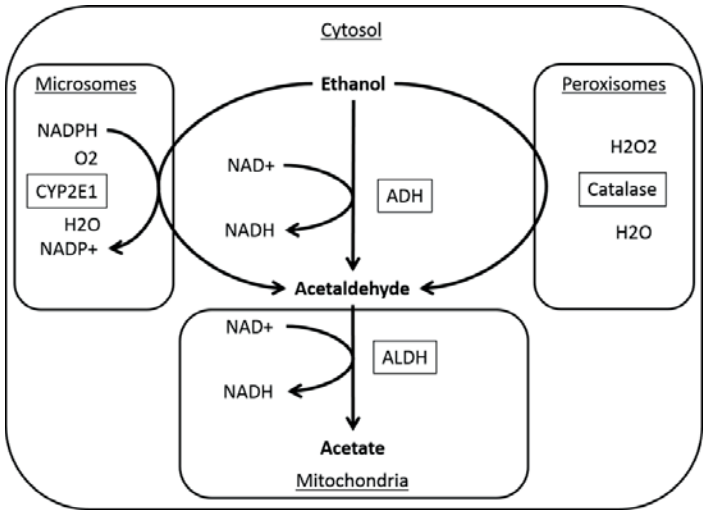

Figure 1. Ethanol metabolism in the hepatocytes, adopted from Rocco et al., 2014. In the cytosol, ethanol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH) and further into acetate by aldehyde dehydrogenase (ALDH) in the mitochondria. Alternatively, ethanol is metabolized to acetaldehyde by CYP2E1 or catalase enzymes in the microsomes and peroxisomes, respectively.

Since liver is the primary organ responsible for ethanol metabolism, it is also one of the key organs suffering damage from ethanol consumption (Rocco et al., 2014). Chronic exposure and ethanol withdrawal symptoms are considered to be important for development of changes in the liver function leading to chronic liver diseases associated with ethanol consumption, e.g. liver cirrhosis. Ethanol oxidation in hepatocytes and the concurrent increase in the nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide ratio, levels of reactive oxygen species and activation of oxidative stress and inflammatory pathways are possible mechanisms mediating these alterations in hepatocytes (Gonzalez-Reimers et al., 2014; Liu, 2014).

Furthermore, ethanol oxidation also seems to occur in the brain in neurons and astroglial cells (Wang et al., 2013). Therefore, the cumulative damage of ethanol to neural tissue is increased due to oxidative damage from free radicals and acetaldehyde toxicity. In rats, chronic ethanol consumption increases the oxidative capacity of astroglial cells, but not

neurons (Wang et al., 2013). This could be associated with neurodegenerative effect of chronic binge ethanol drinking due to activation of oxidative stress and neuroinflammatory pathways downstream of glial cell activation (Collins and Neafsey, 2012). However, details of how and to what extent ethanol metabolism in the astrocytes and neurons is responsible for the observed changes in the CNS still remains to be clarified.

Ethanol metabolism is also an important facet of the treatment of alcoholism. Disulfiram, which inhibits ALDH, is one of the earliest and most widely used medications for alcohol use disorder. If consumed with ethanol, an antabus reaction with multiple adverse events occurs. These include flushing, nausea, vomiting, hypotension, and in more serious cases, cardiovascular effects and even death (Petersen, 1992). The efficacy of the disulfiram treatment seems to be highly dependent on the adherence to the treatment (Hughes and Cook, 1997). This is also reflected in clinical studies where an open trial setting is needed in order to show the clinical effectiveness of disulfiram because the treatment effect is based on the fear of the negative consequences of consuming alcohol (Laaksonen et al., 2008; Skinner et al., 2014; Yoshimura et al., 2014).

The following chapter will review the role of glutamatergic, serotonergic, endocannabinoid and steroid systems in the pathology of alcohol use disorder. The present literary review will focus on these systems, because they have been studied in the experimental part of the thesis. The following review will also discuss the role of other neurotransmitter systems, namely dopamine, opioid and GABAergic, because they are important for understanding the neuropharmacological actions of ethanol.

2.3 ROLE OF DOPAMINE IN THE DEVELOPMENT OF ALCOHOLISM

Dopamine is an important neurotransmitter involved in a wide variety of cerebral functions although it is most often associated with reinforcement learning, motivation and movement (Nutt et al., 2015). Most dopaminergic neurons project to the basal ganglia, where dopamine is necessary for movement and motivational salience. In cortical regions, dopamine has been associated with executive functions, e.g. attention and working memory (Trifilieff and Martinez, 2014; Vijayraghavan et al., 2007).

The mesocorticolimbic system is a key structure in mediating the functions of dopamine. This system includes interconnected brain regions, e.g. the VTA, ventral striatum (nucleus accumbens, NAC), substantia nigra (SN), dorsal striatum (caudate and putamen), amygdala and frontal cortical regions, e.g. ACC (Koob, 2013; Volkow et al., 2012). These brain structures, and especially VTA, NAC and frontal cortex, are considered to comprise an important part of the motivational circuit. VTA and SN are the main sources of dopamine to the striatum and frontal cortices and the theory is that the activation of striatum by dopamine triggers the motivation to carry out both novel and habitual responses (Hyman et al., 2006). Dopamine release from VTA to NAC seems to facilitate reinforcement learning of relationship between behavior and both the rewarding and aversive outcomes of that behavior (Kravitz et al., 2012; Lammel et al., 2012), whereas dopamine release from SN to dorsal striatum seems to be important for habitual learning (Everitt and Robbins, 2013).

Repeated pairing of a natural reinforcer and a cue shifts the dopamine release from the outcome to the predictive cue (Hollerman et al., 1998; Schultz, 2004). If the predictive cue repeatedly fulfils the predicted outcome, there is no longer any increased dopamine release when the outcome is achieved (Schultz, 2004). One interpretation is that dopamine release motivates learning of novel behavior as well as the performance of already learned behaviors.

However, in the context of addiction, dopamine has been traditionally considered to be neurotransmitter for hedonic pleasure, producing a feeling of reward in the brain (Volkow et al., 2011; Volkow et al., 2012). The role of dopamine in the development of addiction was discovered in the now classical experiments where rats would willingly and repeatedly self-stimulate the brain region with dopaminergic neurons and this process could be enhanced

by treating the animals with amphetamine, which increases the dopaminergic tone in the brain (Crow, 1972; Olds and Milner, 1954; Stein, 1964). Further *in vivo* microdialysis studies in the rats associated the release of dopamine in the NAC function of many other drugs (Di Chiara and Imperato, 1988). Dopamine release in the NAC is considered to be the positive reinforcement component in the development of addiction. In animals, this positive reinforcement effect has been blocked by administration of dopamine antagonists, supporting the role of dopamine in reward and motivation (Robinson and Berridge, 1993). The theory was postulated that all addictive drugs release dopamine, directly or indirectly, in the NAC whereas non-addictive psychoactive drugs do not.

2.3.1 Goal-directed and habitual behavior

Two different types of behavioral changes have been associated with reinforcement learning: goal-directed and habitual responding (Adams, 1982). Performance in goal-directed behavior is sensitive to change in the outcome of the behavior. This can be measured with a devaluation test where a rewarding outcome e.g. food is devalued by giving it before the performance of the measured behavior (Hilario and Costa, 2008). If the behavior is goal-directed then the rate of that behavior will decrease after devaluation, because the prize of that behavior has already been achieved. In contrast, habits are insensitive to change (DePoy et al., 2013; Hilario and Costa, 2008). After habit formation, the learned behavior is continued even though the outcome is devalued, similar to the development of tolerance and reward deficiency in alcoholism.

The motivation for goal-directed behavior is considered to arise from the expected outcome, and therefore behavior is changed according to the outcome (Everitt and Robbins, 2013; Hilario and Costa, 2008). Habits are considered to be automated behavioral responses to a stimulus and as such, less responsive to changes in the outcome. This type of inflexible and automated responses is observed with substance use disorders (Everitt and Robbins, 2013).

In rodents, habit formation can be induced by over-training the animals in a particular schedule (Hilario and Costa, 2008). Habit formation is supported by a random interval schedule i.e. a constant outcome is given in response to behavior (e.g. pressing a lever) in random intervals (Adams, 1982; Hilario et al., 2007). In contrast, a random ratio schedule, where the outcome is achieved in consistent intervals but the amount of reward/punishment is random, will produce more goal-directed behavior.

At the neuroanatomical level, goal-directed behavior in rats has been associated with dopamine release in the dorsomedial striatum which is considered to correspond to human caudate nucleus (Hilario and Costa, 2008). In contrast, habit formation has been associated with the rodent dorsolateral striatum, thought to possess a similar function as the human putamen. Stimulants which increase the dopaminergic tone can enhance habit formation (Nelson and Killcross, 2006). Possibly relating to the automatic response of habitual behavior, the dorsolateral striatum has a glutamatergic input from the sensorimotor cortex in contrast to the dorsomedial striatum which receives an input from the associative cortex (Yin et al., 2004; Yin et al., 2005).

In addition to dopamine, also other neurotransmitter systems are considered to be important for habit formation. For example, genetic or pharmacological inhibition of endocannabinoid receptor 1 (CB1) function (Hilario et al., 2007) or striatum-specific deletion of adenosine A2A receptors (Yu et al., 2009) inhibits habit formation, but leaves goal-directed behavior intact. Moreover, ethanol has been recognized to be efficient in enhancing habit formation also for other rewarding stimuli in mice (DePoy et al., 2013; DePoy et al., 2015). Chronic ethanol consumption seems to cause adaptations in the dorsolateral striatum, e.g. down-regulation of CB1 receptor signaling and blockade of CB1 receptor-dependent long-term depression (LTD). These adaptations are considered to prime the animal towards habitual learning (DePoy et al., 2013).

Although habit formation explains some aspects of addiction behavior (Everitt and Robbins, 2013), it is not considered to describe the entire diversity of behavior seen in human addicts (Koob, 2013). In the context of human addiction, habitual behavior has been considered to be important in explaining why alcohol intake is converted into a binge after a couple of drinks (DePoy et al., 2013; DePoy et al., 2015). When considering the motivational role of withdrawal and especially negative symptoms during the aversive state in abstinence, the first drinks will attenuate these feelings and provide the desired relief as well as negative reinforcement (Koob, 2013). Habitual behavior is then considered to take over and drinking continues with a binge session even after the initial urge to use alcohol has passed.

2.3.2 Dopamine and addiction in humans

In humans, the role of dopamine has been studied with both post-mortem and *in vivo* imaging. In the *in vivo* imaging, radioactive ligands can be used to indirectly measure dopamine release using position emission tomography (PET) or single-photon emission computed tomography (SPECT). *In vivo* imaging in healthy subjects has been associated with the release of dopamine in the NAC with administration of stimulants (Laruelle et al., 1995; Volkow et al., 1994), tobacco (Barrett et al., 2004), ketamine (Vollenweider et al., 2000), Δ9-tetrahydrocannabinol (THC) (Bossong et al., 2009) and alcohol (Boileau et al., 2003; Urban et al., 2010). In line with the dopamine theory, all of these substances have a potential for developing addiction. However, there are also drugs like modafinil which increase dopamine release in the NAC (Volkow et al., 2009), but do not produce increased reinforcement behavior or even feelings of enjoyment (Jasinski, 2000). Moreover, in humans, even traditional substances of abuse, like alcohol (Yoder et al., 2007), THC (Barkus et al., 2011; Stokes et al., 2009), and ketamine (Aalto et al., 2002; Kegeles et al., 2002) do not always induce dopamine release. Therefore, the association between dopamine release and the positive effect of these drugs is more complex.

For example, in the case of alcohol, increased impulsivity and intoxication have been associated, but the drug high and drinking habits have not been associated with increased dopamine release in the NAC after consumption of alcohol (Boileau et al., 2003; Yoder et al., 2007). This could be associated with the differences in the dopaminergic system in subgroups of alcoholics (Tupala and Tiihonen, 2004). In post-mortem whole-hemisphere studies, alcoholics who are impulsive and antisocial seem to have an intact dopaminergic system, whereas alcoholics with high harm avoidance display a decreased binding to dopaminergic receptors and transporters (Tupala et al., 2000; Tupala et al., 2001a; Tupala and Tiihonen, 2004).

Furthermore, opioids are a class of drugs which are considered to have a clear addiction potential, but they do not necessarily release dopamine in the NAC in doses that produce euphoric high (Daglish et al., 2008) and treatment with a dopamine receptor antagonist does not block the rewarding effects of opioids (Van Ree and Ramsey, 1987). Even with stimulants, the theory of central role of dopamine has been questioned partly because dopamine receptor antagonists do not seem to be efficient in the treatment of stimulant addictions (Lingford-Hughes et al., 2012).

Interestingly dopaminergic pathways include a parallel function to pain. This is important since physical pain is one of the risk factors predicting relapse of alcohol use in humans (Witkiewitz et al., 2015). Furthermore, while most dopaminergic neurons react to unexpected reward or expectation of reward, there is a large subset of dopaminergic neurons which react to noxious stimuli (Bromberg-Martin et al., 2010). Optogenic activation of different subgroups of dopaminergic neurons can produce rewarding or punishing effects (Lammel et al., 2012). This has also been demonstrated in humans; the reward and pain pathways show a considerable overlap (Jensen et al., 2003). This is in line with the hypothesis that dopaminergic function mainly motivates behavior, detecting novel outcomes (both rewarding and aversive) and then motivating future behavior accordingly either by goal-

directed manner or by habit formation depending on the schedule and repetition of the outcome (Everitt and Robbins, 2013; Hilario and Costa, 2008).

One aspect to be taken into account in interpreting the human imaging studies seems to be that the *in vivo* imaging agents, e.g. ^{11}C-raclopride, used to assess dopamine release binds to dopamine D2 and D3 receptors. Dopamine D2 receptor containing GABAergic cells in the NAC have been associated with punishment effect in rodents (Kravitz et al., 2012). In contrast, positive reinforcement has been associated with D1 containing GABAergic cells. D2 expressing GABAergic cells project to the globus pallidus, where decreased levels of $GABA_A$ receptors have been reported in alcoholics (Laukkanen et al., 2013).

Furthermore, in contrast to the basic idea that high dopaminergic function leads to the formation of dependence, high levels of dopamine receptors in the striatum may even decrease the risk of alcohol dependence in a high-risk population (Volkow et al., 2006). Accordingly, decreased binding to dopamine D2/3 receptors and dopamine transporters (DAT) have been associated with alcoholism (Heinz et al., 2004; Hietala et al., 1994; Martinez et al., 2005; Tupala et al., 2000; Tupala et al., 2001a; Volkow et al., 1996; Volkow et al., 2002; Volkow et al., 2007). In human whole-hemisphere autoradiography studies conducted in late-onset Cloninger type 1 alcoholics, decreased D2/3 receptor and DAT levels have been reported in the extended amygdala, e.g. NAC, caudate, putamen and amygdala, but not in the SN (Tupala et al., 2001a; Tupala et al., 2001b; Tupala et al., 2003a; Tupala et al., 2003b). Interestingly, D2/3 receptor density was also decreased in type 2 alcoholics in the dorsal striatum and globus pallidus, but not in the NAC or amygdala (Tupala et al., 2003a; Tupala et al., 2003b), possibly relating to the effects of ethanol on dorsal striatum function detected in mice (DePoy et al., 2013; DePoy et al., 2015).

Moreover, the reactivity of the dopaminergic system can be studied by using pharmacological challenges by stimulants, e.g. methylphenidate and amphetamine. Alcoholism as well as some other substance use disorders have been associated with the blunted release of dopamine in many (but not all) individuals to a pharmacological challenge by stimulants (Martinez et al., 2005; Martinez et al., 2007; Volkow et al., 2007; Volkow et al., 2014).

However, the complexity of the VTA to NAC projections is not always appreciated. While optogenic studies in rats support the role of dopaminergic neurons in reinforcement learning (Tsai et al., 2009), it should be noted that tyrosine hydroxylase (TH) expressing neurons in the VTA also release other neurotransmitters, including glutamate, GABA, brain derived neurotrophic factor and corticotropin releasing hormone (CRH) (Fields and Margolis, 2015). Furthermore, in addition to the previously discussed role of different dopaminergic neurons in rewarding and aversive stimulus (Lammel et al., 2012), VTA also has other neurocircuitry running through it although the full extent and functionality of these circuits are still unclear (Fields and Margolis, 2015). For example, GABAergic neurons project from VTA to NAC cholinergic interneurons and can increase dopamine levels in the NAC via presynaptic nACh receptors (Brown et al., 2012; Cachope et al., 2012). This could be important in the case of combined alcohol and nicotine use disorders.

Furthermore, dopamine antagonists have not been shown to be effective in the treatment of addiction (Nutt et al., 2015). In the case of alcoholism, the current conclusion from the relatively small body of evidence is that antipsychotics are not useful in the treatment of alcoholism because of poor balance between benefits and harms of these drugs (Kishi et al., 2013; Littlewood et al., 2015).

Moreover, although small, a recent local field potential recording study from the human NAC in patients with pharmaco-resistant partial epilepsy receiving deep brain stimulation has challenged the basic dogma that dopamine release in the NAC is associated with reward prediction error coding in humans (Stenner et al., 2015). In contrast to previous human fMRI studies, local field potential recording did not associate NAC activity to reward prediction error during an economic decision-making task. The authors suggest that the role of NAC is more complex and would be more relevant in tracking multiple signals associated with

behavioral choices (Stenner et al., 2015). Further study is obviously needed to understand the role of NAC activation in human behavior.

2.3.3 Role of dopamine in executive functions

In order to understand the controversy between the classical hypothesis that dopamine promotes addiction formation and the recent evidence that low levels of dopamine release in the striatum seem to be associated with risk of addiction (Nutt et al., 2015) and high dopamine function might even be linked with a decreased risk of alcohol dependence in a high risk population with family history of alcoholism (Volkow et al., 2006), we need to consider the other aspects of dopamine in controlling behavior. The main connection here is that dopamine has an important role in the modulation of executive functions. Executive functions are dysfunctional in alcoholism as well as in many other disorders like attention deficit hyperactivity disorder (ADHD) and schizophrenia. Cortical inhibition is considered as a crucial aspect in executive control and the lack of this inhibition is associated with impulsive behavior.

Similar to alcoholism, pathological gambling is a behavioral addiction associated with executive dysfunction. However, pathological gambling is not associated with altered striatal D2/3 receptor availability (Boileau et al., 2013; Clark et al., 2012). In contrast to many drugs of abuse, pathological gambling is associated with increased dopamine release in pharmacological challenge to amphetamine (Boileau et al., 2014). Furthermore, increased impulsive behavior and behavioral addictions like gambling are a recognized side-effect of the Parkinson's disease medication, L-dopa, which increases striatal dopamine release (O'Sullivan et al., 2011). However, this is in stark contrast to the situation with ADHD, where patients benefit from extra dopamine since this is believed to increase their inhibitory control and reduce impulsivity.

Overall, dopamine seems to display an inverted-U dose response relationship with respect to the association between executive function and impulse control. Both too low and too high levels of dopaminergic function are associated with loss of executive control (Nutt et al., 2015; Vijayraghavan et al., 2007). For example, dopamine D2/3 receptor levels have been reported to be increased in young and impulsive Cloninger type 2 alcoholics compared to their older and less impulsive type 1 counterparts in the frontal cortex as a possible sign of decreased dopaminergic input to the region (Tupala et al., 2004). In contrast, type 1 alcoholics seem to have decreased dopaminergic function in the extended amygdala (Tupala et al., 2001a; Tupala et al., 2001b; Tupala et al., 2003a) and both alcoholic sub-groups seem to have decreased dopaminergic function in the dorsal striatum (Tupala et al., 2003b). Therefore, the dopaminergic function might be differently altered in different brain regions in different alcoholics. Furthermore, these alterations can be associated with increased impulsivity in addition to reinforcement learning and habit formation (Nutt et al., 2015).

However, as in the case of reinforcement learning, also other neurotransmitter systems have been associated with executive dysfunction. The glutamatergic system and especially the NMDA receptor-mediated functions seems to be important for the executive dysfunction caused by alcohol. Ethanol acts as a NMDA receptor antagonist in doses relevant to human alcohol use in brain regions important executive function e.g. hippocampus and PFC (Lovinger et al., 1989; Weitlauf and Woodward, 2008; Xu et al., 2012). Furthermore, administration of NMDA antagonists evokes an executive dysfunction and this effect can be used in investigating other disorders associated with executive dysfunction, e.g. schizophrenia (Bubenikova-Valesova et al., 2008).

Overall, even though dopaminergic function has a crucial role to play in the pathology of addiction, it seems clear that addiction needs to be considered as a complex multiple-neurotransmitter disorder (Nutt et al., 2015). In the case of the alcoholism, there is an important role for the opioid system to play in ethanol induced reinforcement learning.

2.4. THE OPIOID SYSTEM AND ALCOHOL INDUCED REINFORCEMENT LEARNING

In contrast to dopamine antagonists, μ-opioid receptor (MOR) antagonists, naltrexone (Bogenschutz et al., 2009; Kiefer et al., 2008; Rubio et al., 2005) and nalmefene (Aubin et al., 2015; Francois et al., 2015; Gual et al., 2013; Mann et al., 2013a; van den Brink et al., 2013) are being used in the treatment of alcoholism. Furthermore, there seem to be subgroups of alcoholics who benefit more from MOR antagonist treatment. Better treatment results have been associated with family history and early-onset of alcoholism as well as with Cloninger's type 2 (Kiefer et al., 2008; Rubio et al., 2005) and Babor type A alcoholism (Bogenschutz et al., 2009).

The endogenous opioid system has a crucial role in alcoholism, considered to contribute significantly to alcohol induced reinforcement learning (Nutt, 2014; Vengeliene et al., 2008). Decreased self-administration of ethanol has been reported in MOR (Roberts et al., 2000) and κ opioid receptor (KOR) knock-out mice (Kovacs et al., 2005). In contrast, δ opioid receptor (DOR) knock-out mice display an increased alcohol intake (Roberts et al., 2001). MOR antagonists can reduce cue- and priming dose but not stress induced reinstatement of ethanol related behavior (Katner et al., 1999; Le et al., 1999).

β-endorphin is an endogenous opioid peptide acting on MOR and DOR (Lam et al., 2008; Olive et al., 2001). Exposure to ethanol can increase β-endorphin levels (Mitchell et al., 2012). This effect seem to exhibit an inverse U-type shape where higher concentrations of ethanol release less β-endorphin compared to moderate doses (Gianoulakis, 1990). Abstinent alcoholics seemed to display increased MOR binding (Heinz et al., 2005; Weerts et al., 2011), whereas no difference was seen before or after drinking between heavy users compared to controls (Mitchell et al., 2012). In a post-mortem autoradiography study, decreased MOR binding was seen in mostly intoxicated Cloninger type 1 alcoholics in the dentate gyrus (Laukkanen et al., 2015a). In summary, MOR binding might be more affected by alcohol withdrawal than acute or chronic intake of the compound (Nutt, 2014).

A simple hypothesis of opioid system induced reinforcement learning is that the opioid system inhibits GABAergic neurons projecting to VTA and this disinhibition increases dopamine release in the NAC (Fields and Margolis, 2015). The classical explanation for the reinforcing properties of ethanol is that it can both induce β-endorphin release and inhibit the effect of glutamate on GABA neurons, which in turn leads to further decreases in GABA transmission and an overall enhancement of dopamine release (Xiao and Ye, 2008). However, in contrast to the classical dopamine dependent reinforcement theory, opioid induced reinforcement learning seems to be able to take place without a dopaminergic component. The opioid receptor agonist, morphine, induced conditioned place preference (CPP), which is one of the tests used to measure positive reinforcement, is not blocked by prior treatment with a nonselective dopamine receptor antagonist in opioid naïve animals (Bechara et al., 1992; Laviolette et al., 2002). However, in opioid dependent animals, dopamine antagonism is able to block CPP. Moreover, it seems that glutamatergic signaling in the VTA is needed for morphine induced CPP (Harris et al., 2004). This seems to indicate that the dopaminergic system is important for reinforcement learning only after initial dependence for opioid action has been achieved. Moreover, it seems that the dopaminergic system is actually more important in mediating the aversive state in opiate withdrawal (Laviolette et al., 2002). This complexity between the dopaminergic and opioid systems could partially explain the disparity in results from neuroimaging studies alcohol in humans (Nutt et al., 2015).

As discussed above, there are many agents which can cause an increase in striatal dopamine release, but do not produce positive reinforcement learning. These include DOR agonists (Mitchell et al., 2014) and a MOR antagonist (Devine et al., 1993), i.e. the latter produced conditioned place aversion (Shippenberg and Bals-Kubik, 1995). This could be related to dopamine mediated negative reinforcement learning (Lammel et al., 2012). It has therefore been suggested that the primary mechanism by which opioids evoke their initial

reinforcement learning is through inhibition of aversive signals e.g. relief of anxiety or pain (Fields and Margolis, 2015). This could also at least partly be the case with alcohol (Nutt, 2014), although further research is needed.

In addition to MOR, also KOR seems to be important in alcoholism. Interestingly nalmefene has both MOR and KOR blocking activity. In rats, nalmefene is more effective than naltrexone in reducing drinking (Walker and Koob, 2008). However, the KOR antagonist nor-BNI selectively decreased the alcohol intake only in alcohol-preferring animals, evidence for a possible role of genetics in association between KOR in alcohol dependence (Walker and Koob, 2008). Indeed also in humans, associations have been reported between alcoholism and SNPs in the prodynorphin and KOR gene (Flory et al., 2011; Xuei et al., 2006) as well as elevated methylation of prodynorphin CpG-SNPs (Taqi et al., 2011). Acute high doses of ethanol were found to increase endogenous dynorphin levels in the NAC (Marinelli et al., 2006) and the central nucleus of the amygdala (Lam et al., 2008) possibly reflecting the aversive stimulus of high ethanol concentrations (Nutt, 2014). With chronic alcohol consumption, the dynorphin system has been postulated be hyperactive, possibly contributing to the negative emotional state (Koob, 2013). In support of this hypothesis, abstinent alcoholics seem to have increased DOR levels in multiple brain regions, including NAC as observed in a PET study with [^{11}C]methylnaltrindole (Weerts et al., 2011). Interestingly, treatment with a CRH receptor antagonist was able to inhibit the alcohol-induced increase of dynorphin levels in the rat amygdala (Lam and Gianoulakis, 2011), supporting role of CRH in the negative reinforcement part of alcoholism pathology. In humans, the opioid system function has also been associated with HPA axis activation and the abnormal co-function of these systems has been observed in DOR binding in alcoholics using PET imaging (Wand et al., 2013).

Overall, the opioid system is an important component of ethanol induced positive learning. The opioid system seems to participate in both positive and negative reinforcement learning, in association with dopaminergic system. However, also the inputs of other neurotransmitter systems are needed to complete the neuropharmacology of alcohol and the pathology of alcoholism. Therefore, in the next chapters, I will move on to review the role of GABAergic and glutamatergic systems in alcohol-induced changes in the function of the CNS.

2.5 $GABA_A$ RECEPTOR MEDIATED EFFECTS OF ETHANOL

In the 1980's, ethanol was shown to enhance $GABA_A$ receptor mediated inhibition (Allan and Harris, 1986; Suzdak et al., 1986; Suzdak et al., 1986). Since then, the importance of the $GABA_A$ receptor in producing the disinhibiting and sedative effects of alcohol has been widely recognized.

There are two forms of $GABA_A$ receptor mediated inhibitory signaling: "phasic" and "tonic", with some overlap between the two. Phasic inhibition is the traditional form of GABAergic signaling, where vesicular release of GABA induces inhibitory postsynaptic currents when postsynaptic $GABA_A$ receptors are activated. GABA is then rapidly removed from the synaptic cleft by diffusion and uptake by the GABA transporter. Furthermore, also the postsynaptic $GABA_A$ receptors become desensitized. In contrast to phasic inhibition, tonic $GABA_A$ mediated inhibition is more persistent and results from extrasynaptically located $GABA_A$ receptor activation by extracellular GABA (Glykys and Mody, 2007a; Hamann et al., 2002).

Ethanol affects both phasic and tonic GABAergic signaling (Allan and Harris, 1986; Choi et al., 2008; Suzdak et al., 1986; Suzdak et al., 1986). The concentration of extracellular GABA is affected by the balance between uptake and vesicular and non-vesicular release of GABA also from glial cells (Glykys and Mody, 2007a; Lee et al., 2011; Lee et al., 2010; Rossi et al., 2003). Since GABA is mainly released by vesicular release, it is this release rate which is often

the balance defining factor and therefore the level of tonic $GABA_A$ inhibition usually runs in parallel to the changes in the vesicular release (Glykys and Mody, 2007b). However, because tonic GABAergic inhibition is persistent, it has been considered to mediate ~75% of total GABAergic inhibition in the CNS (Hamann et al., 2002).

It has been difficult to pinpoint the exact mechanism to explain how ethanol influences GABAergic function (Helms et al., 2012). In a simplified view, two main mechanisms have been proposed: 1) direct action on $GABA_A$ receptors and 2) enhancement of vesicular release of GABA.

The direct action of ethanol to $GABA_A$ receptors has been associated with especially the δ subunit containing GABAergic receptors. The δ subunit has been associated with extrasynaptically located $GABA_A$ receptors mediating tonic inhibition (Hamann et al., 2002; Rossi and Hamann, 1998; Stell et al., 2003). These δ subunit containing $GABA_A$ receptors are located in hippocampal and cerebellar granule cells as well as in thalamic relay neurons (Wisden et al., 1992) and in smaller amounts in a limited array of cells elsewhere, for example in VTA (Vashchinkina et al., 2014a; Vashchinkina et al., 2014b). Pharmacological modulation of δ subunits causes effects only in the tonic GABAergic inhibition and ethanol concentrations such as those only obtained after consuming moderate to intoxicating amounts (5-50mM) enhance tonic GABAergic inhibition (Glykys et al., 2007; Hanchar et al., 2005; Wei et al., 2004). However, these findings have proved difficult to replicate and several groups have not been able to demonstrate any direct effect of ethanol on $GABA_A$ receptors (Borghese et al., 2006; Botta et al., 2007). The phosphorylation status of $GABA_A$ receptors has been considered one possible explanation for differences between the studies, e.g. $GABA_A$ receptors containing α4δ subunits require phosphorylation by protein kinase Cδ in order to mediate ethanol enhanced tonic inhibition (Choi et al., 2008).

Another possible mechanism of action of ethanol induced enhancement in GABAergic signaling is increased vesicular release of GABA. This mechanism has been observed in many brain regions e.g. cerebellum, VTA, SN and amygdala, but it seems to be lacking in others e.g. cortex and thalamus (Kelm et al., 2011). Mechanisms for ethanol enhanced vesicular release of GABA are yet not known, but there is some evidence for different candidates including ethanol induced activation of the transcription of heat shock factor, resulting in an increase in the levels of vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein, which is required for synaptic vesicle fusion (Varodayan and Harrison, 2013), the balance between expression of neuronal nitric oxide synthase and postsynaptic protein kinase C activity (Kaplan et al., 2013) and direct binding to protein kinase Cε (Das et al., 2009; Pany and Das, 2015). Furthermore, in the rat interneuron-Purkinje cell synapses, the effect of ethanol is dependent of internal calcium release mediated possibly by inositol 1,4,5-trisphosphate receptors (IP(3)Rs) (Kelm et al., 2010). The phospholipase C antagonist, edelfosine, and the protein kinase C antagonists, chelerythrine and calphostin, are able to prevent ethanol induced GABA release indicating that the PLC/IP(3)R/PKC pathway seems to be required for this effect. Moreover, one possibility to explain how ethanol increases GABA release is involvement of different G-protein coupled receptors (GPCRs), such as the $GABA_B$, CB1, CRH1, and 5-HT_{2C} which could mediate the effect (Kelm et al., 2011). Overall, there could be several cellular mechanisms involved in mediating how ethanol can increase GABA release and these may be cell and brain region dependent.

Although the mechanism remains to be clarified, there is a consensus that the ethanol induced increase in the vesicular release of GABA results in increased phasic as well as tonic GABAergic inhibition, because increased vesicular release also increases extracellular levels of GABA (Helms et al., 2012; Kelm et al., 2011). Therefore, a direct effect of ethanol on $GABA_A$ receptor function is not necessary for an ethanol induced effect in GABAergic signaling. Furthermore, even without a direct action, the subunit composition and modulation could influence ethanol induced enhancement of GABAergic inhibition in a brain region specific manner. In line with the increased GABAergic activity caused by alcohol, decreased $GABA_A$ receptor binding has been observed in the post-mortem brains of alcoholics in the

hippocampus and globus pallidus, possibly as a compensatory mechanism (Laukkanen et al., 2013).

2.6 GLUTAMATE SYSTEM IN ALCOHOL DEPENDENCE

In the present chapter, I will focus on the role of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in addiction not only since these receptors are one of the most important glutamatergic receptors in synaptic plasticity, but also because this receptor type has been investigated in the experimental part of the thesis. However, also other glutamatergic receptors will be discussed, since they are considered to be important in alcoholism and could be targets for novel medication (Holmes et al., 2013).

When discussing substance use disorders, it has been speculated that drugs of abuse act to modify normally adaptive motivational circuitry so that it is responsive to the drug related stimuli (by positive and negative reinforcement as discussed above), but also they are less flexible to respond to natural reinforcers (Bowers et al., 2010; Everitt and Robbins, 2013). The glutamatergic system is one of the key players in neural plasticity which is considered to be at least one of the ways for learning (Bowers et al., 2010). In the case of reinforcement learning, it should be noted that the dopaminergic and glutamatergic systems are involved and can influence each other in a complex manner (Balcita-Pedicino et al., 2011; Bowers et al., 2010; Omelchenko et al., 2009; Sesack et al., 2003). For example, the lateral habenula has been shown to inhibit dopaminergic cells in the VTA and SN via a bisynaptic connection from the lateral habenula to the GABAergic cells in the rostromedial mesopontine tegmental nucleus, which then inhibit dopamine release in the VTA and SN (Balcita-Pedicino et al., 2011; Omelchenko et al., 2009). Glutamatergic systems have been considered to have an important role in inducing and maintaining alcoholism (Holmes et al., 2013).

Chronic alcohol consumption induces increased extracellular glutamate levels as well as alterations in glutamate receptors and transporters (Holmes et al., 2013). A positive correlation has been detected between the severity of alcoholism and cerebrospinal fluid glutamate levels (Umhau et al., 2010). Glutamate levels in the ACC seem to be increased during early withdrawal (Hermann et al., 2012), then possibly decrease up to day 9 before returning to their normal range (Mon et al., 2012). Experimental animals also show elevated glutamate levels during acute withdrawal (Rossetti and Carboni, 1995) and the increased extracellular levels of glutamate especially in the NAC correlated with the severity of the withdrawal symptoms (Fliegel et al., 2013).

Ethanol acts as a NMDA receptor antagonist in doses relevant to human alcohol use in brain regions important for addiction pathology, e.g. hippocampus and PFC (Lovinger et al., 1989; Weitlauf and Woodward, 2008; Xu et al., 2012). Furthermore, NMDA receptor antagonists like phencyclidine (PCP) and ketamine have been able to substitute for the discriminative stimulus effects of alcohol in rodents (Hundt et al., 1998; Sanger, 1993) and mimic the subjective feelings of intoxication in humans (Krupitsky et al., 2007; Krystal et al., 1998). Recently the antidepressant like effects of NMDA antagonists have been studied widely (Coyle and Laws, 2015). This suggests that the NMDA antagonist effect could also be one of the desired pharmacological effects when ethanol is used as a self-medication to ease a depressed mood, a speculation in need of further research (Coyle and Laws, 2015; Dickerson et al., 2010).

Overall, the changes in the glutamatergic system associated with the reinforcing properties of alcohol consumption have been studied most extensively. These alterations could be associated with many aspects of alcohol related behavior e.g. alcohol seeking and relapse. However, also changes in the cortical regions are likely to be important but unfortunately there is less published research on the glutamatergic changes in the cortical regions in association with alcohol pathology.

2.6.1 AMPA receptors

AMPA receptors are ionotropic glutamate receptors typically composed of four subunit proteins (GluA1-A4). The subunits can form hetero- or homo-meric complexes. Often GluA2 subunits exist in a complex with either GluA1 or GluA3 subunits (Dingledine et al., 1999). Much of the research on the role of AMPA receptors in addiction has been conducted with stimulants, especially cocaine (Bowers et al., 2010). Furthermore, compared to VTA and NAC, little is known of the role of AMPA receptors in the cortical regions in association with alcoholism (Nimitvilai et al., 2015).

AMPA receptors have been associated especially with re-establishment of drug consumption and drug seeking behavior. A blockade of the AMPA receptors in the NAC results in inhibition of the glutamatergic projections from the PFC to the NAC. Antagonism of AMPA receptors in the NAC is more effective in preventing a re-establsihment of cocaine-seeking than inhibition of the dopaminergic system (Cornish and Kalivas, 2000). Furthermore, inhibition of AMPA receptors prevents drug, cue and stress induced relapse of cocaine seeking behavior (Bachtell et al., 2008; Bäckstrom and Hyytiä, 2003; Bäckstrom and Hyytiä, 2006; McFarland et al., 2004). AMPA receptor agonists have been claimed to promote and antagonists to prevent relapses (Cornish and Kalivas, 2000; McFarland et al., 2004), but this has been considered as an over-simplified hypothesis (Bachtell et al., 2008).

Stimulants seem to increase AMPA receptor function, which is one possible mechanism to explain how motivated behavior is altered in response to stimulant administration. This strengthening or weakening of synapses could be one of the biological mechanisms behind reinforcement learning. AMPA receptors have a key role in many forms of synaptic plasticity (Malinow and Malenka, 2002). In long-term potentiation (LTP), GluA2-lacking AMPA receptors are inserted in order to achieve synaptic strengthening. Compared with GluA2-containing AMPA receptors, GluA2-lacking AMPA receptors have greater channel conductance, are calcium permeable, and can therefore trigger calcium-dependent signaling cascades within the cell (Grueter et al., 2012). The opposite process of LTP, long-term depression (LTD) can be achieved by removal of AMPA receptors from synapses (Malinow and Malenka, 2002). The AMPA receptor trafficking is one of the best known mechanisms by which behavior and drug taking can strengthen and weaken synapses (Grueter et al., 2012). It should however be noted that both LTP and LTD processes also involve other receptors, e.g. TRVP1 mediated LTD in the hippocampus (Chavez et al., 2010).

2.6.2 Reinforcement learning and AMPA receptors in VTA and NAC

Reinforcement learning increases AMPA receptor mediated responses in the VTA dopaminergic neurons in an NMDA receptor dependent manner (Stuber et al., 2008a; Stuber et al., 2008b). Following exposure to natural reinforcers, e.g. food, this synaptic potentiation seems to be transient: increased glutamate transmission in the VTA is observed for up to 7 days after the last training session, but it has waned by day 14 (Chen et al., 2008a). Similarly, potentiation of AMPA receptor mediated responses in VTA is observed following single or repeated injections of cocaine, but this effect fades by day 10 (Borgland et al., 2004).

However, voluntary self-administration of cocaine exerts a major influence on the durability of LTP-like changes in the VTA. Voluntary cocaine self-administration induced LTP in the VTA which persisted for up to 3 months of abstinence (Chen et al., 2008a) extending beyond the pharmacological action of cocaine observed in the involuntary administration experiments (Borgland et al., 2004; Chen et al., 2008a). It has been speculated that these persistent changes are associated with a more active learning process in the voluntary self-administration model (Bowers et al., 2010). Furthermore, the increased glutamatergic action in the VTA can be detected in the VTA even after extinction of drug-seeking behavior (Chen et al., 2008a). This is in contrast with the concept that the synaptic potentiation induced by natural reinforcers is reversed after extinction of the associated behavior (Pan et al., 2008). These results suggest that the changes in the glutamatergic function of the VTA might be more persistent for drugs than for natural rewards.

The role of AMPA receptor mediated synaptic plasticity is somewhat different in the NAC compared to the VTA. Multiple exposures to cocaine are needed to induce synaptic plasticity in the NAC whereas a single injection alone exerts an effect in the VTA (Kourrich et al., 2007). Furthermore, there are sub-region differences between shell and core of NAC. During early withdrawal, there is decreased AMPA receptor function in the NAC shell, compared to the situation with prolonged abstinence, which increases the AMPA receptor function (Kourrich et al., 2007). Re-administration of cocaine during abstinence reverses this process back to reduced AMPA receptor function. In contrast, in the NAC core, there seems to be an increase in the AMPA function during early abstinence which is then suppressed during prolonged abstinence (Kourrich and Thomas, 2009). This could be associated with the distinctive functions associated with NAC core and shell. From work conducted in rodents, a simplified image has emerged that the increased NAC core function can be associated with activation of the motor programs needed for seeking rewards whereas decreased NAC shell activity could be associated with releasing a "brake" on reward seeking behavior (Ghitza et al., 2004; Taha and Fields, 2006; Wood and Rebec, 2004). Thus the changes seen in AMPA receptor levels in the NAC core and shell could summate to lead to increased reward seeking behavior during early withdrawal.

Furthermore, the AMPA subunit composition seems to play a crucial role in the cocaine induced up-regulation of AMPA mediated glutamatergic function during prolonged abstinence. GluA2-lacking AMPA receptors have been associated with cocaine craving (Conrad et al., 2008) whereas interventions which promote the surface expression of the GluA2 subunit inhibit cocaine relapse (Famous et al., 2008). The phosphorylation of GluA2 by phosphokinase C is needed for trafficking of GluA2-containing AMPA receptors away from the cell surface membrane and disrupting this modulation attenuates reinstatement of cocaine seeking. Moreover, AMPA receptor trafficking in both the core and shell of NAC seems to be needed for the re-establishment of cocaine seeking (Famous et al., 2008).

Interestingly, the decreased basal extracellular glutamate concentrations in the NAC observed during abstinence have been considered to be one possible explanation of impaired synaptic plasticity in the NAC in abstinent animals (Martin et al., 2006). This phenomenon has been suggested to be associated with the lack of behavioral flexibility encountered in addictions (Bowers et al., 2010). Impaired function of the glial cysteine-glutamate antiporter (Lewerenz et al., 2013) and glutamate transporter 1 may be responsible for the decreased basal glutamate levels. Restoring glutamate transportation with N-acetylcysteine or ceftriaxone has been proposed as a possible new treatment for drug addictions (Roberts-Wolfe and Kalivas, 2015). However, a double-blind clinical trial in cocaine patients using N-acetylcysteine was a disappointment, suggesting that the positive effect could be seen only in already abstinent patients (LaRowe et al., 2013).

2.6.3 AMPA and alcohol

Although much of the work on AMPA receptors and addiction has been done with stimulants, there are also some studies examining the role of AMPA receptors in relation to alcoholism. It should be noted that alcohol is not considered to directly modulate AMPA receptor function, at least in concentrations usually consumed. The role of AMPA receptors in alcoholism has mostly been studied in limbic brain structures e.g. VTA and NAC, but not in cortical regions.

In rodents, chronic alcohol exposure has enhanced AMPA receptor function in the basolateral amygdala, VTA and NAC (Ary et al., 2012; Chandler et al., 1999; Heikkinen et al., 2009; Lack et al., 2007; Stuber et al., 2008a). Knock-out of the GluA1 or GluA2 subunits has minimal effects on alcohol intoxication, tolerance, or drinking (Cowen et al., 2003; Mead and Stephens, 2003a; Mead and Stephens, 2003b). In contrast, GluA3 subunit deletion has been associated with decreased cue-driven alcohol-seeking (Sanchis-Segura et al., 2006), which could be associated with chronic ethanol induced increase in GluA3 levels in the brain in animals (Bruckner et al., 1997; Dettmer et al., 2003). This is in line with the above discussed

studies with cocaine, in which alterations in the AMPA receptor trafficking in the NAC and VTA were especially associated with drug seeking behavior (Conrad et al., 2008; Famous et al., 2008).

Furthermore treatment with AMPA receptor agonists has increased ethanol consumption in animal studies (Cannady et al., 2013). Systemic or direct NAC or VTA administration of mixed AMPA/kainate receptor (CNQX and NBQX) or specific AMPA receptor (GYKI 52466) antagonists reduced alcohol seeking behavior in rodents (Bäckstrom and Hyytiä, 2004; Czachowski et al., 2012; Sanchis-Segura et al., 2006; Stephens and Brown, 1999). Furthermore, injection of AMPA receptor antagonist DNQX directly into amygdala has reduced the extent of anxiety induced by alcohol withdrawal (Lack et al., 2007). From these results, it seems that AMPA receptor antagonists might be beneficial in the treatment of alcoholism, probably in reducing alcohol seeking and the anxiety associated with alcohol withdrawal.

In humans, increased hippocampal GluA2 and GluA3 immunoreactivity has been observed in the post-mortem brains of alcoholics (Breese et al., 1995). In contrast, autoradiographic evaluations have detected no alterations in AMPA or kainate receptors between alcoholics and controls, although NMDA receptor levels were increased in the FC but not in the ACC (Freund and Anderson, 1996; Freund and Anderson, 1999). However, these studies did not differentiate alcoholics into sub-groups and different types of alcoholics could be affected differently. Therefore, the presence of different subgroups as well as methodological differences in measuring AMPA receptors could be behind these conflicting results. A summary of the studies focusing on AMPA receptor levels in human alcoholics and the effects of chronic alcohol exposure in animals is shown in table 2.

Furthermore, alcohol dependence has been associated with variations in coding for the multi-PDZ gene, which is involved with NMDA-mediated AMPA receptor trafficking. This indicates that differences in AMPA receptor trafficking could be important in determining an individual's vulnerability to develop alcoholism (Karpyak et al., 2012). However, the multi-PDZ protein also interacts with other receptors e.g. $GABA_B$, dopamine D2 receptors as well as 5-HT_{2C} receptors (Balasubramanian et al., 2007; Becamel et al., 2001; Griffon et al., 2003). This highlights the tangled nature of the neurotransmitter systems involved in alcoholism, but also indicates that differences in expression or function of proteins affecting the trafficking of receptors could be important in addiction pathology.

Table 2. Summary of studies of AMPA receptor binding in human alcoholics and AMPA receptor function in animals

Main findings	Reference(s)
Post-mortem brain samples of alcoholics	
Increased GluA2 and GluA3 immunoreactivity in the hippocampus	Breese et al., 1995
No alterations in [^{3}H]AMPA binding in the frontal cortex	Freund and Anderson, 1996
Animal studies	
Chronic ethanol exposure enhances AMPA receptor function in the basolateral amygdala, VTA and NAC	Ary et al., 2012; Chandler et al., 1999; Heikkinen et al., 2009; Lack et al., 2007; Stuber et al., 2008a

2.6.4 Other glutamatergic medications for the treatment of alcoholism

Returning to the broader concept of glutamatergic system, there are several medications already in use or being tested in clinical and preclinical experiments. The most important of these compounds is topiramate, which is used as an off-label treatment for alcoholism (Blodgett et al., 2014; Johnson and Ait-Daoud, 2010). Topiramate has a complex pharmacological profile, including AMPA/kainate receptor inhibition but also facilitation of $GABA_A$ activation. The complete picture of pharmacology of topiramate is still not yet fully understood. At the behavioral level, topiramate treatment has reduced craving, withdrawal

and drinking in alcoholics (Baltieri et al., 2008; Florez et al., 2008; Johnson et al., 2003a; Johnson et al., 2004; Johnson et al., 2007; Krupitsky et al., 2007; Miranda et al., 2008; Paparrigopoulos et al., 2011). Topiramate seems to possess similar efficacy as naloxone in the treatment of alcoholism, possibly being better in reducing alcohol craving (Baltieri et al., 2008; Florez et al., 2008). Topiramate treatment has also been associated with reduced impulsivity in alcoholics (Rubio et al., 2009). A single nucleotide polymorphism (rs2832407) in the kainate GluK1 receptor subunit has been linked with the efficacy of topiramate treatment in heavy users, indicating that the kainate receptor function might be important for the treatment effect of topiramate (Kranzler et al., 2014; Kranzler et al., 2014).

Moreover, the NMDA antagonist memantine treatment has also been associated with reduced alcohol craving, but not drinking, in alcoholics (Krishnan-Sarin et al., 2015). Furthermore, both topiramate and memantine, as well as the glutamate release inhibitor, lamotrigine, have been reported to decrease ethanol withdrawal symptoms in humans (Krupitsky et al., 2007).

In addition, acamprosate (N-acetyl homotaurine) is used in the treatment of alcoholism (Holmes et al., 2013). Acamprosate can modulate glutamate levels and acamprosate administration at the beginning of alcohol withdrawal seems to decrease glutamate levels in humans (Umhau et al., 2010) as well as in rodents (Dahchour and De Witte, 2003). Acamprosate has been considered as an NMDA and metabotropic glutamate 5 (mGluR5) receptor antagonist, however, its action on these receptors at clinically relevant doses has been questioned and the mechanism of action remains unclear (Holmes et al., 2013; Johnson et al., 2003b; Popp and Lovinger, 2000).

Moreover, also many other potential medications affecting the glutamatergic system have been investigated in preclinical experiments for treatment of alcoholism and other substances of abuse (Holmes et al., 2013; Litten et al., 2012). For example, substances which upregulate the glutamate transporter 1 function in the brain e.g. ceftriaxone and N-acetylcysteine, have been claimed to reduce alcohol intake in animal models (Rao et al., 2015; Reissner et al., 2015). At present, a clinical trial is recruiting patients to study the effectiveness of N-acetylcysteine in the treatment of alcoholism as an adjunct therapy with naltrexone (ClinicalTrials.gov: NCT01214083).

Furthermore, also the mGlu5 receptor antagonist, MTEP, was claimed to reduce alcohol seeking behavior (Bäckstrom et al., 2004) and an mGlu2/3 receptor agonist LY379268 has inhibited cue-induced relapse in animals (Bäckstrom and Hyytiä, 2005). Recent whole-hemisphere autoradiography studies have shown that type 1 alcoholics have increased mGlu5 and type 2 alcoholics increased mGlu2/3 receptor levels, indicating that sub-groups of alcoholics might benefit differently from medications affecting these glutamatergic receptors (Kupila et al., 2013; Laukkanen et al., 2015b).

Overall, there is still a need for further research to clarify the role of the glutamatergic system in alcoholism and addiction, especially in cortical regions. Understanding the role of glutamatergic changes in alcoholism, which could be different in different alcoholics (Kupila et al., 2013; Kupila et al., 2015; Laukkanen et al., 2015b), could enable optimization of current and novel treatment options. Furthermore, function of glutamatergic and GABAergic systems is modulated by e.g. endocannabinoid and serotonergic systems, as well as by neuroactive steroids. These will be reviewed in the following chapters.

2.7 ENDOCANNABINOID SYSTEM

Fatty acid amides of amino acids (FAAAs) and related compounds have significant modulating effects on certain CNS functions e.g. stress and emotions (De Petrocellis et al., 2001; De Petrocellis et al., 2004; Kim and Watkins, 2014). Many of FAAAs are endogenous agents of cannabinoid receptors (Devane et al., 1988; Howlett et al., 1986; Mechoulam et al., 1988). Cannabinoid receptor 1 (CB1) (Matsuda et al., 1990) and cannabinoid receptor 2 (CB2)

(Munro et al., 1993), are G protein-coupled receptors (GPCRs) and inhibit adenylate cyclase and increase mitogen-activated protein kinase (MAPK) activity (Mechoulam and Parker, 2013). Anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoyl glycerol (2-AG) were the first two endocannabinoids to be isolated (Devane et al., 1992; Mechoulam et al., 1995). Much less work has been done in examining other endogenous FAAAs (Mechoulam and Parker, 2013).

Unlike many other neurotransmitters, endocannabinoids are synthetized on demand. The classical retrograde endocannabinoid action is displayed by 2-AG. It is first synthetized in the postsynaptic cell and then released into the synapse where it activates the presynaptic CB1 receptors (Howlett et al., 2002; Mechoulam and Parker, 2013). However, the role of AEA is more complex. Similar to 2-AG, AEA is synthetized on demand in the postsynaptic cell but in addition to CB1 it also binds to other targets e.g. TRPV1 receptors which are postsynaptic (Chavez et al., 2010; Felder et al., 1993). Therefore, in addition to modulating the activity of the presynaptic cell, AEA can also influence the properties of postsynaptic cells.

Endocannabinoids are considered to be removed from the synapse by a rapid membrane transport process. However, this process is still unclear since evidence is somewhat contradictory for example for FAAH-like anandamide transporter, which is one of the main candidates (Fu et al., 2011; Leung et al., 2013). This process may be crucial also in mediating AEA's effect on the endovanilloid system, since the binding site for AEA in the TRPV1 receptor is inside the cell.

In the cell, fatty acid amide hydrolase (FAAH) is the primary hydrolyzing enzyme of AEA (Ahn et al., 2009). 2-AG is hydrolyzed by monoacylglycerol lipase (MAGL), FAAH, ABHD6 and ABHD12. MAGL is responsible for the majority (approximately 85%) of 2-AG hydrolysis and this enzyme is colocalized with CB1 receptors (Savinainen et al., 2012). The roles of ABHD6 and ABHD12 have been poorly characterized, but ABHD6 has been associated with controlling endocannabinoid levels in the site of generation and ABHD12 in the microglial cells (Savinainen et al., 2012). An interplay between neurons and astrocytes seem to coordinate 2-AG metabolism by MAGL, thus controlling CB1 activation (Viader et al., 2015). Furthermore, astrocytes seem to be mainly responsible for converting 2-AG into prostaglandins therefore also controlling part of the neuroinflammation process associated with endocannabinoid function.

CB1 is one of the most abundant GPCRs in the brain but is also expressed in many peripheral organs. CB1 receptors are mainly presynaptic and have been associated with modulation of both GABAergic and glutamatergic function (Howlett et al., 2002). CB1 mediated inhibition of adenylate cyclase causes a decrease in the cyclic adenosine monophosphate (cAMP) levels and further inhibition of cAMP dependent protein kinases (Mechoulam and Parker, 2013). Moreover, the CB1 receptor activation induced increase in MAPK activity has been associated with synaptic plasticity and cell migration (Howlett et al., 2002). CB1 receptors influence the maturation of CNS from the embryonal stages to adolescence (Fride et al., 2009). Furthermore, the distribution of CB1 receptors changes during maturation with neonatal brain showing increased levels of CB1 in the white matter compared to the amount in a mature brain (Romero et al., 1997). Furthermore, during adolescence, CB1 receptor levels increase contrary to many other receptor systems (Verdurand et al., 2011). CB1 receptor polymorphisms have been postulated to contribute to the risk of development of addiction, however the importance of these polymorphisms is not yet known (Zhang et al., 2004).

CB2 receptors are also present in the immune system and are expressed also in the CNS in the glial and neural cells although at lower levels than CB1 (Brusco et al., 2008; Nunez et al., 2004). The CB2 receptor has been proposed to be part of the general protective system against non-protein stressors (Mechoulam and Parker, 2013). A CB2-specific agonist has been developed, HU-308 (Hanus et al., 1999) and this group of compounds has been claimed to be useful in a variety of condition including neuropsychiatry and liver diseases since they do not exert the psychopharmacological effects of CB1 agonists (Mechoulam and Parker, 2013).

These two receptors mediate many of the typical pharmacological effects of cannabis use, which include mild euphoria, sedation, relaxation, a sense of hunger, enhanced sensory input, as well as impaired attention, memory, and sense of time (Borgelt et al., 2013). Δ9-tetrahydrocannabinol (THC) is a partial agonist of the CB1 receptor and the main active ingredient associated with typical psychopharmacological effects of *cannabis sativa* (Howlett et al., 2002; Martin et al., 2002). One other major active principle of cannabis, cannabidiol (CBD), is an anti-inflammatory agent with a complex pharmacology which counteracts many of the effects of THC (Campos et al., 2012). It has been reported that CBD acts as a CB1 antagonist and a CB2 inverse agonist (Thomas et al., 2007), but interestingly it exerts effect also on other neurotransmitter systems, e.g. acting as an agonist of TRPV1 (Bisogno et al., 2001) and 5-HT_{1A} receptors (Russo et al., 2005).

2.7.1 Role of the endocannabinoid system in anxiety, neurogenesis and reinforcement learning

The endocannabinoid system is important for several brain functions which are associated with alcoholism (Henderson-Redmond et al., 2015; Mechoulam and Parker, 2013). These include stress, neurogenesis, reinforcement and cognition processes.

The endocannabinoid system is one of the major stress modulating systems in the CNS e.g. in the homeostatic regulation of the HPA axis (Mechoulam and Parker, 2013). Classical cannabinoid receptor agonists and endocannabinoids exert a biphasic action. Depending on dose and personal characteristics, cannabinoids can produce euphoria and relaxation or alternatively dysphoria and anxiety (D'Souza et al., 2004; Wade et al., 2003). In rodents, low dose injection of methanandamide (a stable form of AEA) into the PFC has an anxiolytic effect, whereas a larger dose evokes an anxiogenic response (Rubino et al., 2008a; Rubino et al., 2008b). AEA's ability to stimulate TRPV1 has been considered to be one possible explanation for its anxiogenic effects (Moreira et al., 2012). At the cellular level, CB1 activation decreased whereas TRVP1 activation increased intracellular calcium levels (Moreira et al., 2012).

FAAH inhibition can also be used to increase AEA levels to produce anxiolytic effect which can be blocked by CB1 inhibition (Kathuria et al., 2003). However, it should be remembered that FAAH inhibition leads to increased levels also of other fatty acid amides, such as n-palmitoyl ethanolamine (PEA) (Moise et al., 2008). Furthermore, also blocking the MAGL in order to increase 2-AG levels has been shown to have anxiolytic effects (Sciolino et al., 2011). Moreover, the only CB1 antagonist/inverse agonist, rimonabant, that was on the market, had to be withdrawn because it enhanced anxiety and suicidal tendencies in patients even though psychiatric disorders were indicated as exclusion criteria for its use as a weight-reducing agent (Christensen et al., 2007).

Furthermore, chronic consumption of alcohol has been associated with decreased neurogenesis, which is another important aspect of CNS function associated with the endocannabinoid system (Galve-Roperh et al., 2009; Mechoulam and Parker, 2013; Oudin et al., 2011). As mentioned above, CB1 receptors are expressed in the developing brain and their activation is needed for normal axonal growth and cell migration (Fride et al., 2009; Williams et al., 2003; Zhou et al., 2015). However, endocannabinoid function is also important for adult neurogenesis in conjunction with EGF and FGF signaling (Sutterlin et al., 2013). Endocannabinoid signaling has been associated with the promotion of proliferation and astrocyte differentiation of neural progenitor cells (Aguado et al., 2005; Aguado et al., 2006; Palazuelos et al., 2006). Both CB1 and TRPV1 knock-out mice show evidence of dysfunctional adult neurogenesis (Jin et al., 2004). Furthermore, both synthesizing diacylglycerol lipases (DAGLs) and metabolizing MAGL enzymes are needed for the axonal growth promoting and neural guidance action of 2-AG (Keimpema et al., 2010).

Moreover, the endocannabinoid system has a complex role in motivational processes such as reinforcement learning (Mechoulam and Parker, 2013). As stated above in chapter 2.3.2.,

in humans there are conflicting reports on the ability of THC to increase dopamine release in the striatum (Barkus et al., 2011; Bossong et al., 2009). Similarly to its biphasic effect on stress modulation, depending on study design and dose used THC can be either rewarding or aversive for animals (Gardner et al., 1988; Harris et al., 1974; Lepore et al., 1995; Mallet and Beninger, 1998; Parker and Gillies, 1995). Low-dose pre-exposure or very low dose paradigms seem to be required for conditioned place preference or self-administration to develop in rodents (Tanda et al., 2000; Valjent and Maldonado, 2000).

In relation to its role in addiction, the endocannabinoid system has been most convincingly associated with cue and drug-induced relapses. In humans, cannabis cues activate the reward circuit including NAC, and alterations in the CB1 and FAAH genes have been associated with enhanced activation (Filbey et al., 2010). CB1 antagonism was shown to decrease gradually the release of dopamine to NAC reducing the positive reinforcement properties of rewarding stimuli (Trujillo-Pisanty et al., 2011). One possible mechanism is modulation of the function of GABAergic projections from the rostromedial tegmental nucleus which then inhibits the activity of dopaminergic neurons in the VTA (Melis et al., 2014). Furthermore, 2-AG seems to function as a negative regulator of LTP process in glutamatergic synapses in the VTA (Kortleven et al., 2011). Moreover, there seems to be gender differences in the 2-AG's modulation of dopamine release in the VTA, adding the possibility of some interplay between endocannabinoids and sex hormones (Melis et al., 2013).

Moreover, endogenous cannabinoids AEA and 2-AG are self-administered by squirrel monkeys (Justinova et al., 2005; Justinova et al., 2011). AEA increases dopamine release in the NAC in a CB1 mediated manner (Justinova et al., 2005; Justinova et al., 2011; Solinas et al., 2006). However, the FAAH inhibitor UR597 has not been self-administered on its own but does increase AEA induced dopamine release and decreases the dose needed for self-administration behavior to develop with AEA administration (Justinova et al., 2008; Solinas et al., 2007). In line with these results, FAAH inhibition alone does not produce conditioned place preference (Gobbi et al., 2005).

2.7.2 Endocannabinoid system and cognition

The hippocampus is one of the brain areas with most abundant levels of CB1 receptors; it is also a key brain structure for acquiring, storing and retrieving memories (cognition). Acute administration of CB1 agonist THC has been associated with transient impairment of short-term episodic and working memory, but does not seem to influence the retrieval of old memories (Ranganathan and D'Souza, 2006). In line with human data, CB1 agonists have disrupted short-term or working memory in animals, but not retrieval of previous memories (Lichtman and Martin, 1996; O'Shea et al., 2004). Cognition impairing effect seemed to be more long lasting in adolescent rats that adults, which is in line with the evidence that the endocannabinoid system plays a major role in the maturation of the CNS (Nakamura et al., 1991; O'Shea et al., 2004). However, the literature about the effects of chronic cannabis exposure to memory is more contradictory (Mechoulam and Parker, 2013). Polydrug use and preexisting differences between users and non-users make comparisons difficult. One of the best studies of exclusively cannabis user concluded that current heavy users have cognitive impairments, but former heavy users do not show cognitive impairment (Fried et al., 2005). Long term abstinence (at least 3 months) has been considered to lessen the degree of cognitive impairment caused by cannabis use.

In contrast to THC, endogenous cannabinoids do not produce consistent impairment of cognition. FAAH inhibition, but not MAGL inhibition, has been shown to disrupt consolidation of fear and object recognition memory (Busquets-Garcia et al., 2011). A recent study showed that impaired MAGL function in the hippocampus increased anxiety-like behavior but did not affect the formation of aversive memories (Guggenhuber et al., 2015). However, FAAH inhibition has also been associated with improvement of working memory, possibly in a PPAR-α mediated manner (Mazzola et al., 2009; Varvel et al., 2007) i.e. increasing the levels of PPAR-α agonists e.g. PEA.

CB1 agonist induced inhibition of glutamatergic activity in the hippocampus could be one possible mechanism for impaired working memory (Heifets and Castillo, 2009; Mechoulam and Parker, 2013). Endocannabinoid-induced LTD can be achieved via multiple mechanisms. Simultaneous NMDA and CB1 receptor activation leads to decreased cAMP-PKA activity and LTD (Heifets and Castillo, 2009). Furthermore, activation of mGluR5 and further release of AEA are necessary for TRPV1 mediated LTD in the hippocampus (Chavez et al., 2010).

Moreover, the results from effects of CB1 antagonism on memory have been mixed. In simplified terms, CB1 antagonism should be able to prolong the duration of memory but it does not seem to facilitate learning (Varvel et al., 2009). In the basolateral amygdala, inhibition of CB1 receptor function prevented the acquisition of associative fear memory but did not affect the recall or consolidation of memories (Tan et al., 2011). Furthermore, an interaction with the glucocorticoid system seems to be important for memory function of endocannabinoids in the amygdala (Ramot and Akirav, 2012)

Moreover, the endocannabinoid system has been associated with extinction learning of aversively motivated learning behavior (Marsicano et al., 2002). In rats, treatment with an FAAH inhibitor enhanced extinction of fear-potentiated startle and spatial memory (Chhatwal et al., 2005; Varvel et al., 2007). Furthermore, this enhanced extinction seemed to be specific to aversive stimuli and was not observed in response to positive reinforcement from food (Holter et al., 2005). For this reason, enhancing endocannabinoid system function has been proposed to be a possible way to enhance the treatment of post-traumatic stress disorder (Mechoulam and Parker, 2013).

2.7.3 Alcohol and endocannabinoid system

The function of the endocannabinoid system is believed to be important in the pathology of alcoholism (Henderson-Redmond et al., 2015; Pava and Woodward, 2012). One of the key points has been considered to be the endocannabinoid regulation of stress response (Ruehle et al., 2012) and stress-enhanced negative reinforcement learning (Campolongo et al., 2009; Ramot and Akirav, 2012). However, also the role of endocannabinoid function in the ethanol induced habit formation has attracted interest in recent years (DePoy et al., 2013; DePoy et al., 2015). One important point is that there are some clear differences in results from animal and human studies on the role of endocannabinoid system in alcohol consumption.

In animals, the activity of endocannabinoid system has been associated with ethanol consumption and positive reinforcement. CB1 knock-out mice show decreased ethanol consumption and reduced ethanol induced conditioned place preference (Houchi et al., 2005; Poncelet et al., 2003). In addition, pharmacological blockade of CB1 has been associated with reduced ethanol consumption in animals and CB1 agonism with increased ethanol intake (Colombo et al., 1998; Colombo et al., 2002). Administration of the CB2 agonist JWH 015 enhanced the development of ethanol preference in stressed mice (Onaivi et al., 2008). Pharmacological blockade or genetically knocking out CB1 receptor prevents ethanol induced dopamine release in the NAC in mice (Femenia et al., 2010; Hungund et al., 2003). This effect on dopamine firing might be dependent on cannabinoid activity induced suppression of inhibition from rostromedial tegmental nucleus GABAergic neurons to VTA neurons (Melis et al., 2014). Moreover, cocaine induced conditioned place preference appeared to be normal in CB1 knock-out mice, indicating that dopamine function is relatively normal in this animal model (Houchi et al., 2005). Therefore, CB1 mediated effects of ethanol seem to be especially important for ethanol induced positive reinforcement.

However, in rats, FAAH inhibition which increases the levels of AEA and other fatty acid amines did not affect ethanol drinking behavior although it has been associated with decreased symptoms of anxiety during ethanol withdrawal (Cippitelli et al., 2008). In contrast, FAAH inhibition and knock-out increased ethanol consumption in mice in a sex specific manner (Basavarajappa et al., 2006; Blednov et al., 2007; Vinod et al., 2008). Furthermore, FAAH knock-out mice had no alterations in ethanol induced conditioned place preference (Blednov et al., 2007). Moreover, astrocyte glutamate transporter (EAAT1) knock-

out mice, which display decreased endocannabinoid signaling, exhibited decreased ethanol consumption and conditioned place preference (Karlsson et al., 2012). At the present time, there are no published studies evaluating the effect of an MAGL inhibitor on ethanol consumption.

Ethanol consumption has been reported to exert variable effects on the endocannabinoid system; these might well be animal model, brain region and cell type specific. In the case of acute ethanol administration, there are conflicting reports showing both increased and decreased AEA levels in rats (Ceccarini et al., 2013; Ferrer et al., 2007). In hippocampal neurons, acute ethanol administration seemed to increase endocannabinoid activity (Basavarajappa et al., 2008). Increased AEA levels in the hippocampus inhibited LTP, learning and memory formation in the mice and this could contribute to the cognitive impairment caused by ethanol (Basavarajappa et al., 2014).

Chronic ethanol administration increased AEA levels in the cortex of mice (Vinod et al., 2006). In contrast, increased 2-AG but not AEA levels were seen in the striatum of mice after chronic ethanol exposure (DePoy et al., 2013). Furthermore, chronic ethanol exposure seemed to increase AEA and 2-AG levels in the striatum and limbic forebrain of rats (Gonzalez et al., 2004; Vinod et al., 2012). Chronic ethanol treatment has been associated with decreased CB1 and CB2 receptor levels in rodents (Ceccarini et al., 2013; Coelhoso et al., 2013; Vinod et al., 2006).

Moreover, withdrawal was described to decrease endocannabinoid levels in the limbic forebrain of rats (Gonzalez et al., 2004). Furthermore, decreased gene expression of FAAH, MAGL, CB1 and CB2 has been reported, especially after repeated ethanol withdrawals in the rat amygdala (Serrano et al., 2012). However, these effects might be brain region specific since in the hippocampus, endocannabinoid levels were increased and CB1 expression decreased after repeated withdrawals (Mitrirattanakul et al., 2007). Persistently increased CB1 levels after withdrawal have also been described in the striatum, possibly contributing to endocannabinoid-mediated re-establishment of ethanol consumption (Ceccarini et al., 2013; Coelhoso et al., 2013). CB2 receptor up-regulation has also been associated with ethanol withdrawal in mice (Coelhoso et al., 2013).

Furthermore, differences in the function of endocannabinoid system have been found between alcohol preferring and alcohol non-preferring rat lines. These included increased levels of CB1 receptors and endocannabinoids AEA and 2-AG as well as decreased FAAH levels (Malinen and Hyytiä, 2008; Vinod et al., 2012). Chronic ethanol consumption has been claimed to exert a sex specific effect in alcohol preferring AA rats (Malinen et al., 2009). After chronic but limited access to ethanol, subsequently abstinent female AA rats showed increased concentrations of AEA. In this group, reintroduction of ethanol decreased AEA levels and increased 2-AG levels . In contrast, ethanol consumption after abstinence increased AEA levels in male AA rats (Malinen et al., 2009).

The CB1 agonists like WIN 55,212-2, 2 and THC have potentiated the re-establishment of ethanol consumption in rats (Alen et al., 2008; McGregor et al., 2005). However, this effect was not selective and CB1 increased appetitive effect has been considered to be important for this phenomenon. Interestingly CB1 antagonists seem to decrease cue-induced but not stress-induced reinstatement of ethanol consumption in rats (Cippitelli et al., 2005; Economidou et al., 2006). The reduction of cue-induced reinstatement of ethanol consumption may be associated with the habit formation inducing property of ethanol, which has been associated with endocannabinoid function in the dorsolateral striatum (DePoy et al., 2013; DePoy et al., 2015; Hilario et al., 2007). Compared to ventral or dorsomedial striatum, dorsolateral striatum has more CB1 receptors (Herkenham et al., 1991). The dorsolateral striatum receives a dopaminergic input mainly from SN and it interacts with the sensorimotor cortex and is considered to be important for development of habitual behaviors (Hilario et al., 2007; Moore et al., 2001; Yin et al., 2004; Yin et al., 2005).

CB1 receptor activation is connected through cAMP and MAPK activity with the cAMP response element-binding protein (CREB) and extracellular signal-regulated kinase (ERK)

pathways. Acute ethanol inhibited ERK and CREB signaling, reducing levels of active phosphorylated forms in the rat brain (Chandler and Sutton, 2005). Furthermore, ethanol withdrawal decreased the levels of CREB gene expression in the rat PFC (Pandey et al., 2001).

Decreased CB1 levels and activity have been described in the post-mortem NAC brain samples of alcoholics (Vinod et al., 2010). In the same study, suicidal alcoholics had increased FAAH activity in the NAC whereas non-suicidal alcoholics had reduced FAAH levels compared to controls. Moreover, there is a report that alcoholics seem to have hyperfunctional CB1 receptors in the caudate nucleus and cerebellum compared to controls (Erdozain et al., 2015a). Furthermore, decreased AEA levels have been detected in the post-mortem brain NAC, ACC and FC samples of late onset Cloninger type 1 alcoholics, but not in early onset type 2 alcoholics, in comparison to non-alcoholic controls (Lehtonen et al., 2010). In addition, an altered function of the endocannabinoid system has been described in the post-mortem PFC of alcoholic subjects, especially those who committed suicide. For example, the decreased levels of the phosphorylated active forms of CREB and ERK were observed in the PFC of all alcoholics and decreased CB1 gene expression was seen in suicidal alcoholics compared to controls (Erdozain et al., 2015b). When measured with PET imaging, acute alcohol exposure seems to increase CB1 receptor availability (Ceccarini et al., 2014). In contrast, CB1 receptor availability seems to decrease after chronic exposure as well as during abstinence when measured with PET imaging agents [^{18}F]MK-9470 and [^{18}F]FMPEP-d_2 (Ceccarini et al., 2014; Hirvonen et al., 2013). However, when measured with another PET imaging agent, [^{11}C]OMAR, abstinent alcoholics showed increased CB1 receptor binding (Neumeister et al., 2012). The differences between the *in vivo* PET imaging studies in abstinent alcoholics could be due to use of different imaging agents or in the selection of study populations. Table 3 shows a summary of the findings in the endocannabinoid system in human alcoholics.

Table 3. Summary of reported alterations in the endocannabinoid system in human alcoholics.

Main findings	Reference(s)
Post-mortem brain samples	
Decreased anandamide levels in the NAC, ACC and frontal cortex of Cloninger type 1 alcoholics	Lehtonen et al., 2010
Decreased CB1 receptor activity in the NAC of alcoholics	Vinod et al., 2010
Increased CB1 receptor activity in the caudate nucleus and cerebellum of alcoholics	Erdozain et al., 2015a
Increased FAAH activity in the NAC of suicidal alcoholics	Vinod et al., 2010
Decreased CB1 gene expression in the frontal cortex of suicidal alcoholics	Erdozain et al., 2015b
In vivo imaging	
Increased CB1 availability after acute alcohol exposure ([^{18}F]MK-9470)	Ceccarini et al., 2014
Decreased CB1 availability after chronic alcohol exposure ([^{18}F]MK-9470)	Ceccarini et al., 2014
Decreased CB1 receptor availability in abstinent alcoholics([^{18}F]MK-9470 and [^{18}F]FMPEP-d_2)	Ceccarini et al., 2014, Hirvonen et al., 2013
Increased CB1 receptor availability in abstinent alcoholics ([^{11}C]OMAR)	Neumeister et al., 2012

Studies in humans have concentrated on the striatal and frontal cortical regions. However, consumption of ethanol has also been associated with alterations in 2-AG and AEA levels in both amygdala and hippocampus in rodents (Gonzalez et al., 2004; Malinen et al., 2009; Rubio et al., 2007). Chronic ethanol consumption decreased the effect of CB1 receptors to

GABAergic signaling in the amygdala in a non-direct manner (Varodayan et al., 2015). Furthermore, activation of CB1 receptors inhibited GABA efflux in the mouse hippocampus (Ando et al., 2012) and CB1 knock-out mice displayed signs of increased neuroinflammation in the hippocampus and this effect was associated with CB1 receptor function in the GABAergic interneurons (Albayram et al., 2011). Interestingly, CB1 knock-out mice displayed enhanced spatial learning at an early age but reduced spatial learning when they were elderly (Albayram et al., 2012). However, because there are controversies in the human and animal data regarding endocannabinoid function, there is also a need to study the changes in endocannabinoid levels in the hippocampal and amygdala in human alcoholics.

In conclusion, the endocannabinoid system has a major function is modulating cognition, stress and reinforcement learning. The results of animal and human experiments emphasize the importance of the endocannabinoid system in alcoholism, even though some results are not identical between humans and rodents. Moreover, many of the changes observed in the endocannabinoid system in response to ethanol consumption and withdrawal seem to be brain region and even cell type specific. Modifying the activity of the endocannabinoid system might also be relevant as a way to decrease the harmful effects of alcohol on the CNS via modulation of neurodegeneration and neuroinflammation (Collins and Neafsey, 2012). In addition to 2-AG and AEA, also other AEA-like molecules e.g. docosahexaenoylethanolamide and PEA could have important roles in the alcohol induced CNS pathology because they modulate neuroinflammatory processes (Kim et al., 2011a; Kim and Watkins, 2014; Mattace Raso et al., 2014). Furthermore, medication affecting the endocannabinoid system might be beneficial in reducing alcohol induced habit formation perhaps being able to break the cue induced relapse in alcohol abuse re-establishment (Cippitelli et al., 2005; DePoy et al., 2013; DePoy et al., 2015; Economidou et al., 2006).

2.8 SEROTONERGIC REGULATION OF BEHAVIOR

The serotonergic system has a wide role in regulating many bodily functions, e.g. body temperature and sleep. Serotonin (5-hydroytryptamine, 5-HT) has also been associated with a modulation of the psyche, e.g. mood and cognition. In the brain, serotonergic projections from raphe nuclei project to many brain regions associated with addiction, including the striatum, hippocampus, amygdala, and both frontal and posterior cortical regions (Parent, 1981; Parent et al., 1981; Varnäs et al., 2004).

The serotonergic system has an important role in the regulation of alcohol intake and decreased serotonergic tone has been associated with alcoholism (Cloninger, 1987; Marcinkiewcz, 2015; Sari et al., 2011). Acute administration of ethanol increases extracellular 5-HT levels, possibly one of the reasons why people with low serotonergic tone could show a preference for alcohol use. However, chronic alcohol consumption has decreased serotonergic tone (Sari et al., 2011). Furthermore, one possible cause of the imbalanced serotonergic function in the brains of alcoholics is early life stress which affects both 5-HT concentrations as well as levels of some serotonin receptor subtypes e.g. 5-HT_{1A} (Berglund et al., 2013; Lanzenberger et al., 2007; Spinelli et al., 2010). Therefore, the changes in the serotonergic system in alcoholics could both precede the alcohol use disorder, e.g. early life stress, or could be produced by chronic consumption (Marcinkiewcz, 2015; Sari et al., 2011). It is plausible that both processes will affect the final outcome.

Alcohol has a direct pharmacological action on serotonin 3 receptors (5-HT_3) (Lovinger and Zhou, 1994). 5-HT_3 is an ionotropic excitatory cell membrane receptor e.g. found on inhibitory GABA interneurons, modulating the activity of these neurons in different neurocircuits (Celada et al., 2013). This serotonergic modulation of GABAergic interneurons can influence the inhibition of glutamatergic neurons which in turn can influence alcohol induced behavioral changes.

While the serotonergic system is widespread throughout the brain, there are distribution differences between serotonergic receptors, e.g. 5-HT_{1B} are expressed in the NAC, whereas the 5-HT_{1A} receptors are expressed in the cortex but not in the NAC (Storvik et al., 2009; Storvik et al., 2012). Therefore different serotonergic receptors influence behavior differently. For example, inhibitory control over behavior can be influenced by 5-HT_{1A} and 5-HT_{2A} receptors in the PFC which can disinhibit glutamatergic activity by inhibiting local GABAergic interneurons (Puig et al., 2010). On the other hand, 5-HT_{1B} receptor mediated activity in presynaptic side of glutamatergic cells can inhibit their activity on the medium spiny neurons in the NAC (Muramatsu et al., 1998). Moreover, 5-HT_{2C} receptor activation can increase the activity of striatal interneurons including cholinergic as well as fast spiking and low-threshold spike GABAergic interneurons which further inhibit glutamatergic input to medium spiny neurons in the striatum (Blomeley and Bracci, 2005; Blomeley and Bracci, 2009; Cains et al., 2012).

Furthermore, in addition to glutamatergic and GABAergic systems, there is also a complex interaction between the serotonergic and endocannabinoid systems. CB1 activation in mouse brain cortical slices inhibited 5-HT release (Nakazi et al., 2000) and the CB1 antagonist SR141716A administration increased 5-HT levels in the PFC and NAC (Darmani et al., 2003; Tzavara et al., 2003). One possible mechanism involves inhibition of dorsal raphe glutamate release by CB1 receptor activation by AEA (Haj-Dahmane and Shen, 2009). Furthermore, endocannabinoid release can be induced by serotonergic activity and this effect has been associated with the 5-HT_2 activation induced activation of phosphatidylinositol-specific phospholipase e.g. leading to release of 2-AG (Best and Regehr, 2008; Haj-Dahmane and Shen, 2009; Parrish and Nichols, 2006).

Moreover, serotonergic drugs have been studied to determine whether they are beneficial in the treatment of alcoholism. Several targets have been used, including serotonin transporter (SERT, 5-HTT) and serotonergic receptors e.g. 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_3 receptors (Johnson, 2008; Litten et al., 2012). However, with respect to the serotonergic receptors, only 5-HT_7 gene polymorphism has been associated with alcoholism in genome wide association studies, suggesting that changes seen in alcoholics in other serotonergic target proteins could be more likely due to the interaction of environmental factors with genetics, including epigenetic modulations (Kim et al., 2014b; Zlojutro et al., 2011; Zuo et al., 2014). The present literary review will concentrate on SERT, but also the role of above mentioned serotonergic receptors in alcoholism will be reviewed shortly in order to obtain an overview of the complex role of serotonergic system in alcoholism.

2.8.1 Serotonin transporters

SERT belongs to the SLC6 family of transporters and is a transmembrane monoamine neurotransmitter sodium symporter which is sodium and chloride dependent. SERT controls serotonin concentrations in the synaptic cleft in both the CNS and periphery. Raphe nuclei are the primary source of serotonergic projections in the brain.

There is a positive correlation between ethanol intake and SERT availability in the raphe nuclei in non-human primates (Heinz et al., 2003). Furthermore, voluntary ethanol consumption increased SERT gene expression in the raphe nuclei in rats (Oliva and Manzanares, 2007). Interestingly, treatment with the MOR antagonist, naloxone, after ethanol consumption seems to induce small decrease in SERT gene expression in the raphe nuclei pointing to a possible role for the opioid system in alcohol induced serotonergic changes (Oliva and Manzanares, 2007). Moreover, ethanol exposure also increases SERT expression and function in human dendritic cells in a cAMP mediated manner (Babu et al., 2009). This effect might be associated with modulation of neural immune responses, which are also associated with endocannabinoid and neuroactive steroid function, since SSRI treatment reduced ethanol induced inflammation and tissue damage in rats (Hu et al., 2013).

The results of ethanol to increase SERT levels in animals and cell models are in general opposite to those obtained from human imaging studies, where decreased or unaltered SERT

levels have been reported. In post-mortem brains of non-abstinent human alcoholics, [^{3}H]citalopram binding to SERT has been reported to be decreased in the ACC, dorsal amygdala and caudate nucleus (Mantere et al., 2002; Storvik et al., 2006a; Storvik et al., 2006b; Storvik et al., 2007; Storvik et al., 2008). In agreement, a SPECT study with [^{123}I]β-CIT and a PET study with [^{11}C](+)McN5652 have reported decreased SERT binding in the brainstem, thalamus, amygdala, anterior and posterior cingulate cortex, frontal cortex and cerebellum of abstinent alcoholics (Heinz et al., 1998; Szabo et al., 2004). Furthermore, decreased SERT binding in the brainstem and ACC was detected in violent offenders and subjects with impulsive aggression as measured with [^{11}C]McN5652 and [^{123}I]b-CIT (Frankle et al., 2005; Tiihonen et al., 1997).

However, when using the PET tracer [^{11}C]DASB, no significant differences were detected in SERT binding between abstinent alcoholics and controls and more specifically between abstinent Cloninger type 2 alcoholics and controls (Brown et al., 2007; Martinez et al., 2009). Compared to previous SPECT and PET studies, [^{11}C]DASB has higher specificity and a faster washout time, which could partially explain differences between the studies. In addition, genetic polymorphism in the SERT gene affects SERT binding, which could in part explain the disparity between the SERT binding studies in humans (Praschak-Rieder et al., 2007). Furthermore, in the *in vivo* imaging studies, all alcoholics were abstinent and had been subjected to detoxification treatment. This difference could account for some of the binding differences between *in vivo* imaging (Brown et al., 2007; Heinz et al., 1998; Martinez et al., 2009; Szabo et al., 2004) and the post-mortem studies in which most alcoholics had been intoxicated just prior to death (Mantere et al., 2002; Storvik et al., 2006a; Storvik et al., 2006b; Storvik et al., 2007; Storvik et al., 2008). The main findings of the SERT-imaging studies in humans are summarized in table 4.

Table 4. Summary of human imaging studies measuring SERT binding in alcoholics

Main findings	Reference(s)
Post-mortem whole-hemisphere autoradiography	
Decreased [^{3}H]citalopram binding to SERT has been reported in the ACC, dorsal amygdala and caudate nucleus of non-abstinent human alcoholics	Mantere et al., 2002, Storvik et al., 2006a, Storvik et al., 2007
In vivo imaging	
Decreased [^{123}I]β-CIT binding to SERT in dorsal brain stem (raphe nuclei) of abstinent alcoholics	Heinz et al. 1998
Decreased [^{11}C](+)McN5652 binding to SERT in pons, midbrain, thalamus, amygdala, orbitrofrontal cortex, ACC, PCC and cerebellum of abstinent alcoholics	Szabo et al. 2004
No significant differences in [^{11}C]DASB binding to SERT in abstinent alcoholics	Brown et al., 2007; Martinez et al., 2009

Serotonin transporter polymorphism and alcoholism

A SERT gene polymorphism has been associated with alcoholism. SERT transcription can be altered by a functional polymorphism in the 5-HTT-linked promoter region (5-HTTLPR). 5-HTTLPR has two functional polymorphisms denoted as long (L) and short (S) alleles. Two copies of L-allele (LL) have been associated with increased SERT activity and increased mRNA density than LS or SS genotypes. The 5-HTTLPR polymorphism has been associated with alcohol craving, withdrawal and alcohol induced neurotoxicity (Ait-Daoud et al., 2009; Johnson et al., 2008). Interestingly, both the short (Feinn et al., 2005; Sander et al., 1997) and long (Heinz et al., 2000; Johnson et al., 2008) alleles have been associated with alcoholism. However, it should be noted that not all studies have found associations between 5-HTTLPR polymorphism and alcoholism (Choi et al., 2006; Kohnke et al., 2006).

In more detail, the L-allele has been associated with increased alcohol craving and fewer negative effects with first exposure as well as lower scores in socialization (Ait-Daoud et al., 2009; Herman et al., 2011). In contrast, the S allele has been linked with Cloninger type 2 alcoholism (Hallikainen et al., 1999), larger intake (Johnson et al., 2008), increased withdrawal symptoms (Kohnke et al., 2006) as well as tolerance (Turker et al., 1998), binge drinking (Herman et al., 2005), earlier onset of alcoholism (Johnson et al., 2008) and relapses (Pinto et al., 2008). LL-carriers have higher SERT binding in the raphe nuclei in comparison to SS subjects, and importantly LL-carrier alcoholics show decreased SERT binding compared to LL-controls, which was not the case between SS-alcoholics and SS-controls, as measured with [^{123}I]β-CIT SPECT (Heinz et al., 2000).

In meta-analyses, alcoholics were estimated to be 15-18% more likely to have an S allele (Feinn et al., 2005; McHugh et al., 2010). Due to weakness of this association, there might not be any direct link between 5-HTTLPR polymorphism and alcoholism. The observed association could be indirect, since SERT function has also been associated with stress and emotional regulation (McHugh et al., 2010). Early life stress has been associated with serotonergic dysfunction and with an increased risk of alcohol dependence (Berglund et al., 2013). The 5-HTTLPR polymorphism could be part of the personal genetic makeup that makes an individual vulnerable to the effects of early life stress. In line with this reasoning, the 5-HTTLPR S-allele was especially associated with early onset of alcoholism and with those alcoholics with comorbid psychiatric disorders (Feinn et al., 2005). Importantly, also antisocial behavior has been associated with 5-HTTLPR polymorphism (Johnson et al., 2008). In line with this profile, S-allele has been associated with Cloninger type 2 alcoholics (Hallikainen et al., 1999), who often have experienced strong adverse childhood experiences, have an antisocial personality and develop alcohol dependency early and seem to portrait decreased serotonergic function in the brain (Leggio and Addolorato, 2008).

In the case of L-carriers, who seem to have increased SERT activity and decreased synaptic 5-HT levels compared to S-carriers, chronic alcohol consumption maybe the cause of the decreased SERT binding and activity compared to LL-controls (Heinz et al., 2000). This may increase synaptic 5-HT levels and elevate autoreceptor activity which in turn may cause a downregulation of the serotonergic system in chronic L-carrier alcoholics (Ait-Daoud et al., 2009; Sari et al., 2011), plausibly due to a serotonergic influence on different neurotransmitter systems. For example, the LL genotype has been associated with reduced sensitivity to dopaminergic activity, possibly associated with 5-HT$_3$ activity in the activity of dopaminergic nerves, linking 5-HTTLPR polymorphism to the previously reviewed role of dopamine in alcoholism pathology described in chapter 2.3 (Budde et al., 2010; Campbell and McBride, 1995; Minabe et al., 1991). This is a possible scenario to explain how alcohol consumption could induce a down-regulation of the serotonergic system, which could be more important for the changes seen in Cloninger type 1 alcoholics (Mantere et al., 2002; Storvik et al., 2006a; Storvik et al., 2007). However, it is likely that both the social psychical environment and alcohol consumption contribute to the changes seen in the serotonergic system of alcoholics, and furthermore the contribution of these effects is likely to differ between individuals.

In addition, a single nucleotide A to G substitution at rs25531 in the 5-HTTLPR L-allele causes a functional polymorphism, with Lg-allele shown to cause decreased expression patterns compared to La-allele, similar to S-allele. This also affects SERT binding, with Lg/Lg-carriers showing decreased binding compared to La homozygotes (Praschak-Rieder et al., 2007). This could affect the previously reviewed binding experiments and could in part account for the contrasting results. The low activity alleles have been associated with a risk of substance use disorders (Enoch et al., 2011). This polymorphism has not been studied in earlier genetic studies and therefore drawing conclusions from these works is challenging. Newer studies have taken this polymorphism into account. For example, in a recent study of an Italian population, early onset of alcoholism was associated with S and Lg alleles in males (Pascale et al., 2015), in line with previous findings (Johnson et al., 2008). In a pilot study, La homozygotes with late-onset of alcoholism seemed to benefit from sertraline (SSRI)

treatment, whereas S or Lg carriers displayed no effect to treatment and La homozygotes with early-onset of alcoholism actually drank less while they were being treated with the placebo than with sertraline treatment (Kranzler et al., 2011) suggesting additional components to the treatment effect in addition to the 5-HTTLPR polymorphism.

Furthermore, 5-HTTLPR contains a TATA-like domain and binding sites for transcription factors, e.g. the transcription factor AP2 (TFAP2B). The high function allele of TFAP2B has been associated with alcoholism in women (Nordquist et al., 2009); this has been linked with gene expression of SERT as well as MAO-A, which is an important enzyme for monoamine metabolism, including serotonin and dopamine. MAO-A has also been associated with alcoholism, especially antisocial alcoholism, in some (Guindalini et al., 2005; Samochowiec et al., 1999) but not in other studies (Parsian et al., 2003; Saito et al., 2002). Furthermore, MAO-A polymorphism has been associated with alcohol intoxication related behaviors like violence (Tiihonen et al., 2015). It seems that the relationship between MAO-A polymorphism and alcoholism is modulated by environmental factors such as severe childhood stress, quality of family relations and sexual abuse (Nilsson et al., 2011).

Treatment outcomes and SERT

There are conflicting reports on the efficacy of selective serotonin transporter inhibitors (SSRIs) in the treatment of alcoholism with some describing putative benefits (Naranjo et al., 1984; Naranjo et al., 1987) or no treatment effect (Kranzler et al., 1993; Kranzler et al., 1995). For example, treatment with the SSRI fluoxetine has been associated with a reduced urge to drink, a modest decrease in total alcohol intake and decreased short term relapse risk (Janiri et al., 1996; Naranjo et al., 1990; Naranjo et al., 1994). In contrast, some other studies have not found that fluoxetine would be effective in heterogeneous groups of alcoholics, even though it seemed to decrease depressive symptoms (Kranzler et al., 1993; Kranzler et al., 1995). In line with this observation, fluoxetine treatment decreased anxiety and increased the levels of neuroactive steroid allopregnenolone during alcohol withdrawal (Romeo et al., 2000). Some of these effects might be associated with genetic polymorphisms, a factor which was not taken into account in earlier studies. One problem with SSRI medication is that the side-effects of the treatment tend to decrease adherence to therapy (Kranzler et al., 1993; Kranzler et al., 1995; Naranjo et al., 1984).

Another SSRI drug, sertraline, has also been reported to be effective in reducing drinking in sub-groups of alcoholics. Sertraline treatment seems to be effective in late-onset alcoholics, and this effect seem to endure for at least 6 months (Dundon et al., 2004; Kranzler et al., 2012; Pettinati et al., 2000). However, the efficacy of sertraline in late-onset alcoholics seems to be dependent on the 5-HTTLPR polymorphism and only late-onset alcoholics with LL-genotype are believed to benefit from the treatment (Kranzler et al., 2011). There appears to be a gender difference, since women with the SS/SL 5-HTTLPR genotype who were treated with sertraline drank less than sertraline receiving LL women (Kenna et al., 2014b). In this study, results for men were not statistically significant, further highlighting the possible role of steroid sex hormones.

In contrast to late-onset alcoholics, sertraline treatment actually increased alcohol consumption in early-onset alcoholics (Dundon et al., 2004; Kranzler et al., 2012; Pettinati et al., 2000). It has been suggested that early-onset alcoholics have a dysfunctional serotonin metabolism, and thus treating early-onset alcoholics with SSRIs could cause an overstimulation of the serotonergic system, especially 5-HT_3 receptors which modulate dopamine release (Budde et al., 2010; Campbell and McBride, 1995; Minabe et al., 1991). This is considered as a possible mechanism to explain why SSRI treatment could cause an increase in alcohol intake in early-onset alcoholics (Sari et al., 2011).

2.8.2 Other serotonergic targets associated with alcoholism

Serotonergic receptors, with the exception of 5-HT_3 receptors, are G protein coupled receptors with seven transmembrane domains. As mentioned above, even though many

serotonergic receptors have been associated with the pathology of alcoholism, GWA studies have identified only 5-HT$_7$ gene polymorphism as being associated with alcohol dependence (Kim et al., 2014b; Zlojutro et al., 2011; Zuo et al., 2014).

Serotonin 1A receptors

The function of 5-HT$_{1A}$ receptors has been associated with many bodily functions, e.g. regulation of sexual behavior, pain, sleep and body temperature (Ahlenius and Larsson, 1989; Gudelsky et al., 1986; Tissier et al., 1993; Zemlan et al., 1988). The 5-HT$_{1A}$ receptor has also been associated with alcohol consumption and withdrawal as well as with depression and anxiety which are common comorbidities of alcoholism (Kenna, 2010).

5-HT$_{1A}$ receptors are distributed in cortical regions as well as in limbic regions, e.g. hippocampus, but not in the striatum (Storvik et al., 2009; Varnäs et al., 2004). 5-HT$_{1A}$ receptors are located both on the postsynaptic membrane as well as in the cell bodies and dendrites of serotonergic neurons, in which they act as autoreceptors (Verge et al., 1985). Therefore, 5-HT$_{1A}$ receptor activation can modulate the release of other neurotransmitters e.g. dopamine as well as inhibiting the release of serotonin itself (Benloucif and Galloway, 1991).

Alcohol preferring rats seem to have increased 5-HT$_{1A}$ levels, considered to be a compensatory action caused by deficient serotonergic tonus (Wong et al., 1990). Furthermore 5-HT$_{1A}$ agonists like buspirone decrease alcohol consumption in animals (Collins and Myers, 1987; Kostowski and Dyr, 1992; Meert, 1993; Privette et al., 1988; Svensson et al., 1993). However, there are conflicting reports on the effectiveness of buspirone as a treatment for alcoholism in clinical studies (Bruno, 1989; George et al., 1999; Kranzler et al., 1994; Tollefson et al., 1991). Alcoholics with comorbid anxiety seem to benefit more from buspirone treatment, although this effect might be related more to reduced anxiety (Kenna, 2010).

The heterogeneity of alcoholics could be a partial explanation and subgroup differences could be associated with the disparities noted in clinical trials. Decreased [^{3}H]WAY-100635 binding to 5-HT$_{1A}$ receptors has been reported in the frontal regions of post-mortem brain samples of alcoholics (Storvik et al., 2009). However, in a PET imaging study, no significant differences were detected in [^{11}C]WAY-100635 binding between early-onset alcoholics and controls (Martinez et al., 2009). Furthermore, blood alcohol concentration has been associated with a down-regulation of 5-HT$_{1A}$ receptor binding sites in post-mortem samples, an effect that seemed to be brain region specific (Dillon et al., 1991; Gross-Isseroff and Biegon, 1988). Overall, there is disparity between results from animal studies and human studies regarding 5-HT$_{1A}$ receptor and alcoholism. Therefore, there is a need for more research in order to understand the role of 5-HT$_{1A}$ receptor in alcoholism.

Serotonin 1B receptors

5-HT$_{1B}$ receptors act as a serotonergic autoreceptors decreasing the release of 5-HT as well as a heteroreceptor inhibiting the release of other neurotransmitters (Morikawa et al., 2000). 5-HT$_{1B}$ receptors have distinctly different expression patterns from the 5-HT$_{1A}$ receptors i.e. high densities of 5-HT$_{1B}$ receptors have been observed in the globus pallidus, ventral striatum, SN and dorsal subiculum, while more moderate expression has been seen in the dorsal striatum and hippocampus (Storvik et al., 2012; Varnäs et al., 2004). 5-HT$_{1B}$ receptors have been associated with many disorders including migraine, aggressive behavior, depression and anxiety, and in relation to alcoholism, 5-HT$_{1B}$ function has been associated with the reinforcing and intoxicating effect of ethanol (Sari, 2013).

However, the role of 5-HT$_{1B}$ receptors in ethanol consumption is complex. Even though 5-HT$_{1B}$ receptor agonists increased dopamine release from the VTA (Yan et al., 2004), they inhibited ethanol intake in rats (Tomkins and O'Neill, 2000; Wilson et al., 1998). Treatment with 5-HT$_{1B}$ antagonists was able to reduce the ethanol induced increase in dopamine release in rodents (Yan et al., 2005). 5-HT$_{1B}$ knock-out mice tend to have increased ethanol intake (Crabbe et al., 1996), but also overexpression of 5-HT$_{1B}$ receptors in the ventral striatum has increased ethanol intake in rats (Furay et al., 2011; Hoplight et al., 2006).

In humans, upregulation of 5-HT_{1B} receptors in the ventral striatum has been associated with alcoholism (Hu et al., 2010). However, in a post-mortem whole-hemisphere autoradiography study, no statistically significant differences were seen between alcoholics and controls in 5-HT_{1B} binding (Storvik et al., 2012). There are also functional polymorphisms in the 5-HT_{1B} receptor gene but the reports of associations between these changes and alcoholism have been inconsistent. It has been speculated that the 5-HT_{1B} receptor gene polymorphism would be associated with antisocial alcoholism rather than alcoholism as a whole (Cao et al., 2013; Hasegawa et al., 2002; Lappalainen et al., 1998; Lee et al., 2009).

Serotonin 2 receptors

In contrast to the 5-HT_1 receptors, 5-HT_2 receptors mediate excitatory neurotransmission. Of the three subtypes 5-HT_{2A} and 5-HT_{2C} receptors have been the most extensively studied in association with alcoholism.

In general, activation of most serotonergic receptors e.g. the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_3 receptors increases dopamine release (Alex and Pehek, 2007). For example, activation of 5-HT_{2A} receptors in the prefrontal cortex has been associated with increased cortical release of dopamine and this effect seemed to be mediated by glutamatergic projections to VTA, possibly contributing to the psychedelic properties of 5-HT_2 agonists (Pehek and Hernan, 2015). In addition, 5-HT_{2C} receptor activation in the median prefrontal cortex was claimed to increase dopamine release in the NAC (Leggio et al., 2009a).

However, in contrast, the activation of 5-HT_{2C} receptors in the striatum reduced the release of dopamine and this effect was thought to be mediated by increased $GABA_A$ receptor activity in the SN (Burke et al., 2014). Furthermore, 5-HT_{2C} receptor activation inhibited also tonic dopamine release, possibly via a GABAergic mechanism; this also contrasts to the effects of other serotonergic receptors which mainly influence the induced dopamine release (Alex and Pehek, 2007).

Interestingly both 5-HT_{2A} agonist (psychedelics) and antagonists (antipsychotics) have been associated with reduced alcohol-drinking behavior (Bogenschutz et al., 2015; Kishi et al., 2013; Krebs and Johansen, 2012; Litten et al., 2012). However, it should be noted that even though 5-HT_{2A} activation is considered to be necessary for the action of classical psychedelic compounds and that this receptor's antagonism is a property encountered in many antipsychotics, these drugs are by no means specific agonists or antagonists for 5-HT_{2A} receptor and they can affect many other serotonergic receptor and neurotransmitter systems as well.

Meta-analysis of randomized controlled clinical trials from 60's and 70's found a beneficial effect of LSD in the treatment of alcoholism (Krebs and Johansen, 2012). Furthermore, also a recent small proof-of-concept study with psilocybin showed increased abstinence in alcoholics after psilocybin treatment (Bogenschutz et al., 2015). Imaging studies with humans have associated psilocybin function with decreased activation of brain regions associated with the default mode network e.g. posterior cingulate cortex (PCC) as well as decreased connectivity between PCC and hippocampus (Carhart-Harris et al., 2012; Roseman et al., 2014). These brain regions seem to be important for consciousness and social cognition and several theories have been proposed to explain the benefit of psychedelics in the treatment of alcoholism including the formation of new associations which might promote a disruption of compulsive behaviors (Carhart-Harris et al., 2014; Lebedev et al., 2015; Mayseless et al., 2015), perhaps including the habit formation encountered with chronic alcohol use.

In addition, antipsychotic medication has been claimed to reduce ethanol consumption in animal models. For example in rats, the 5-HT_2 antagonist ritanserin reduced ethanol intake and another 5-HT_{2A} antagonist, amperozide, reduced both ethanol and total fluid consumption (Lankford et al., 1996; Overstreet et al., 1997; Panocka and Massi, 1992). However, clinical studies in humans have been a disappointment. For example, eventhough olanzapine, a nonspecific inverse agonist of 5-HT_{2A}, achieved a dose dependent reduction in alcohol craving the cost-benefit balance has not been considered to favor clinical use of

olanzapine in the treatment of alcoholism (Littlewood et al., 2015). A meta-analysis of clinical trials did not find any clear evidence that antipsychotic medications would be beneficial in treatment of alcohol use disorder (Kishi et al., 2013).

Serotonin 3 receptors

The 5-HT_3 receptor is the only ligand gated ion channel within the family of serotonin receptors. The 5-HT_3 receptors are Na^+, K^+ and Ca^{2+} permeable which when activated, induce depolarization and possibly also an increase in the cytosolic Ca^{2+} levels and activation of Ca^{2+} controlled cell responses (Derkach et al., 1989). 5-HT_3 receptors are located in both cortical and limbic brain structures (Barnes et al., 1990; Gehlert et al., 1991; Marazziti et al., 2001). Interestingly, both 5-HT_3 agonists and antagonists seem to increase the expression of 5-HT_3 receptors (Morton et al., 2015).

Ethanol can activate 5-HT_3 receptors in the CNS e.g. leading to increased dopaminergic activity in the NAC (Lovinger and Zhou, 1994; Lovinger, 1999) even though the 5-HT_3 receptor concentration is low in VTA and NAC (Campbell and McBride, 1995). Local perfusion of a 5-HT_3 antagonist into both VTA and NAC was able to prevent the ethanol induced increase in extracellular dopamine in VTA and NAC (Campbell and McBride, 1995). In agreement, treatment with 5-HT_3 antagonists has decreased ethanol intake in rats, but this seemed to be dependent on the ethanol access schedule (Fadda et al., 1991; McKinzie et al., 1998; McKinzie et al., 2000). For example, the 5-HT_3 antagonist, MDL 72222, was effective in reducing ethanol intake only when ethanol was presented in a random interval manner (Fadda et al., 1991; McKinzie et al., 2000). As mentioned above in chapters 2.3.1., random interval operant training has been associated with habit formation which seems to be enhanced by ethanol administration (DePoy et al., 2013; DePoy et al., 2015; Hilario and Costa, 2008). Furthermore, co-administration of ethanol and the 5-HT_3 antagonist ICS 205-930 into posterior VTA prevented the ethanol induced operant response suggesting that 5-HT_3 function might be important for alcohol induced reinforcement learning (Rodd-Henricks et al., 2003). In addition, genetic polymorphism in the 5-HT_3 gene has been associated with alcohol dependence (Enoch et al., 2011).

Furthermore, the 5-TH_3 antagonist, ondansetron, has been shown to exert a modest effect especially in the treatment of early-onset alcoholics (Johnson et al., 2000; Johnson et al., 2002; Kranzler et al., 2003; Sellers et al., 1994). However, more recent studies have observed that the 5-HTTLPR polymorphism seem to affect the efficacy of ondansetron in reducing drinking. In a laboratory setting, ondansetron reduced drinking in alcoholics with the LL 5-HTTLPR genotype (Kenna et al., 2014a). Furthermore, women with the LL 5-HTTLPR genotype receiving ondansetron drank less than SS/SL women receiving ondansetron (Kenna et al., 2014b). The results for men were not statistically significant indicating also a possible involvement of sex steroid hormones.

Serotonin 7 receptors

The 5-HT_7 receptor is the only subtype of serotonergic receptors which has been associated with alcoholism in the GWA studies (Kim et al., 2014b; Zlojutro et al., 2011). More specifically, 5-HT_7 polymorphism was associated with deficits in event-related brain oscillations which have previously been associated with an increased risk of developing alcoholism (Zlojutro et al., 2011). In an early Finnish study, 5-HT_7 polymorphism was postulated to be important in a subgroup of alcoholic offenders with multiple behavioral problems (Pesonen et al., 1998). However, as a whole, the role of 5-HT_7 receptor in alcoholism has not been extensively studied and many questions remain open (Hauser et al., 2015).

5-HT_7 receptors can be detected in many brain regions, including brain stem nuclei e.g. raphe nuclei, VTA and SN, as well as thalamus, hypothalamus, hippocampus and cortical regions like ACC and PFC (Varnäs et al., 2004). 5-HT_7 receptors seem to be located on GABAergic interneurons and glutamatergic terminals (Harsing et al., 2004; Lovenberg et al.,

1993; Tokarski et al., 2011). 5-HT$_7$ antagonists are able to block serotonin induced increase in activity of GABAergic interneurons in the medial PFC, hippocampus and globus pallidus (Chen et al., 2008b; Fan et al., 2011; Tokarski et al., 2011). In addition, the activity of glutamatergic neurons in the medial PFC and hippocampus can be enhanced in a 5-HT$_7$ mediated manner (Fan et al., 2011; Tokarski et al., 2003). Moreover, activation of 5-HT$_7$ receptors in the astrocytes has been associated with antagonism of the generation of dopaminergic neuron in proliferating neurospheres of mesencephalic precursors (Parga et al., 2007). Treatment with a 5-HT$_7$ antagonist also was able to inhibit the amphetamine induced reduction of firing of VTA dopaminergic cells (Mnie-Filali et al., 2007). There seems to be a brain region specific effect also relating to dopamine interactions, since 5-HT$_7$ antagonists decreased dopamine and serotonin turnover in the amygdala but increased efflux in the PFC (Takeda et al., 2005; Wesolowska and Kowalska, 2008).

In mice, ethanol vapor exposure increased 5htr7 mRNA expression in the lateral hypothalamus, NAC and caudate, but treatment with a 5-HT$_7$ receptor antagonist did not alter ethanol drinking behavior (Yoshimoto et al., 2012). This indicates that the effect of 5-HT$_7$ receptor on alcohol consumption might be associated with other behaviors rather than direct drinking behavior. Thus, the function of the 5-HT$_7$ receptors has been associated with cognitive function, especially location related memory and attention in rodents (Ballaz et al., 2007a; Ballaz et al., 2007b; Sarkisyan and Hedlund, 2009). Perhaps more importantly for addiction, 5-HT$_7$ receptors have also been associated with impulsivity in a study where treatment with a 5-HT$_7$ antagonist was able to decrease some of the effects of methylphenidate on the projections from orbital PFC to NAC (Canese et al., 2011; Leo et al., 2009). No definitive conclusions can be drawn from the present limited amount of evidence. However, it seems plausible that in the case of alcoholism, the 5-HT$_7$ function could be associated with cognitive dysfunction and impulsivity (Ballaz et al., 2007a; Ballaz et al., 2007b; Canese et al., 2011; Leo et al., 2009; Sarkisyan and Hedlund, 2009), and further, that alcohol consumption might influence the function of the 5-HT$_7$ receptors (Yoshimoto et al., 2012).

2.8.3 Conclusion of the role of serotonergic activity in alcoholism

In conclusion, the serotonergic system is obviously associated with the neuropharmacological function of ethanol and the pathology of alcoholism (Leggio and Addolorato, 2008; Lovinger, 1999; Marcinkiewcz, 2015; Sari et al., 2011). However, the relationship is far from simple, probably because the serotonergic system modulates so many neural systems associated with alcoholism from reinforcement learning to social interactions (Marcinkiewcz, 2015). Social interactions are reinforcing in themselves (Panksepp and Lahvis, 2007) and these are considered to contribute to the reinforcing properties of alcohol in humans (Muller and Schumann, 2011). Recent studies investigating the interaction between serotonin and oxytocin in mice (Dolen et al., 2013) and humans (Mottolese et al., 2014) have detected an interaction between these two neurotransmitter systems which seems to be essential for social reward. In summary, more research is needed to clarify the role of serotonergic alterations in alcoholism, especially considering their impact on social functions.

2.9 STEROID HORMONES AND ALCOHOLISM

As described above in chapter 2.1, changes in the reinforcement and stress systems are considered to be important factors in the development of alcoholism (Koob, 2013). Steroid hormones are important for regulation of both of these systems, especially stress-related responses. Stress has been described as an adaptive response to environmental challenges (Selye, 1950). One of the most important stress responses is activation of the hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin releasing hormone (CRH) released from the hypothalamus leads to release of adrenocorticotrophic hormone (ACTH) from the pituitary

gland which then triggers steroid hormone release from the adrenal cortex. ACTH and endogenous opioid β-endorphin are co-released from the anterior pituitary gland and have the same precursor protein (Guillemin, 1978). Importantly, the release of steroids exerts a negative feedback effect on the HPA axis and steroid receptors in the hypothalamus, pituitary and other brain regions are important for modulating and terminating stress responses.

This classical stress response is important for maintaining homeostatic balance but repetitive activation has been considered to exert a cumulative load on the system and induce a maladaptive allostatic state (Koob, 2013). One important consideration in this theory of the pathogenesis of addiction is that in parallel to allostatic changes in the HPA axis, there are also allostatic changes in the brain stress circuitry in crucial brain regions like the central amygdala, noradrenergic locus coeruleus and PFC (Koob, 2013). The negative affective state experienced by the alcoholic during abstinence is considered to be affected by both increased stress vulnerability and dysfunctional reward processes in the brain and steroid hormones can influence both of these processes.

The negative affective state has been considered to be important in the development of escalated drinking during addiction pathology (Edwards et al., 2015; Koob, 2013). CRH is important for both the activation of the HPA axis and the brain stress system. Administration of a CRH receptor 1 antagonist was able to prevent the development of the ethanol withdrawal induced escalation phase in a rat model of alcoholism (Roberto et al., 2010). However, in a recent open label clinical trial with the CRH1 antagonist, pexacerfont, no improvements were achieved in alcohol craving, anxiety or neural responses to alcohol cues (Kwako et al., 2015). This might indicate that the earlier preclinical literature highlighting putative benefits of CRH1 antagonism might not translate into viable clinical treatment options in humans.

In association with alcoholism, the glucocorticoid system has been the most extensively studied steroid hormone system (Edwards et al., 2015). However, also other steroid hormones, e.g. sex hormones, dehydroepiandrosterone and pregnenolone, have been claimed to play an important role in the pathology of alcoholism (Helms et al., 2012; Lenz et al., 2012; Maninger et al., 2009; Sanchez et al., 2010). Furthermore, one important aspect is that plasma steroid levels do not necessarily reflect brain levels of steroid hormones (Little et al., 2008).

2.9.1 Role of glucocorticoid steroid system in alcohol induced brain alterations

Cortisol is the primary glucocorticoid steroid in humans, in contrast to corticosterone in rodents. Acute alcohol use as well as alcohol withdrawal both lead to an increase in the cortisol levels in humans (Adinoff et al., 1990; Adinoff et al., 2003). In rodents, corticosterone in itself is reinforcing (Piazza et al., 1993) and an increase in the corticosterone levels seems to mediate some of the reinforcing effects of ethanol (Fahlke and Hansen, 1999). Furthermore, increased corticosterone levels seem to be associated with the stress induced increase in ethanol consumption (Edwards et al., 2013; Logrip and Zorrilla, 2012). In rats, treatment with the glucocorticoid steroid receptor (GRs) antagonist, mifepristone, reduced ethanol consumption in stressful situations, but this effect was not seen with mineralocorticoid steroid receptors antagonists (Koenig and Olive, 2004). Therefore, the glucocorticoid steroid systems seems to be more important than its mineralocorticoid counterpart in the modulation of ethanol consumption.

Moreover, corticosterone levels are elevated in the plasma and brain during ethanol intoxication in rodents (Little et al., 2008). However, in times of prolonged abstinence, corticosterone levels seem to be elevated only in specific brain regions e.g. PFC but not in the plasma. Furthermore, escalation of the ethanol consumption during the development of dependence can be blocked by administration of GR antagonist mifepristone (Vendruscolo et al., 2012).

In comparison to the situation in low-drinking rats, a blunted reaction to ethanol administration induced corticosterone increase is seen in ethanol dependent rats as a sign of tolerance (Richardson et al., 2008). Ethanol dependent rats express decreased GR expression in cortical and limbic regions (Vendruscolo et al., 2012). This tolerance is observed to last also in prolonged periods of abstinence in rodents (Zorrilla et al., 2001). However, during prolonged abstinence, also increased GR expression has been observed in some key brain regions including CeA and NAC.

In human alcoholics, alcohol cues increase cortisol release thought to be evidence of a conditioned sensitized response (Fox et al., 2007; Sinha et al., 2009). In contrast, stress cues have a blunted ability to release cortisol in alcoholics compared to controls. Furthermore, alcoholics have decreased plasma ACTH and β-endorphin levels and increased cortisol levels (Gianoulakis et al., 2003). The reduced ACTH and increased cortisol levels seen in adult patients with substance use disorder have been associated with childhood trauma, neglect and poor parent-child attachment (Gerra et al., 2008; Schafer et al., 2010). This could be associated with an epigenetic modulation of GR gene promoter expression, because at least in rats, maternal behavior can introduce this epigenetic modulation which then affects the function of HPA axis in the offspring (Weaver et al., 2004). It has been suggested that alcohol consumption further damages the heritable low HPA function in individuals at risk of developing alcoholism (Edwards et al., 2015; Gianoulakis et al., 2005).

As mentioned above, cognitive deficits are associated with alcoholism (Riege et al., 1981; Zorumski et al., 2014); possible mechanisms include thiamine deficiency and the resulting Korsakoff's syndrome, endocannabinoids (discussed in chapter 2.7.2) and glucocorticoids (Ridley et al., 2013; Rose et al., 2010). In the case of glucocorticoid steroids, the severity of cognitive impairment during alcohol withdrawal has been associated with increased cortisol levels (Errico et al., 2002). Furthermore, treatment with the GR antagonist, mifepristone, has reduced the overall withdrawal severity as well as ethanol withdrawal induced memory deficits in mice (Jacquot et al., 2008; Sharrett-Field et al., 2013). In hippocampal slices, administration of corticosterone increased ethanol withdrawal evoked damage (Mulholland et al., 2005) and the GR antagonist, mifepristone, has exerted neuroprotective effects in the dentate gyrus in binge-drinking rodents (Cippitelli et al., 2014). Overall, glucocorticoids seem to enhance the neural damage and cognitive impairment caused by alcohol withdrawal (Rose et al., 2010).

Moreover, a recent double blind placebo controlled clinical study detected decreased alcohol cue induced craving in the group of alcoholics receiving mifepristone (Vendruscolo et al., 2015). Furthermore, there are several on-going clinical trials examining the use of mifepristone in the treatment of alcoholism (e.g. ClinicalTrials.gov Identifiers NCT02243709 and NCT02179749). These clinical studies will doubtless clarify the benefits (or failures) of GR antagonists in the treatment of alcoholism.

2.9.2 Neuroactive steroids and effects of ethanol

Other steroid hormones in addition to the glucocorticoids influence the function of the CNS. These neuroactive steroids affect specific receptors which influence cell function as well as many of the same receptor systems as ethanol e.g. $GABA_A$ and NMDA receptors (Helms et al., 2012; Leishman et al., 2013; Lenz et al., 2012). The extent of the increase in the levels of some neuroactive steroids after alcohol consumption has been linked to subjective evaluation of alcohol preference (Pierucci-Lagha et al., 2006; Wemm et al., 2013). For example, acute alcohol consumption seemed to increase plasma pregnenolone levels and decrease progesterone levels (Pierucci-Lagha et al., 2006). However, at least in rats, chronic consumption of alcohol depressed the level of impact that acute ethanol consumption exerted on steroid levels (Boyd et al., 2010), suggesting that in chronic use, the effect of alcohol consumption on steroid levels might be attenuated. Testosterone (T) has been the most widely studied of these steroids and metabolites. However, as a whole, the actions of many neuroactive steroids in the context of alcoholism is still largely unclear.

Sex steroids

The sex steroids (sex hormones, gonadal steroids) include androgens, estrogens and progestogens e.g. T, estradiol (E2) and progesterone (P4). In addition to activating the HPA axis which produces many neuroactive steroids, ethanol consumption can also activate *de novo* steroidogenesis in the brain and affect the release of sex steroids via the hypothalamic-pituitary-gonads axis (HPG) (Agis-Balboa et al., 2006; Kimoto et al., 2001; King et al., 2002; Mellon and Deschepper, 1993; Sanchez et al., 2014; Sanna et al., 2004; Zwain and Yen, 1999). The hypothalamus releases gonadotropin-releasing hormone (GnRH) which induces the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release from the anterior pituitary. Sex hormones are then generated from cholesterol in a series of enzymatic steps in the male testes and female ovaries with smaller amounts synthesized in the adrenal glands and other tissues, including the brain (Lenz et al., 2012; Munetsuna et al., 2009; Wehrenberg et al., 2001).

Steroids have at least two mechanisms with which they regulate cellular activity: activation of nuclear receptor and modulation of "non-genomic" cell protein function. In the classical "genomic" mechanism, sex steroids bind to their own receptors; T to androgen receptor (AR), estrogens to estrogen receptors 1 (ER1) and 2 (ER2), and P4 to the progesterone receptor (PR). These receptors are found in many regions of the brain, including areas important for addiction pathology like VTA, SN and hippocampus (Beyenburg et al., 2000; Creutz and Kritzer, 2002; Shughrue et al., 1997). Steroid binding changes the conformation of the nuclear receptor protein and the complex becomes translocated into the cell nucleus (O'Malley and Tsai, 1992). In the nucleus, the complex binds to hormone response elements on the DNA and affects the transcription of specific genes. However, activation of transcription is further modified by multiple different co-activators. These co-activators bind to specific DNA sequences and can regulate the transcription by enabling the sex hormone complex to induce gene expression independent from their specific hormone-response elements (Uht et al., 1997). This complexity makes possible a wide array of different functions to be activated or inhibited in the cell.

However, some of the sex hormone induced effects do not follow this slow schedule of nuclear receptor activation. Membrane receptors are the second mechanism through which sex steroid can function and in this case they have a more acute effect on the function of many cell proteins. Membrane estrogen receptor (Gq-mER) activation by E2 leads to activation of phospholipase C which triggers an up-regulation of protein kinases A and C (Roepke et al., 2009). Furthermore, Gq-mER interacts with metabotropic glutamate receptors to modulate behavior (Boulware et al., 2013; Dewing et al., 2007; Micevych and Mermelstein, 2008). In addition, androgens have binding sites on membrane receptors and these can influence the phosphorylation of cell proteins (Braun and Thomas, 2004; Gu et al., 2014). Moreover, dopamine induced activation of secondary messenger signaling in the cell has been demonstrated to influence co-activators of P4 steroid hormone pathways, especially the non-classical types (Mani and Oyola, 2012).

Organizational effects of sex steroids

Sex hormones affect the organizational changes in the brain during the prenatal period and early life, slowly declining thereafter and lasting until early adulthood (Schulz et al., 2009). In contrast to organizational effects, steroid hormones have also activational effects that are transient and reversible. However, this distinction is not absolute.

A decreased ratio between index finger (2D) length and ring finger (4D) length has been considered as a biomarker for prenatal T exposure. Both men and women with alcohol use disorder tend to display a decreased 2D/4D ratio which indicates increased prenatal T exposure compared to controls (Kornhuber et al., 2011). However, there was no association between severity of alcohol dependence or craving. Therefore it seems that the prenatal T

exposure might only be a risk factor but not contribute to the disease pathology after its initiation.

Adolescence is associated with the development of alcoholism (Lavikainen et al., 2008; Schulte et al., 2009; Spear, 2000) and those teenagers who mature early are more likely to drink to intoxication (Bratberg et al., 2005). The brain development in puberty differs between boys and girls, which is considered to be associated with T activity. However, these differences are also caused by E2, since T can be aromatized to E2 in the brain. Interestingly one of the most prominent features used to categorize alcoholics into subgroups is the age of onset of alcoholism (chapter 2.1.2). Early onset alcoholics are predominantly male, whereas late onset alcoholics are both males and females, emphasizing the possible role of the organizational changes induced by sex steroids in the pathology of early onset alcoholism.

Sex steroid levels and alcohol consumption

Human males generally drink more than females and health harms attributable to alcohol also predominantly affect the male gender (World Health Organization, 2014). This is in contrast to rodents, were female animals drink more ethanol and this difference can be eliminated by gonadectomy (Almeida et al., 1998). These differences could be associated with species differences, but also with social and environmental factors like gender roles and expectations. The social aspect of alcohol consumption is one important species difference, because in humans, alcohol facilitates social interaction which is considered to be major explanation for non-dependent use of alcohol in social contexts (Kuntsche et al., 2005; Van Den Abbeele et al., 2015).

At the hormonal level, high T levels have been associated with increased alcohol consumption and an increased risk of developing the alcoholism (Eriksson et al., 2005; La Grange et al., 1995). Furthermore, high T and E2 levels have been linked with current alcohol use in adolescent and pre- and post-menopausal women (Martin et al., 1999; Muti et al., 1998; Purohit, 1998). However, both acute and chronic alcohol consumption influence peripheral sex hormone levels. A low dose of alcohol increased T levels (Sarkola and Eriksson, 2003), whereas high intoxicating doses reduced peripheral T levels in human males (Mendelson et al., 1977). In contrast, high levels of ethanol have been associated with increased T levels in the rat brain (Alomary et al., 2003). In contrast to human males, in women, both low and high doses of acute alcohol intake have been associated with increased peripheral T levels (Frias et al., 2000; Frias et al., 2002; Sarkola et al., 2000). Moreover, P4 levels seem to decrease in women after alcohol consumption, whereas alcohol's effect on E2 is believed to be dependent on the use of oral contraceptives (Sarkola et al., 1999). Furthermore, the luteal phase of menstrual cycle, characterized by increased P4 levels, has been associated with decreased positive reinforcement (Dreher et al., 2007).

Chronic alcohol use has been associated with reduced peripheral T and P4 levels and increased E2 levels in males (Maneesh et al., 2006; Muthusami and Chinnaswamy, 2005) as well as reduced fertility (Kucheria et al., 1985). Possible mechanisms include ethanol consumption evoked inhibition of T synthesis via opioid (Emanuele et al., 1998), nitric oxide (Adams et al., 1993) and adrenergic dependent signaling (Rivier, 1999). In women, chronic alcohol consumption has been associated with irregularities in the menstrual cycle (Mello et al., 1989). Alcohol withdrawal can reverse these effects (Välimäki et al., 1984) and increased levels of T, E2 and LH have been reported in abstinent patients with alcoholism (Hasselblatt et al., 2003; King et al., 1995). At the onset of alcohol withdrawal, there does not seem to be any difference in peripheral T levels between alcoholics and controls, but increased T levels are seen only after prolonged abstinence (Walter et al., 2007).

In addition, alterations in the metabolism of sex steroids have been associated with alcohol consumption (Purohit, 2000). For example, metabolism of T to E2 is catalyzed by aromatase (CYP19A1). A polymorphism in the aromatase gene which leads to an increased E2 concentration and decreased T levels in males (Peter et al., 2008) has been associated with craving during alcohol withdrawal in male alcoholics (Lenz et al., 2011). Estrogens seem to

exert a greater response in modulating alcohol induced activity of the HPA axis compared to androgens (Lenz et al., 2012). Moreover, T has been claimed also to play a role in psychological traits like aggression (von der Pahlen et al., 2002) and antisocial behavior (Yildirim and Derksen, 2012) which are also differently presented in sub-types of alcoholics (Cloninger, 1995). Increased T levels in the cerebrospinal fluid have been associated with both alcoholism and antisocial personality (Virkkunen et al., 1994).

Furthermore, both androgens and estrogens are able to trigger reinforcing responses in the brain (de Souza Silva et al., 2009; Di Paolo et al., 1985). One example of this is anabolic-androgenic steroids (AAS) which have a high potential to cause dependence, approximately one third of all users may become dependent (Kanayama et al., 2010). AAS use has also been associated with an increased risk of having another substance use disorder such as alcoholism (DuRant et al., 1993). Moreover, again bearing similarities to alcohol, also sex steroid induced reinforcement has been considered to be associated with the opioid system. The opioid system can influence the steroid levels in the periphery and in the brain (Katz and Mazer, 2009) and similarly steroids can also influence levels of endo-opioids and opioid receptors (Hammer and Bridges, 1987; Pluchino et al., 2009). Administration of opioid receptor agonists led to an inhibition of adrenal function which in turn depressed the peripheral levels of steroids (Pirnik et al., 2001). An opposite effect was seen after the administration of the opioid receptor antagonist, naloxone, which increased the plasma levels of ACTH with this effect being more robust in females than males (Mangold et al., 2000; Roche et al., 2010; Uhart et al., 2006). Furthermore, E2 and P4 increase the levels of both MOR expression and β-endorphins (Hammer and Bridges, 1987; Pluchino et al., 2009). Moreover, administration of the opioid antagonist, naltrexone prevented the reinforcing effects of testosterone in hamsters (Peters and Wood, 2005). In male rats, AAS nandrolone elevated β-endorphin levels and decreased dynorphin levels in the VTA and NAC, respectively (Johansson et al., 1997; Johansson et al., 2000). Nandrolone treated rats also exhibited increased ethanol consumption.

Overall, the role of sex hormones in alcoholism should be considered as both organizational, i.e. increasing the risk of developing alcoholism, and activational, i.e. modulating the acute and chronic effects of alcohol consumption and the pathology of alcoholism (Lenz et al., 2012). Alcohol and sex hormones exist in a bidirectional relationship with each other, where alcohol consumption can influence sex hormone levels and sex hormones can influence alcohol consumption as well as the consequences of alcohol consumption. One example of this effect is the association between the increased risk of breast cancer and even moderate alcohol consumption which has been considered to be associated with alcohol induced disruption of sex hormone homeostasis (Park et al., 2014). In contrast, sex hormones have also been studied as a possible treatment option for reversing ethanol caused tissue damage. For example, in the prostate microenviroment study, T treatment was able to reverse ethanol evoked damage (Mendes et al., 2015). In conclusion, even though much is known about the interaction between sex hormones and alcohol there are still many unknowns to be clarified.

Other neuroactive steroids

Similar to ethanol (chapter 2.5), in the 1980's neuroactive steroids alphaxalone, allopregnenolone and 3α,21-dihydroxy-5α-pregnan-20-one were also shown to enhance $GABA_A$ receptor activation (Harrison and Simmonds, 1984; Majewska et al., 1986). Furthermore, also similar to ethanol, some neuroactive steroids modulate the action of many other neurotransmitter systems e.g. NMDA receptor function (Wu et al., 1991).

The rate limiting step in steroid synthesis, whether in the periphery or CNS, is the transportation of cholesterol to the inner mitochondrial membrane by an inducible chaperone steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996). Once in the mitochondrial membrane, cholesterol is synthetized to pregnenolone. This reaction is catalyzed by P450 side-chain cleavage (P450scc, CYP11A1). Activation of the HPA axis and

release of ACTH increases the expression of StAR and CYP11A1, resulting in increased production of pregnenolone (Lavoie and King, 2009). All other steroids are then synthetized from pregnenolone (figure 2).

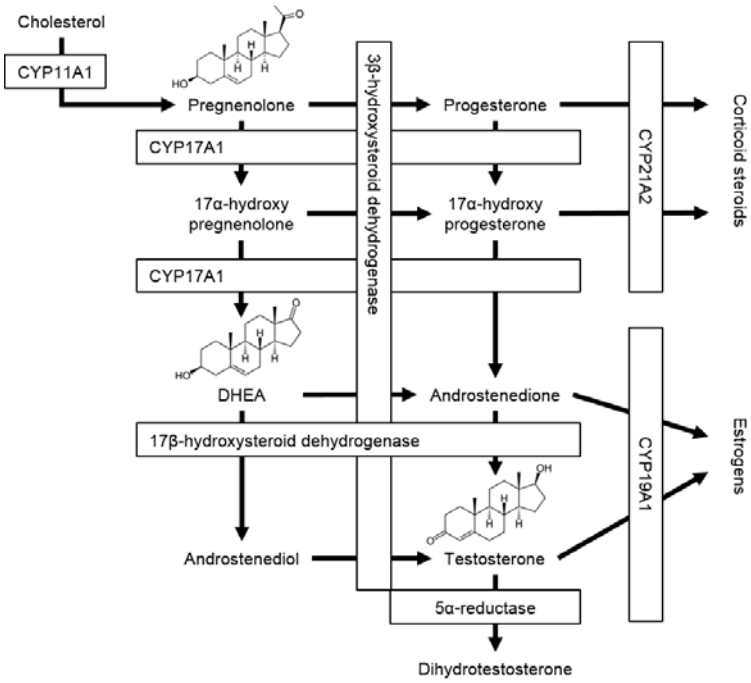

Figure 2. Simplified flow chart of steroid synthesis. Cholesterol is transported to the inner mitochondrial membrane by steroidogenic acute regulatory protein where cholesterol is converted into pregnenolone by CYP11A1. All other steroids are then synthetized from pregnenolone by enzymes in the smooth endoplastic reticulum.

One important group of enzymes participating in synthesis of steroid hormones are hydroxysteroid dehydrogenases e.g. 3β-hydroxysteroid dehydrogenase (3β-HSD). As is the case also in ethanol metabolism, hydroxysteroid dehydrogenases require nicotinamide adenine dinucleotide phosphate or nicotinamide adenine dinucleotide as a co-factor (Krause and Karavolas, 1980). The depletion of these co-factors by chronic ethanol consumption has been postulated to alter steroid metabolism (Crabb et al., 2004; Helms et al., 2012), however, this speculation need further study to be confirmed.

Steroidogenesis modulates the excitatory and inhibitory balance in the CNS and has been considered as an important regulator of behavior and reactivity to environmental stimuli (Helms et al., 2012). Like ethanol, neuroactive steroids modulate the $GABA_A$ activity i.e. they do not activate the receptors in concentrations usually seen in the brain but increase the efficacy of GABA to induce receptor activation (Helms et al., 2012). Neuroactive steroids with different isomeric configurations seem to exert a different modulatory action on the $GABA_A$ receptor. Precursor transformation first by 5α-reductase followed with 3α-hydroxysteroid dehydrogenase (3α-HSD) evoked a positive modulation of $GABA_A$ receptor whereas metabolism first by 5β-reductase and then by 3β-HSD or sulfation by sulfotransferase seemed to produce negative modulators of the $GABA_A$ receptor (Helms et al., 2012; Park-Chung et al., 1999; Puia et al., 1990; Wang et al., 2002). In contrast to negative modulation of $GABA_A$ receptors, sulfated steroids seem to be able to modulate both positively and negatively the activity of the NMDA receptor (Helms et al., 2012). Furthermore, again similar to ethanol, neuroactive steroids also seem to be able to increase the release of neurotransmitters. For example, allopregnenolone increased GABA release in rat neurons

(Haage et al., 2002) and pregnenolone sulfate increased glutamate release in VTA and hippocampal neurons (Meyer et al., 2002; Whittaker et al., 2008).

Ethanol and neuroactive steroids

Ethanol increases the peripheral levels of many neuroactive steroids (Porcu et al., 2010). This effect does not seem to be a direct influence on the steroidogenic cells, but is at least partly mediated by ethanol induced release of ACTH, CRH and vasopressin, of which ACTH mediated activation of adrenal steroid genesis has been considered as the most important (Lee et al., 2004; Porcu et al., 2004).

Furthermore, there is also *de novo* steroidogenesis in the CNS (Chisari et al., 2010). Astroglial cells were considered to be the primary location for CNS steroidogenesis in early studies (Mellon and Deschepper, 1993; Zwain and Yen, 1999), but later also neurons were shown to possess the cellular machinery for *de novo* steroidogenesis, including StAR (Kimoto et al., 2001; King et al., 2002), CYP11A1 (Kimoto et al., 2001) and 5α-reductase (Agis-Balboa et al., 2006). Ethanol also seems to influence *de novo* steroidogenesis. For example, in rat hippocampal slices, 50mM ethanol produced both an acute as well as a delayed increase in GABAergic activity (Sanna et al., 2004). The latter increase in GABAergic activation was inhibited by 5α-reductase inhibitor finasteride, indicating that this later increase was due to altered steroid metabolism (Sanna et al., 2004). Accordingly, ethanol has been reported to increase 5α-reductase mRNA expression in the rat PFC (Sanchez et al., 2014). However, there seem to be species differences in brain steroid synthesis and it has been reported that CYP11A1 and CYP17A1 enzymes might not be expressed in the adult human brain (Steckelbroeck et al., 2010).

In human studies, there are numerous variables which are difficult to monitor. For example, many steroids undergo a diurnal rhythm and drinking alcohol in the morning has increased plasma levels of pregnenolone and decreased allopregnanolone levels (Pierucci-Lagha et al., 2006), whereas the same dose in the evening did not seem to change pregnenolone or allopregnanolone levels compared to baseline (Porcu et al., 2010). In animal studies, these effects can be controlled better. In male rhesus macaques, ethanol self-administration lowered ACTH levels, however the effect on different steroids was selective: deoxycorticosterone was associated with blood alcohol concentration and mineralocorticoids were considered to compensate for adrenocortical adaptation among heavy drinkers (Helms et al., 2014).

Furthermore, neuroactive steroids influence $GABA_A$ function in a relatively ubiquitous manner, since subunit composition seems to be less important than for some other compounds. Therefore, the most obvious and acute interaction between ethanol and neuroactive steroids happens in those brain regions where ethanol enhances GABA release (chapter 2.5) and neuroactive steroids modulate the affinity of GABA to $GABA_A$ receptor (Helms et al., 2012).

Dehydroepiandrosterone and pregnenolone as examples of the complex interaction between ethanol and neuroactive steroids

The interactions between ethanol consumption and neuroactive steroids are complicated and extend beyond $GABA_A$ function. For example, dehydroepiandrosterone can enhance NMDA and decrease $GABA_A$ receptor function, but also increases catecholamine synthesis and monoamine oxidase (MAO) activity (Imamura and Prasad, 1998; Maninger et al., 2009; Perez-Neri et al., 2009). High levels of dehydroepiandrosterone have been associated with decreased MAO activity in the NAC of rats (Perez-Neri et al., 2009), and with a subsequent decrease in dopamine turnover (Perez-Neri et al., 2008). Moreover, dehydroepiandrosterone administration was found to reduce the amount of ethanol consumed by rats (Gurkovskaya et al., 2009).

Pregnenolone has also been associated with enhanced activation of NMDA receptors and an inhibition of $GABA_A$ receptor activity (Majewska et al., 1990; Majewska, 1992). However,

pregnenolone has also been associated with feed-back control of the endocannabinoid system functions (Vallee et al., 2014) and a reduction of acute alcohol self-administration in rodents (Besheer et al., 2010; Rezvani and Levin, 2014). Moreover, as mentioned above, one function of the opioid system is to modulate neuroactive steroid levels. For example, administration of the MOR antagonist, naloxone, increased plasma levels of pregnenolone in cynomolgus monkeys (Porcu et al., 2006).

In humans, alcohol consumption increased plasma dehydroepiandrosterone and pregnenolone levels (Pierucci-Lagha et al., 2006; Välimäki et al., 1984). Furthermore, the plasma steroid levels displayed a correlation to some of the subjective effects of alcohol. For example, plasma pregnenolone levels were correlated with the degree of liking of the effects of alcohol consumption (Pierucci-Lagha et al., 2006). Furthermore, high saliva levels of dehydroepiandrosterone have been associated with drinking to cope with stress in women (Wemm et al., 2013). However, overall, the role of dehydroepiandrosterone and pregnenolone in human alcohol consumption is still largely unknown.

2.9.3 Conclusions about the role of steroid hormones in alcoholism

In conclusion, steroid hormones interact with many pharmacological sites mediating the actions of ethanol and play a role in the pathology undermining alcoholism. The main findins of studies investigating changes in steroid levels in human body fluids in association with alcohol consumption are summarized in table 5. However, the understanding of the complex relationships between different steroid hormones, other transmitter systems and alcohol still remains to be clarified in the future studies (Lenz et al., 2012). Furthermore, most of the human studies have been analysed from plasma or saliva samples, which might not fully represent steroid hormone levels in the brain because of *de novo* steroidogenesis and active metabolism in the CNS (Alomary et al., 2003; Little et al., 2008). Therefore, there is a need to elucidate the alterations in the steroid system in the brains of human alcoholics.

Table 5. Summary of the main effects of alcohol consumption on the concentrations of testosterone, dehydroepiandrosterone and pregnenolone in human body fluids.

Main findings	Reference(s)
Testosterone	
Acute alcohol consumption increases levels in women	Frias et al., 2000; Frias et al., 2002; Sarkola et al., 2000
Acute low dose alcohol consumption increases levels in males	Sarkola and Eriksson, 2003
Acute high dose alcohol consumption decreases levels in males	Mendelson et al., 1977; Välimäki et al., 1984
Chronic alcohol consumption decreases levels in males	e.g. Maneesh et al., 2006; Muthusami and Chinnaswamy, 2005
Alcohol withdrawal and abstinence increase levels	Hasselblatt et al., 2003; King et al., 1995; Walter et al., 2007
High levels have been associated with alcoholism	Eriksson et al., 2005; La Grange et al., 1995; Martin et al., 1999; Muti et al., 1998; Purohit, 1998; Virkkunen et al., 1994
Dehydroepiandrosterone	
Acute alcohol consumption increases plasma levels	Pierucci-Lagha et al., 2006; Välimäki et al., 1984
High saliva levels associated with drinking to cope with stress in women	Wemm et al., 2013
Pregnenolone	
Acute alcohol consumption increases plasma levels	Pierucci-Lagha et al., 2006

2.10 CONCLUSION OF THE LITERARY REVIEW

At the end of this literary review, it is hoped that I have convinced the reader that the action of ethanol to the human nervous system is an extremely complicated affair. The interactions between different transmitter and hormone systems in producing the behavior associated with alcohol consumption are even more complicated than the direct pharmacological actions of ethanol. For example, even the most classical phenomenon of positive reinforcement due to ethanol exposure can be affected at least to some extent by all the above reviewed transmitter and hormone systems in the brain. However, although much of the research in the field has focussed on this reinforcing aspect, even this is not sufficient to explain all of the behavioral aspects associated with alcoholism. Understanding the malfunction of executive processes and social behavior are also of major importance if one wishes to understand the pathology of human alcoholism. The function of the AMPA receptor is important for learned behavior including many aspects of executive function. Similarly the endocannabinoid system is crucial for modulating memory processes and learning in limbic regions e.g. hippocampus and amygdala. Moreover, serotonergic function is important in many aspects of social behavior which are compromised in many alcoholics. Furthermore, neuroactive steroids undergo important interactions with alcohol in many of the above mentioned receptor systems and could therefore influence both a predisposition to and the subsequent development and maintenance of alcoholism. Furthermore, more studies are needed in human alcoholics, keeping in mind the spectrum of alcoholics, since some aspects of the animals studies do not seem to translate into human pathology. Therefore, in the experimental part of the present thesis, AMPA receptor binding, endocannabinoid levels, SERT binding and neuroactive steroid levels were studied in the post-mortem brain samples of Cloninger type 1 and 2 alcoholics in order to advance our knowledge of the pathology of alcoholism in humans.

3 Aims

The main objective of the present thesis was to increase our understanding of the neural correlates of alcohol use evoked changes in the reinforcement and stress systems as well as the neural correlates of predisposing factors, especially of the impulsive and antisocial behavior seen in the type 2 early onset alcoholics. In order to achieve this goal, the neurochemical changes in the post-mortem brain samples of Cloninger type 1 and type 2 alcoholics were investigated and compared to samples from non-alcoholic controls.

The study was divided into following four studies with more specific aims:

1. To investigate differences in [^{3}H]AMPA binding to AMPA receptors in the post-mortem brain samples of Cloninger type 1 and type 2 alcoholics compared to non-alcoholic controls.

2. To measure differences in endocannabinoid profiles in the amygdala and hippocampus in post-mortem brain samples of Cloninger type 1 and type 2 alcoholics.

3. To examine differences in [^{3}H]citalopram binding to SERT in brain regions associated with social cognition in post-mortem brain samples of Cloninger type 1 and type 2 alcoholics compared to non-alcoholic controls.

4. To determine differences in ketosteroid levels in the post-mortem brain samples of Cloninger type 1 and type 2 alcoholics compared to non-alcoholic controls.

4 Methods

4.1 SUBJECTS AND BRAIN SAMPLES

Twenty-seven post-mortem brain left hemispheres were obtained during clinical autopsy from the Department of Forensic Medicine, University of Oulu, Finland, and the Department of Forensic Medicine, University of Eastern Finland, Kuopio, Finland. This portion of the study was approved by the Ethics Committees of the University of Oulu and the National Board of Medico-legal Affairs, Helsinki, Finland. The brains were removed, cleaned of the dura and divided at the midsagittal plane. The left hemisphere was placed on a glass plate before freezing at − 75 °C. Brain samples were cryo-sectioned into 100-μm cantomeatal slices that were allowed to air dry before storage at − 25 °C with dehydrating agents until use. A post-mortem analysis for drugs, which included alcohol, and the normal necropsy protocol were performed. None of the hemispheres exhibited damage or gross neuroanatomical abnormalities as judged from a series of Nissl stained sections. Medical records on the cause of death, previous diseases and medical treatments were also collected and available for all 27 subjects.

All 27 subjects were Finns. The study groups consisted of type 1 alcoholics (N=9), type 2 alcoholics (N=8) and non-alcoholic controls (N=10) (Table 6). All of the controls were free of psychiatric diagnoses. Six of the eight type 2 alcoholics had a criminal record or a history of violent offences (physical or sexual). Alcoholism (alcohol dependence) was diagnosed according to DSM-IV criteria (APA, 1994). Furthermore, the alcoholic subjects were sub-classified as type 1 or type 2, according to the criteria established by Cloninger (Cloninger, 1987). Diagnoses were made from the medical records by two physicians independently of each other (Storvik et al., 2009; Tupala et al., 2001a). The kappa coefficient of diagnostic agreement for the subjects was 0.9 i.e. one type 2 alcoholic was diagnosed as a type 1 alcoholic by the second physician. Otherwise, diagnoses were unanimous. Subjects having a psychotic disorder or any neurological diseases (such as epilepsy), or those taking a medication that could affect the CNS (such as neuroleptics or antidepressants, including the SSRIs) were excluded. Subjects with severe inflammation as a cause of death (i.e. acute pancreatitis and pneumonia) were excluded in the study IV, because severe inflammation causes alterations in steroid levels.

Cryosectioning and autoradiography were performed at the School of Pharmacy, University of Eastern Finland, Kuopio, Finland, as previously described (Mantere et al., 2002). Individual variations in brain size were also considered when selecting representative sections. Each cryosection was coded to allow a subsequent blind analysis of the data.

Compared to *in vivo* methods, the evaluation of post-mortem brains enables use of methods not available when using live subjects. Limitations include ante- and post-mortem effects, and measurement at only one time point. In the case of human post-mortem brain samples, the single time point measurement means that one cannot draw any conclusions about whether any alterations detected preceded the studied phenomenon or were due to it.

Table 6. The study subjects: age at time of death, post-mortem interval (PMI), blood alcohol concentration (BAC) and cause of death.

Subjects	Sex	Age (years)	PMI (h)	BAC (‰)	Cause of death
Non-alcoholic controls					
1	Male	55	5.5	0.0	Acute myocardial infarction
2	Male	45	9.5	0.0	Acute myocardial infarction
3	Male	77	7.5	0.0	Acute myocardial infarction
4	Female	57	11.0	0.0	Acute myocardial infarction
5	Male	50	18.5	0.0	Acute myocardial infarction
6	Female	60	12.0	0.0	Acute myocardial infarction
7	Male	49	33.0	0.4	Acute myocardial infarction
8	Male	53	29.0	0.0	Acute myocardial infarction
9	Male	53	11.0	0.0	Acute myocardial infarction
10	Male	36	11.0	0.0	Dissection of aorta
Type 1 alcoholics					
1	Male	39	12.5	0.0	Pneumonia
2	Male	48	4.5	0.1	Acute pancreatitis
3	Male	45	12.0	1.5	Suicide by hanging
4	Male	42	14.8	0.8	Acute myocardial infarction
5	Male	76	10.5	3.2	Acute myocardial infarction
6	Female	56	19.0	4.1	Ethanol intoxication
7	Male	48	6.5	1.4	Pneumonia
8	Male	69	16.0	4.7	Ethanol intoxication
9	Female	57	11.0	2.0	Right subdural hemorrhage
Type 2 alcoholics					
1	Male	49	12.0	1.7	Fibrotic degeneration of myocardium
2	Male	37	9.5	3.0	Gunshot wound
3	Male	47	15.5	3.0	Knife wound
4	Male	20	14.5	1.3	Knife wound
5	Male	46	18.0	0.0	Suicide by hanging
6	Male	18	9.5	1.5	Heart rupture (car accident)
7	Male	32	16.5	3.6	Suicide by hanging
8	Male	28	17.5	0.0	Suicide by hanging

4.2 WHOLE-HEMISPHERE AUTORADIOGRAPHY

One of the first and most influential studies using radioactive ligands to detect receptor binding was conducted by Pert and Snyder in 1973. In this classical study, they revealed the existence of μ-opioid peptide receptors (MOR) in the brain using tritiated naloxone (Pert and Snyder, 1973). The basic idea of an autoradiography study is that a ligand with high affinity to the studied receptor protein stays attached to the receptor even during rapid washing periods which rinses away any non-specific background interactions. In their study, Pert and Snyder demonstrated that [^{3}H]naloxone binding sites were of limited number and exhibited required pharmacological specificity which was demonstrated by using morphine to compete with the [^{3}H]naloxone binding. Applying this method to anatomically more intact tissues developed this binding assay further.

A whole-hemisphere autoradiography of post-mortem brain tissue was used in the present study but the basic idea is still the same as devised by Pert and Snyder. High affinity ligand binds to target receptor, extensive and rapid washing is used to rinse away background interactions and non-specific binding is determined by use of non-radioactive ligand which displaces the radioligand from the binding site. Compared to the current *in vivo* imaging techniques e.g. PET imaging, the resolution of whole-hemisphere autoradiography is better. On the other hand, compared to post-mortem techniques with higher spatial

resolution, e.g. immunohistochemistry techniques, whole-hemisphere autoradiography method is less expensive and enables larger sets of study samples to be processed simultaneously, thus decreasing the technical variation between samples. The limitations of autoradiography include ante- and post-mortem effects, specificity of the used ligands and most importantly, the fact that one makes only one measurement at one time point, which as stated, makes it difficult to determine whether any changes detected were present before onset of alcoholism or took place subsequently.

The assay protocols used in the present research for whole-hemisphere autoradiography are shown in table 7. Tissue samples were pre-incubated to remove endogenous transmitters from the samples before the main incubations in which the radioactive ligand was exposed to the specimen. After washing, the samples were dried at room temperature. In study I, brain slices were exposed to, phosphor imager plates (BAS IP-TR 2040, Fuji, Japan) before scanning (Storm 860 PhosphorImager scanner, Amersham). In study III, brain sections were exposed to radiation-sensitive film ([^{3}H]-Hyperfilm, discontinued, Amersham, Buckinghamshire, UK). The autoradiograms were analysed by computerized densitometry (ImageJ, 1.45s, National Institute of Health, USA) and the resulting pixel values were mathematically transformed into physical tissue values (pmol/g) via [^{3}H]calibrating standard scales (cat. no. RPA 507, Amersham).

Table 7: Protocols for receptor binding assays.

Target	Ligand	Displacer	Incubation buffer	Pre-incubation	Main incubation	Final rinsing
AMPA	26nM [^{3}H]AMPA	10µM CNQX	50mM Tris-citrate buffer containing 1M citric acid (+100 mM potassium thiocyanate for main incubation), pH 7.4	30 min 4 °C	45 min 4°C	3 x 2 min buffer + brief dip in water 4 °C
SERT	1.2nM [^{3}H]citalopram	10 µM fluoxetine, 0.1% ascorbic acid	137mM sodium chloride, 2.7mM potassium chloride, 1.8mM potassium phosphate and 10.1mM hydrosodium phosphate; pH 7.4	15 min 20 °C	90 min 20 °C	3 x 10 min buffer + brief dip in water 4 °C

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SERT, serotonin transporter.

4.3 MASS SPECTROMETRY ANALYSES

The liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods applied in the measurement of ketosteroids (Keski-Rahkonen et al., 2011) and endocannabinoids (Lehtonen et al., 2011) have been previously described. Briefly, the frozen post-mortem brain tissue samples (1–3 mg for endocannabinoids and 5-10 mg for ketosteroids) were removed from glass slides with a scalpel and transferred to a pre-weighed Eppendorf tube, then weighed again to determine the precise weight of the samples.

In the measurement of endocannabinoids, methanol (500 µl) and the deuterated internal standards AEA-d8 (50 µl, 50 nM) and 2-AG-d8 (50 µl, 460 nM) were added to the tissue samples, which were then homogenized by ultrasonification. Lipids were extracted by

adding chloroform and water to yield a final methanol/chloroform/water ratio of 1:2:1 (v/v/v), before centrifugation at 1500 × g for 10 min at 10 °C. The upper aqueous layer was discarded and the lower organic layer was transferred to a screw capped glass test tube. The liquid extraction was repeated; the chloroform phases were combined and then evaporated to dryness under nitrogen at 20 °C. The residue was reconstituted in 70 μl of an ice-cold acetonitrile water solution (5:2, v/v).

In the measurement of ketosteroids, the dry frozen brain samples were cryo-ground in 2 mL microcentrifuge tubes using a TissueLyser II (Qiagen Finland, Helsinki, Finland) with 5-mm stainless steel beads in a precooled adapters, and shaken for 30 seconds at 30Hz. The deuterated internal standards were added to the tissue samples, which were then homogenized in 200 μL of deionized water, and a 150 μL aliquot was taken for LC-MS/MS analysis.

After sample preparation, in both studies the samples were then transferred to vials for separation by liquid chromatography (Agilent 1200 Series Rapid Resolution LC System, Agilent Technologies, Waldbronn, Germany). Endocannabinoids and ketosteroids were measured by electrospray ionization (ESI) triple quadrupole mass spectrometric detection (Agilent 6410 Triple Quadrupole LC/MS, Agilent Technologies, Palo Alto, CA, USA) as previously described (Keski-Rahkonen et al., 2011; Lehtonen et al., 2011).

4.4 STATISTICAL ANALYSES

The measured values were standardized to the same mean and distribution within each region and across the study groups by subtracting the mean of all the values from the measured value and then dividing by the standard deviation of all the values. Standardized scores were used for statistical analyses, because the main study interest was to measure differences between the study groups. Standardized scores also enable comparison of changes seen in different receptors, transporters and neurochemicals to each other in comparison to controls. In most cases, group means with 95% confidence intervals (95% CI), which were obtained with bias-corrected bootstrapping, are shown. Furthermore, in most studies, statistical significance was evaluated by a permutation type analysis of variance (Monte-Carlo p-values), followed by multiplicity adjustment with an appropriate method (e.g. Holm's and Hommel's methods). Pearson's and Spearman's methods were used to calculate correlations. Cohen's method was used to calculate effect sizes: d effect sizes to compare two groups and f effect size to compare three groups. The α level was set at 0.05. STATA (release 13.1, College Station TX) was used for statistical analyses. The resulting figures in the present thesis were created with GraphPad Prism (version 5.03, GraphPad Software Inc.).

5 Results

The results of the individual studies are shown in detail in the individual articles (Studies I-IV). Only the key finding discussed in the next chapter will be presented here. Because most of the results were demonstrated as standardized values in the articles, only the actual measured binding values or concentrations from the brain regions in which significant differences were observed between the study groups will be shown here. This presentation in combination with the data shown in the individual articles should allow the reader to judge better the individual key results of the present thesis.

5.1. [^{3}H]AMPA BINDING TO AMPA RECEPTORS

[^{3}H]AMPA binding was observed in all the measured brain regions i.e. ACC, NAC, FC, hippocampus and DG (Study I). Increased [^{3}H]AMPA binding was observed only in the ACC between type 2 alcoholics and non-alcoholic controls (Figure 3).

The other key finding of this study was the negative Spearman correlation between [^{3}H]AMPA binding and the previously published DAT density (Tupala et al., 2006) in the ACC in type 2 alcoholics (R=-0.952). This correlation was not observed in the other study groups and the difference between the groups was statistically significant (χ^2=13.84, p=0.001).

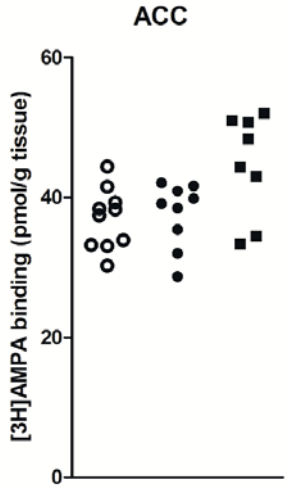

Figure 3. [^{3}H]AMPA binding in the anterior cingulate cortex. Significant difference was observed between [^{3}H]AMPA binding between type 2 alcoholics and controls in the ACC (Monte Carlo p-value=0.011). Legend: ◦ = non-alcoholic controls; ● = type 1 alcoholics; ▪ = type 2 alcoholics; ACC, anterior cingulate cortex.

5.2 ENDOCANNABINOID LEVELS IN THE HIPPOCAMPUS AND AMYGDALA

All six measured endocannabinoids were detected in the hippocampus and amygdala. MANOVA comparison showed that the endocannabinoid profile between the study groups was different in the amygdala (p=0.037). This difference was further localized to differences in the docosahexaenoyl ethanolamide levels between type 1 alcoholics and non-alcoholic controls (Figure 4).

The other key finding of this study was the negative Spearman correlation between AEA levels in hippocampus and previously published mGluR1/5 receptor concentrations (Kupila et al., 2013) in the hippocampal CA1 region (R=-0.88) in type 1 alcoholics, which was not seen in type 2 alcoholics or control. The difference between the study groups was significant (χ^2=9.403, p=0.009).

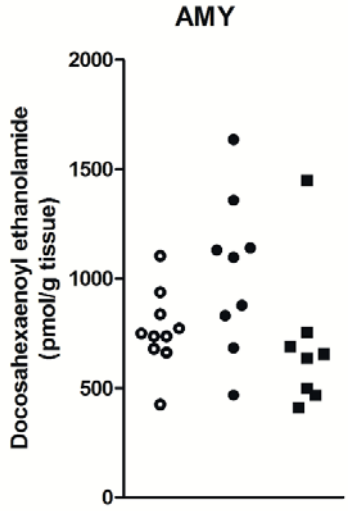

Figure 4. Docosahexaenoyl ethanolamide levels in the amygdala. Significant difference between the study groups was observed in docosahexaenoyl ethanolamide levels between type 1 alcoholics and controls in the amygdala (Monte Carlo p-value=0.022). Legend: ◦ = non-alcoholic controls; ● = type 1 alcoholics; ▪ = type 2 alcoholics; AMY, amygdala.

5.3 [^{3}H]CITALOPRAM BINDING TO SEROTONIN TRANSPORTERS IN THE POSTERIOR BRAIN REGIONS

[^{3}H]Citalopram binding was observed in all measured brain regions. However there were almost tenfold differences between the brain regions (table 2 of study III). Significant differences between the study groups were observed in the PINS, PHG and PCC (Figure 5).

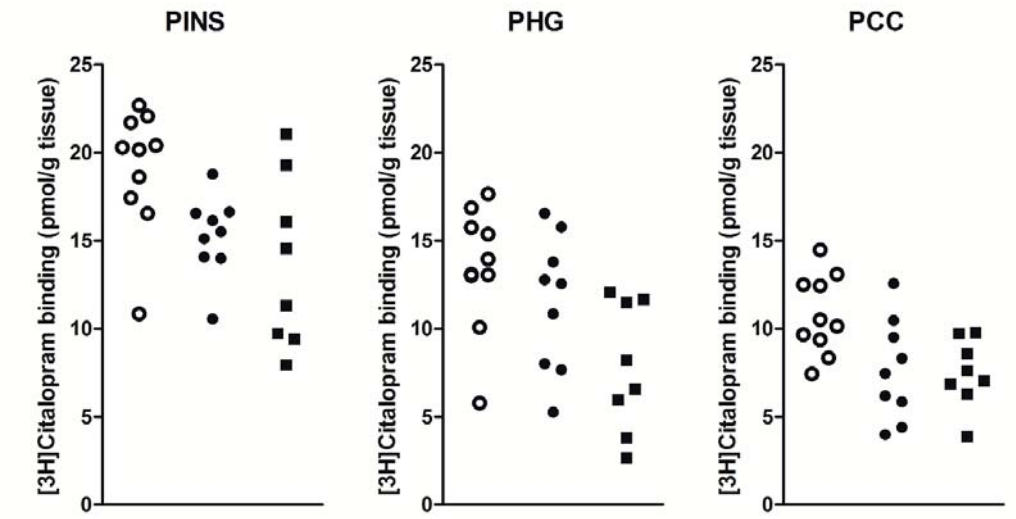

Figure 5. [^{3}H]Citalopram binding in the posterior insula, parahippocampal gyrus and posterior cingulate cortex. Significant differences between the study groups were observed in the PINS (Monte Carlo p-value=0.014), PHG (p-value=0.011) and PCC (p-value=0.011). Legend: ◦ = non-alcoholic controls; ● = type 1 alcoholics; ▪ = type 2 alcoholics; PINS, posterior insula; PHG, parahippocampal gyrus; PCC, posterior cingulate cortex.

5.4 STEROID HORMONE LEVELS IN THE POST-MORTEM BRAIN SAMPLES

Dehydroepiandrosterone and pregnenolone levels were quantifiable in almost all brain samples by the LC-MS/MS method. T levels were quantifiable in all male subjects, but in only one female subject (a type 1 alcoholic) who was excluded from the statistical analyses for T results (table 2 of study IV). Overall inclusion or exclusion of female subject did not have any major effect on mean dehydroepiandrosterone or pregnenolone levels (table 2 of study IV). Other ketosteroids e.g., progesterone and androstenedione, were below the quantitation limit of the assay in most of the samples from non-alcoholic controls and were therefore excluded from further analysis.

Alcoholic groups had increased dehydroepiandrosterone and pregnenolone levels compared to non-alcoholic controls (figure 2 and table 2 of study IV). The steroid hormone concentrations in the NAC are shown as an example in figure 6. One other key finding of this study was the negative Spearman correlations between previously published [^{3}H]naloxone binding (Laukkanen et al., 2015a) and tissue pregnenolone levels in the AINS, ACC, NAC and FC (figure 3 of study IV; R-values between -0.64 and -0.80; p-values between 0.002 and <0.001).

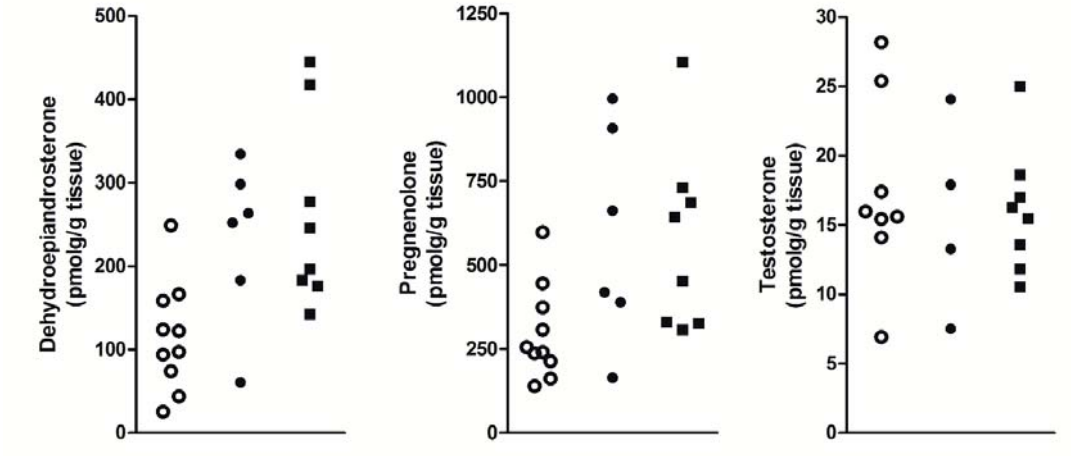

Figure 6. Dehydroepiandrosterone, pregnenolone and testosterone levels in the nucleus accumbens. Significant differences were seen between the study groups in post-mortem brain samples in dehydroepiandrosterone and pregnenolone levels, but not in T levels, as described in sub-study IV. Here steroid levels in the NAC are shown as an example of steroid levels in the post-mortem brain samples. Differences between the study groups can be observed also here. Legend: ◦ = non-alcoholic controls; ● = type 1 alcoholics; ▪ = type 2 alcoholics; DHEA, dehydroepiandrosterone; PREGN, pregnenolone; T, testosterone

6 Discussion

The individual studies included in the present thesis are discussed in depth in the attached articles (Studies I-IV). Therefore, I will highlight the main points and discuss more about the general aspect related to the results of the present thesis. Furthermore, I will discuss some limitations of the present research and how these could be overcome in future experiments. I will also discuss some questions that arise from the present results and how these questions could be answered in future experiments.

6.1 CHANGES IN THE BRAIN ASSOCIATED WITH ALCOHOLISM

The studies in the present thesis show alterations observed in the post-mortem brain samples which are associated with alcoholism in general (Study III and IV) as well as sub-type specific changes according to Cloninger's typology (Study I and II). Furthermore, study IV also included results on pregnenolone levels which divided alcoholics into two sub-groups, but this division did not follow Cloninger's typology. Overall, this demonstrates the main reason (and drawback) for using typologies of alcoholism: alcoholics are a heterogeneous group of individuals, and even though alcoholics share some aspects of the disease there are certain aspects in which there are more individual differences. Furthermore, typologies of alcoholism capture part, but not all, of these individual differences (Leggio et al., 2009b). The main findings from the set of post-mortem brain samples which were used in the study have been summarized in figure 7.

6.1.1 Impulsive type 2 alcoholics

Study I showed increased [^{3}H]AMPA binding in the ACC in type 2 alcoholics compared to non-alcoholic controls. More recent autoradiography studies in these same subjects have detected also increased [^{3}H]LY341495 binding to mGluR2/3 receptors in the ACC and decreased [^{3}H]ifenprodil binding to NR2B in the NAC in type 2 alcoholics (Kupila et al., 2015; Laukkanen et al., 2015b). As mentioned in chapter 2.6, there has been very little research on the role of cortical AMPA receptors in alcoholism. There seems to be a positive correlation between AMPA GluA1 subunit expression in the PFC and ethanol self-administration in rats (Pickering et al., 2007). Furthermore, chronic intermittent ethanol exposure seems to increase the AMPA/NMDA receptor ratio in the lateral orbitofrontal cortex in mice (Nimitvilai et al., 2015). Overall, the observed high [^{3}H]AMPA binding in the ACC in type 2 alcoholics could be a predisposing factor or a group specific response to chronic alcohol exposure or a combination of these factors.

The results of study I could be associated with the impulsivity seen in type 2 alcoholics. Impulsive behavior has been associated with activity of the ACC (Croxson et al., 2009; Kennerley et al., 2006; Kennerley and Wallis, 2009; Prevost et al., 2010). The increased synaptic activity in the ACC could explain the observed increased [^{3}H]AMPA binding in the type 2 alcoholics (Barry and Ziff, 2002). Synaptic strengthening by LTP has even been considered to be a permanent change in cortical regions, and being responsible for long lasting changes in behavior (Gold et al., 1996; Kessels and Malinow, 2009; Petralia and Wenthold, 1992). Moreover, also the negative Spearman correlation between [^{3}H]AMPA binding and the previously published DAT density (Tupala et al., 2006) in the ACC only in type 2 alcoholics could be contributing to the symptoms of executive dysfunction, because dopamine action in the frontal cortical regions has been associated with inhibitory control over impulsive behavior (Nutt et al., 2015; Vijayraghavan et al., 2007). These observations are in line with the theoretical framework that type 2 alcoholics have an intact reward system

but have dysfunctional executive control which results in excessive alcohol consumption and other impulsive behaviors (chapter 2.1.3.).

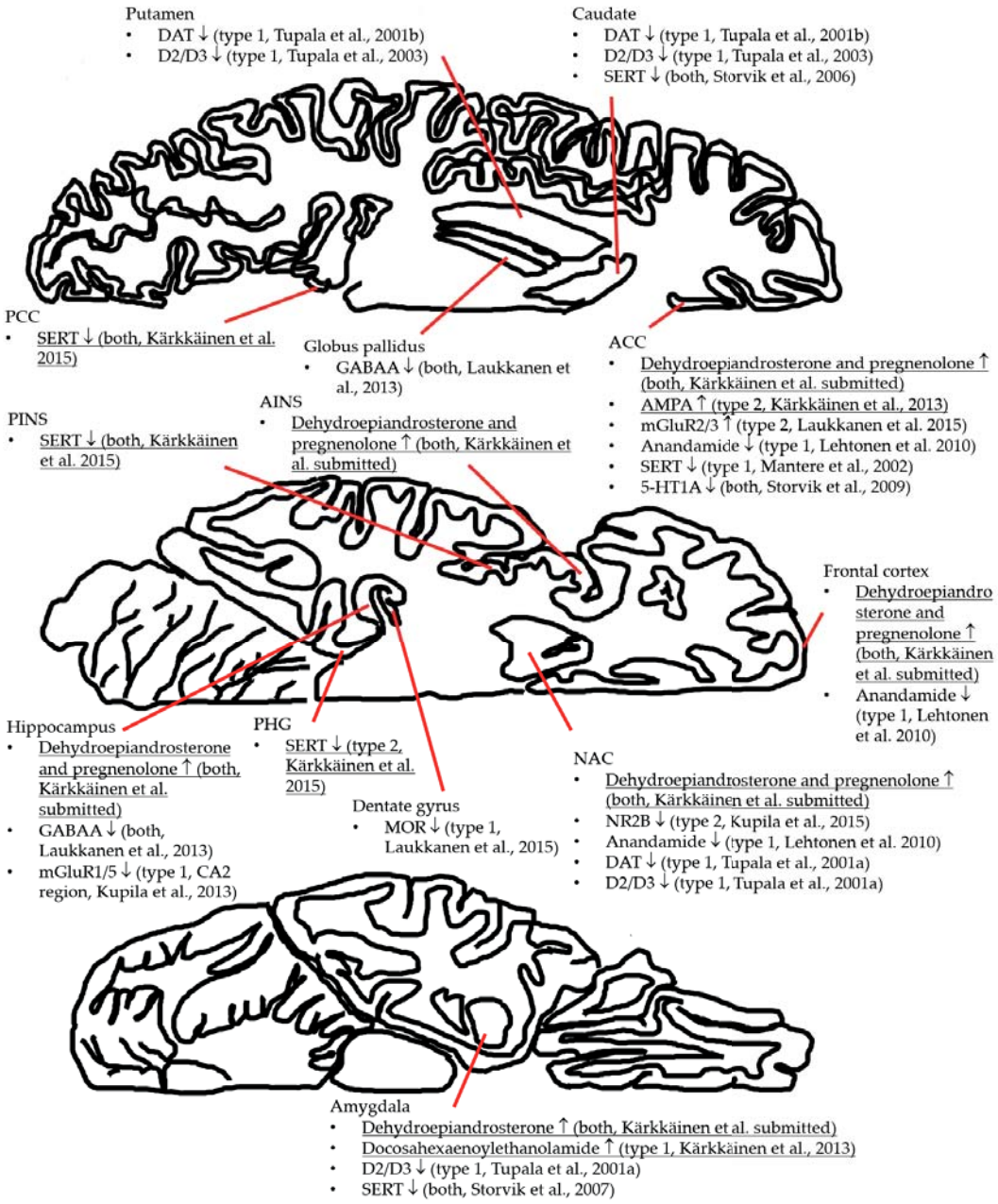

Figure 7. Summary of the findings from the set of post-mortem brain samples of Cloninger type 1 and 2 alcoholics examined in the study. Findings from the present thesis are underlined. Arrows refer to significantly increased/decreased binding or compound levels when compared to samples from non-alcoholic controls.

In future studies, other aspects of glutamatergic system, e.g. EAAT1 levels, but also the composition of AMPA receptor subunit levels should be measured since this is important for the function of these receptors e.g. determining calcium permeability after AMPA receptor activation (Grueter et al., 2012). It is plausible that the entire function of the glutamatergic system including transporters and metabolizing enzymes could be altered in type 2 alcoholics.

6.1.2 Altered endocannabinoid system in type 1 alcoholics

In the present study II, there were no significant differences between post-mortem samples of amygdala and hippocampus in the main endocannabinoids AEA and 2-AG between samples from alcoholics and controls. Previously, decreased AEA levels have been reported from these same subjects in the NAC, ACC and FC (Lehtonen et al., 2010). These results are in line with more recent results of altered CB1 receptor function in the caudate nucleus but not in the hippocampus of post-mortem samples of alcoholics compared to controls (Erdozain et al., 2015a).

In addition to the type 2 specific changes described above, also changes specific to type 1 alcoholics have been reported in the glutamatergic system, namely in mGluR1/5 binding (Kupila et al., 2013). These changes could be associated with the function of the endocannabinoid system. In sub-study II, a negative correlation was detected in type 1 alcoholics between previously published mGluR1/5 binding in the CA1 region (Kupila et al., 2013) and AEA levels in the hippocampus. This could be associated with TRPV1 receptor mediated LTD process in the hippocampus (Chavez et al., 2010). There is a possibility that the hippocampus of type 1 alcoholics is vulnerable to the effects of ethanol and this could explain, at least to some extent, their cognitive decline. This speculation needs to be studied further.

The other main finding emerging from study II was increased docosahexaenoylethanolamide levels in the amygdala in type 1 alcoholics. However, very little is known about the role of docosahexaenoylethanolamide in the CNS, especially in the amygdala. Docosahexaenoylethanolamide is a derivative of the *omega*-3 fatty acid docosahexaenoic acid (DHA) and has a weak affinity for cannabinoid receptors (Felder et al., 1993). However, there is a report that docosahexaenoylethanolamide can increase CB1 expression and improve glucose uptake in C2C12 myoblast cells (Kim et al., 2014a). Moreover, docosahexaenoylethanolamide has been found to promote synaptic development and glutamatergic synaptic activity in hippocampal neurons (Kim et al., 2011a; Kim et al., 2011b). Docosahexaenoylethanolamide oxidative metabolism products have also been associated with anti-inflammatory and neuroprotective properties (Yang et al., 2011). In conclusion, more work will be needed to clarify the role of docosahexaenoylethanolamide in the CNS in association with ethanol consumption.

6.1.3 Decreased SERT binding in alcoholics

One of the main hypotheses based on Cloninger's typology of alcoholism is that only type 2 alcoholics have a deficient serotonergic system (Cloninger, 1988; Cloninger, 1995). The results of whole-hemisphere autoradiography studies do not confirm this hypothesis since alterations in the serotonergic system have been observed in both type 1 and 2 alcoholics (Leggio and Addolorato, 2008; Mantere et al., 2002; Storvik et al., 2009; Storvik et al., 2012).

The main finding from study III was that alcoholics seem to have decreased [^{3}H]citalopram SERT binding in the posterior brain regions PCC and PINS (figure 5). The PINS has been associated with alcohol-cue reactivity and addiction pathology (Dager et al., 2014; Naqvi et al., 2007). Decreased activity in the PINS has also been associated with cost-benefit analysis of alcohol consumption in a bar laboratory setting (Mackillop et al., 2014). Furthermore, PINS has an important role in interoception and emotion recognition (Cauda et al., 2012; Paulus and Stewart, 2014). If one considers the entire spectrum of social behavior, then the PCC has been associated with social cognitive processing, e.g. perception taking, observing social

interactions and self-related processing in social interactions (Sperduti et al., 2012; van Veen et al., 2009). The left PCC in particular has been claimed to be involved in determining how the individual evaluates negative social situations (Arsenault et al., 2013) and with an inability to describe sad feelings (Lemche et al., 2013), which could be related to alexithymia-like symptoms seen in alcoholics (Cloninger, 1987). The observed decreased [^{3}H]citalopram binding in the PCC and PINS in alcoholics could be linked with a dysfunctional serotonergic regulation of social cognitive processes, possibly resulting in social anxiety. Similar to the differences in behavioural traits observed in Cloninger type 1 and type 2 alcoholics (Cloninger, 1987) also in social-anxiety patients, two types of behaviour are observed: social withdrawal and antisocial behaviour (Kashdan and McKnight, 2010).

Moreover, the present finding of decreased [^{3}H]citalopram binding to SERT in the PHG, only in type 2 alcoholics is the first observation of type 2 specific changes in the serotonergic system (figure 5). This alteration could be associated with social context recognition (Aupperle et al., 2013; Rapp et al., 2013; Shi et al., 2013; Silk et al., 2013). However, the overall picture is that the serotonergic system is altered in all alcoholics, although individual differences might be seen in a brain region specific manner (Sari et al., 2011). Furthermore, the reasons for these changes cannot be elucidated i.e. are they attributable to alcohol consumption or are they pre-existing factors making an individual susceptible to becoming an alcoholic?

Future studies should also measure if the levels of serotonergic receptors are altered in these brain regions in alcoholics. For example, brain imaging studies in healthy volunteers using the non-specific 5-HT$_{2A}$ agonist psilocybin have identified the PCC as one of the key brain regions for the function of the drug which include ego-dissolution and are suggested to enable altering stagnated brain circuits (Carhart-Harris et al., 2012; Carhart-Harris et al., 2014; Lebedev et al., 2015). The decreased SERT binding detected in the PCC might reflect a depressed serotonergic tone which could inhibit the cerebral flexibility required for the individual to appreciate the risks of excessive alcohol consumption (Carhart-Harris et al., 2014). Future research should measure if also other components of the serotonergic system are altered in the posterior brain regions associated with interoception and social cognitive processes.

6.1.4 Dehydroepiandrosterone and pregnenolone levels

The increased dehydroepiandrosterone and pregnenolone levels observed in the post-mortem brain samples of alcoholics in study IV could be associated with alcohol induced alterations in the synthesis and metabolism of steroids (Helms et al., 2012). Dehydroepiandrosterone and pregnenolone can inhibit many of the pharmacological actions of ethanol (chapter 2.9.2). Therefore, increased dehydroepiandrosterone and pregnenolone levels in the brain of alcoholics could contribute to the allostatic changes associated with the development of alcoholism (Helms et al., 2012; Koob, 2013; Vendruscolo et al., 2015). This is in line with the theoretical framework that chronic alcohol consumption induces alterations in the brain which reduce the positive effects of alcohol.

The other main finding of study IV was the negative correlation between pregnenolone levels and previously published [^{3}H]naloxone binding (Laukkanen et al., 2015a). This indicates that the opioid system might be involved in regulating pregnenolone levels also in the CNS, similar to the situation in the periphery (Porcu et al., 2006). Interestingly, the pregnenolone levels in alcoholics did not follow Cloninger's typology of alcoholism. Alcoholics with high pregnenolone levels had also low levels of MOR binding. This might translate into poorer treatment outcomes if these individual receive drug therapy with MOR antagonists such as naltrexone and nalmefene. Furthermore, changes in peripheral pregnenolone levels following MOR antagonist administration could be a possible biomarker for treatment efficiency of MOR antagonists. Future studies should clarify the role of steroid hormones in the CNS and the associations between steroid hormones and neurotransmitter systems important for alcoholism pathology.

6.2 POSSIBLE CLINICAL IMPLICATIONS

Typologies of alcoholism are intended to guide clinical treatment and therefore one important aspect of this discussion is how the present results could influence clinical studies and the future treatment of alcoholism. It should however be noted that no clinical recommendation can be provided from the present results. Therefore, the aim of the following discussion is that it should be considered more as an inspiration for future studies rather than providing direct clinical recommendations.

In relation to Cloninger's typology of alcoholism, the present study has found both supporting and contrasting evidence. Two of the studies (Study I and II) found type specific changes in the post-mortem brain samples, whereas two studies detected similar changes in all alcoholics (Study III and dehydroepiandrosterone results from study IV). However, study IV also found evidence of sub-groups of alcoholics which did not follow the Cloninger's typology of alcoholism (pregnenolone results from study IV).

The increased AMPA receptor binding in type 2 alcoholics could indicate that a sub-group of alcoholics with early onset of alcoholism and impulsive tendencies might benefit from treatment with AMPA receptor antagonists (Cloninger, 1988; Holmes et al., 2013); some compound in this class are currently undergoing preclinical trials. The present results indicate that subgroup composition should be taken into account when designing clinical trials for AMPA antagonists. Moreover, the nonselective AMPA/kainate receptor antagonist topiramate is used as an off-label treatment for alcoholism (Blodgett et al., 2014; Johnson and Ait-Daoud, 2010). In addition to reducing alcohol consumption (Baltieri et al., 2008; Florez et al., 2008; Johnson et al., 2003a; Johnson et al., 2004; Johnson et al., 2007; Krupitsky et al., 2007; Miranda et al., 2008; Paparrigopoulos et al., 2011) topiramate treatment has also been associated with reduced impulsivity in alcoholics (Rubio et al., 2009). This is in line with the concept of using topiramate in impulsive alcoholics. However, kainate receptor function might be more important than AMPA function in the treatment effect of topiramate, because a polymorphism in the kainate GluK1 receptor subunit has been associated with the efficacy of topiramate treatment in heavy alcohol users (Kranzler et al., 2014; Kranzler et al., 2014).

The role of docosahexaenoylethanolamide in the pathology of alcoholism is too unclear to make any clinical inferences from the detected increased docosahexaenoylethanolamide levels in the amygdala of type 1 alcoholics. Previous results of decreased AEA levels in the NAC, ACC and FC in Cloninger type 1 alcoholics however might indicate that this sub-group of alcoholics might benefit from some kind of medication increasing the AEA levels (Lehtonen et al., 2010). FAAH inhibition which increases levels of AEA and other fatty acid amines has been claimed to decrease symptoms of anxiety during ethanol withdrawal in rats (Cippitelli et al., 2008). However, FAAH inhibition does not seem to decrease drinking. Therefore, FAAH inhibitors might only be useful as an adjunct therapy to decrease the anxiety of alcoholics during withdrawal and abstinence.

The present studies indicate that both alcoholic subtypes could benefit from some form of mediation normalizing the SERT function in the brain (Study III). However, results from SSRI clinical trials have shown that only late-onset alcoholics with LL polymorphism seem to benefit from SSRI medication and early-onset alcoholics actually drink more if treated with SSRI drugs (Dundon et al., 2004; Kranzler et al., 2011; Kranzler et al., 2012; Pettinati et al., 2000). The reasons behind this disparity needs to be studied further. It has been suggested that early-onset alcoholics have a dysfunctional serotonin metabolism and SSRIs could overstimulate the serotonergic system, especially 5-HT_3 receptors, causing increased alcohol consumption in this sub-group of alcoholics (Budde et al., 2010; Campbell and McBride, 1995; Minabe et al., 1991). One way to clarify these apparently conflicting results would be to examine the function of other components of serotonergic system, e.g. 5-HT_2, 5-HT_3 and 5-HT_7 receptors.

One of the more interesting clinical prospects emerges from study IV, where pregnenolone levels were shown to negatively correlate with MOR binding and alcoholics could be

subdivided into two sub-groups according to their pregnenolone levels. Furthermore, previously plasma pregnenolone levels have been associated with a preference or hedonistic liking for the effects of alcohol consumption (Pierucci-Lagha et al., 2006). Overall, one could postulate that monitoring plasma pregnenolone levels after naltrexone or nalmefene challenge may be used to assess the function of the opioid system and possibly the efficacy of MOR antagonist treatment (Porcu et al., 2006). This hypothesis should be evaluated first in preclinical studies before conducting full-scale clinical trials.

6.3 LIMITATIONS

The main limitation of the present study is the relatively small number of samples in the study groups. This is especially true in study IV, in which the alcoholic subjects who died of inflammatory diseases where excluded because inflammation influences steroid hormone levels. Furthermore, the use of post-mortem brain samples does not enable any determination of causality i.e. are the measured differences seen between alcoholics and controls caused by alcohol consumption or do they pre-exist and make the individual susceptible to suffer the alcoholism?

Moreover, from the medical records it is not possible to rule out the possibility that some of the non-alcoholic controls might have had some alcohol related problems. However, these problems would have been minor compared to the alcohol dependent subjects, because medical records of the non-alcohol dependent controls did not indicate any alcohol-related medical appointments.

Furthermore, it is very challenging to obtain post-mortem brain samples from older type 2 alcoholics or younger controls who would meet the inclusion criteria. Therefore, another inevitable limitation is that type 2 alcoholics will tend to die at a younger age than type 1 alcoholics or non-alcoholic controls because of their antisocial and impulsive behavior (Repo-Tiihonen et al., 2001). From a statistical point of view, using age at the time of death as a co-variant is not a valid procedure because age of onset of alcoholism is an integral part of the studied phenomenon (Miller and Chapman, 2001). However, it is possible that the frontal cortical regions are still maturing in the youngest type 2 alcoholics (Li, 2013; Sowell et al., 1999). This cannot be excluded as potential alternative explanation for some of the present findings, especially those from study I.

Moreover, ante- and post-mortem effects could influence the results. The ante-mortem effect can only be controlled by strict selection of samples. Limitations here include years of alcohol abuse which cannot be reliably determined from medical records. If post-mortem effects may influence the measured variable, then this can be partially compensated between the samples by using post-mortem interval as a co-factor during statistical analysis. Endocannabinoid levels have been shown to be altered by the post-mortem interval (Palkovits et al., 2008) and therefore post-mortem interval was used as a co-factor in the statistical analysis conducted in study II. Post-mortem interval was not used as a co-factor in studies I, III and IV because there were no consistent correlations between the post-mortem interval and the measured variables.

Moreover, in the autoradiography studies, ligand binding was measured which might not fully represent the receptor density in the brain. Furthermore, the currently used autoradiography methods do not detect whether or not the receptors are functional. This limitation could be resolved by adopting functional autoradiography procedures (Aaltonen et al., 2014).

One limitation of study IV is that not all steroids and steroid metabolites could be measured with the analytical method. This may be important since different metabolites of pregnenolone and dehydroepiandrosterone are thought to exert different modulatory functions on neurotransmitter systems, e.g. some enhancing and others inhibiting $GABA_A$ receptor function (Helms et al., 2012). Moreover, the spatial resolution of the mass

spectrometry studies II and IV could be improved in the future by applying the recent methodological advances in mass spectrometry imaging for measuring endocannabinoids and neuroactive steroids (Ivanisevic et al., 2014; Shariatgorji et al., 2014; Vaikkinen et al., 2014). However, currently these methods are better suited for measurement of small sample sizes e.g. mouse whole hemisphere not large human specimens.

6.4 FUTURE DIRECTIONS

One of the most obvious future goals would be to undertake these types of experiment in a larger set of post-mortem brains. This can be achieved by accessing international brain banks, which hold also post-mortem brain tissue from alcoholics, e.g. New South Wales brain bank in Australia. In particular, the concentrations of the steroid hormones should be measured in a larger sample set (Study IV). Moreover, also other aspects of the glutamate, endocannabinoid, serotonergic and neuroactive steroid systems need to be measured in order to gather a complete picture of the changes occurring in the brains of alcoholics. This would include not only different receptors but also the machinery needed for synthesis and metabolism of the neurotransmitters and hormones.

However, I consider the main limitation of the present study to be that it is impossible to draw inferences of causality from the observed differences between alcoholics and controls in assays conducted in post-mortem brain samples. This raises the main unanswered question: which of the changes are caused by chronic alcohol consumption and which precede alcoholism? If both alcoholic sub-groups show similar changes, e.g. increased dehydroepiandrosterone levels in study IV, it seems reasonable to speculate that this change might be due to chronic alcohol consumption. However, this might not always be the case. For example, the results from study III, where both alcoholic groups exhibited decreased SERT binding, might be due to a combination of both chronic alcohol consumption and factors preceding alcoholism. Clinical studies have demonstrated that early and late-onset alcoholics react in opposite ways to SSRI medication (Dundon et al., 2004; Kranzler et al., 2011; Kranzler et al., 2012; Pettinati et al., 2000) indicating that other components in addition to SERT density are important for treatment outcomes. Furthermore, if only one group of alcoholics shows a difference compared to controls, as was the case in sub-studies I and II, then these changes could be caused by non-alcohol related factors but also possibly by differential responses to alcohol.

The importance of understanding the difference between alcohol evoked changes and changes preceding alcoholism is related to treatment and prevention of the health harms associated with alcohol consumption (Lim et al., 2012; World Health Organization, 2014). Understanding the mechanism by which alcohol causes health harms could enable the development of methods to reduce the undesired effects of alcohol. This could also influence political decisions, for example less taxation on those alcoholic beverages which are less harmful. Furthermore, treatment of alcoholism should be able to address both alcohol evoked malfunction in the CNS, but also the pre-existing susceptibility factors which are crucial for the development of the alcoholism. In both categories there are likely to be individual variations and possible subgroups which could benefit from different types of treatment options. In addition, understanding the pre-existing factors making an individual vulnerable to alcoholism could enable determination of biomarkers and treatment of these factors before escalation of alcohol consumption. In summary, it is therefore important to understand the mechanisms behind the changes occurring in the brains of alcoholics.

Many of the published experimental studies have been done using animals as research tools. In order to understand why certain humans become alcoholics, one possible experimental tool would be to exploit a novel technology - induced pluripotent stem cells collected from alcoholics and controls. Induced pluripotent stem cells are generated from adult cells by exposing the cells to specific transcription factors (Takahashi and Yamanaka,

2006). Under specific differentiation conditions, these cells can be programmed to form, in theory, any kind of somatic cell with the genome of the original donor. This would make it possible to conduct research on human non-cancerous cells from donors with different phenotypes. Already some preliminary studies have been published describing the advantages of using human induced pluripotent stems cells in alcoholism research (Lieberman et al., 2012). In the future, when the methods to generate cerebral organoids (neural tissue for pluripotent stem cells in a petri dish) develop further (Lancaster et al., 2013), one may be able to examine how more complex human CNS structures are affected by ethanol in the laboratory and whether cells from substance abusers are affected differently than the general population. These types of methods might also make it possible to elucidate how ethanol exposure induces fetal alcohol syndrome-like changes in the CNS. In combination with animal research, this evolution of research methods will hopefully provide answers to many of today's open questions e.g. how alcohol consumption is associated with the differences in the brain chemistry between alcoholics and non-alcoholic controls observed in the present studies.

Furthermore, one of the major differences between the experimental studies done in the laboratory and the situation in the real world is the difference in consumed alcohol beverages. In the laboratory, usually pure ethanol in combination with water is used in order to detect only the effect of ethanol on the system being studied. However humans drink many different kinds of alcoholic beverages. This is important, because a preference of beer and/or wine has been associated with better health outcomes compared to spirits and these differences are not simply in cardiovascular risks but extend to mental health (Adjemian et al., 2015; Athyros et al., 2007; Razvodovsky, 2015; Ronksley et al., 2011; Smart, 1996; Wald and Jaroszewski, 1983). Part of this effect is likely social and related to preferences for different alcoholic beverage types in different populations. However, there is also evidence from animal studies that the beverage types themselves produce different health outcomes (Landmann et al., 2015; Vilahur et al., 2012). The biomedical mechanisms behind these effects, especially in the CNS, are not yet fully understood. Other compounds present in alcoholic beverages in addition to ethanol are likely to be behind these differences between alcoholic beverage types. Metabolomics is a technique to measure the net effect of exposure both in the whole body as well as at the organ specific level, including the CNS (Hanhineva et al., 2013; Ivanisevic et al., 2014; Pekkinen et al., 2013). This methodology is especially well suited for the study of the net effect of consumption of different alcoholic beverages, which contain many active compounds in addition to ethanol. Although different animal research tools could be used, one rather obvious choice would be the pig which has a similar metabolism and brain function as humans (Hanhineva et al., 2013; Sauleau et al., 2009; Val-Laillet et al., 2011; Vodicka et al., 2005). The methods used in the present thesis could also be applied to the study of pig brain tissues after chronic ethanol exposure in order to elucidate the differences between changes attributable to ethanol and pre-existing differences in the brain as risk factors for elevated vulnerability to the effects of alcohol. Because of similar metabolism and brain function, use of pig as a research tool would also enable controlled analysis of the whole body function in relation to ethanol consumption e.g. role of gut-brain axis in ethanol induced changes in the brain (Gonzalez-Reimers et al., 2014; Leggio et al., 2011).

7 Conclusions

The main aim of the present thesis was to increase our understanding of the neural correlates of alcohol use and predisposing factors. Some neurochemical parameters in the brains of alcoholics were investigated by comparing measurements from post-mortem brain samples of Cloninger type 1 and type 2 alcoholics to samples from non-alcoholic controls. Although preliminary because of the relatively small number of subjects, the present results indicate the following main conclusions:

1. The [^{3}H]AMPA binding to AMPA receptors in post-mortem brain samples is increased in the ACC of impulsive type 2 alcoholics as compared to non-alcoholic controls.
2. Docosahexaenoylethanolamide levels are increased in the post-mortem amygdala in the Cloninger type 1 alcoholics as compared to controls
3. The [^{3}H]citalopram binding to SERT is decreased in the PCC and PINS in the post-mortem brain samples of alcoholics in comparison with non-alcoholic controls. When compared to controls, the [^{3}H]citalopram binding is decreased only in the antisocial type 2 alcoholics in the PHG.
4. Dehydroepiandrosterone levels are higher in the post-mortem brain samples of alcoholics than in non-alcoholic controls.
5. The pregnenolone levels negatively correlate with [^{3}H]naloxone binding in the measured brain regions and furthermore post-mortem brain sample levels of pregnenolone are increased only in a sub-group of alcoholics with low [^{3}H]naloxone binding as compared to controls.

In summary, the neurochemical profile of post-mortem brain samples is altered in alcoholics. The present results provide evidence that AMPA receptors, endocannabinoids, SERT and neuroactive steroids are involved in the pathology of alcoholism. Some of the changes were seen in both alcoholics sub-groups compared to controls and were likely to be associated with chronic alcohol consumption e.g. increased dehydroepiandrosterone levels. Some of the changes were sub-type specific and might be associated with factors predisposing to the alcoholism e.g. increased AMPA receptor levels in the ACC in the impulsive type 2 alcoholics. In addition, changes in sub-groups of alcoholics which did not follow the Cloninger's typology were observed i.e. increased pregnenolone levels in alcoholics with decreased [^{3}H]naloxone binding. Overall, the results of present thesis highlight the need to understand alcoholics as a spectrum of individuals with regards to both treatment and research into their etiology. More research will be needed before the causes of the observed neurochemical changes in the post-mortem brains of alcoholics will be elucidated.

8 References

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OLLI KÄRKKÄINEN

Alcoholics are a heterogeneous group with a spectrum of health problems. Alcohol influences the function of many of the brain's messaging systems. This thesis investigated the post-mortem brain samples of late-onset Cloninger type 1 and early-onset type 2 alcoholics. Increased dehydroepiandrosterone and pregnenolone levels, and decreased serotonin transporter binding, were observed in the alcoholics when compared to controls. Moreover, only antisocial type 2 alcoholics had increased AMPA receptor binding in the anterior cingulate cortex and only anxiety-prone type 1 alcoholics showed increased docosahexaenoylethanolamide levels in the amygdala.

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