Thesis for the degree of Doctor of Medicine

Brain glycine receptors as a common target for alcohol and the relapse-preventing drug acamprosate - a preclinical study

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Till kvinnorna i mitt liv…..

”I am among those who think that science has great beauty. A scientist in her laboratory is not only a technician: she is also a child placed before a natural phenomena which impress her like a fairy tale”

– Originally by Madame Marie Curie (modified by PeiPei Chau)

Abstract

Brain glycine receptors as a common target for alcohol and the relapse-preventing drug acamprosate – a preclinical study

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Alcohol abuse and dependence make up the most prevalent categories of substance use disorders in the world. Converging evidence from the current research group has identified two receptor populations, the glycine (GlyRs) and nicotinic acetylcholine receptors (nAChRs) in the mesolimbic dopamine system, as two potentially important targets for the development of new medication to treat alcohol dependence. It is suggested that ethanol primarily acts via GlyRs in the nucleus accumbens (nAc) with a secondary and indirect effect on nAChRs in the ventral tegmental area (VTA), subsequently activating dopaminergic neurons leading to an increase of extracellular dopamine in the nAc. Pharmacological modulation of these receptors alters the activity of the suggested nAc-VTA-nAc circuitry with prominent effects on ethanol-induced dopamine elevations as well as ethanol intake. The general aim of this thesis was to further investigate the role of these receptors for regulating ethanol-induced dopamine and consummatory actions, by using ethanol and substances with possible anti-alcohol effects in the rat. Measurements of extracellular dopamine and amino acid levels in the nAc were made using *in vivo* brain microdialysis in awake, freely-moving male Wistar rats. In addition, a voluntary ethanol consumption paradigm with limited access was used to measure ethanol intake. The results indicate that the anti-relapse substance acamprosate has a similar dopamine-modulating profile as previously observed with ethanol and the endogenous GlyR ligand taurine. The acamprosate-induced dopamine elevation was demonstrated to be inhibited by pre-treatment with GlyR or nAChR-antagonists (Paper I). At a behavioral level, the ethanol intake-reducing effect of acamprosate was *reversed* by GlyR antagonism in the nAc (Paper II). In addition, the loss of the ethanol intake-reducing effect of chronic administration of acamprosate is potentially linked with its' dopamine-modulating property (Paper III). The influence of acamprosate-related substances, the metabotropic glutamate type 5 receptor (mGluR5) antagonist MPEP and taurine, were also investigated. We found that mGluR5 and GlyR may have a joint mechanism to activate the dopamine output (Paper IV). Also, an augmentation of extracellular taurine levels is required in order to obtain an ethanol-induced dopamine increase (Paper V). The findings of this thesis have revealed a new mechanism of action for the anti-relapse agent acamprosate. But, most importantly, the results have further confirmed the relevance of the nAc-VTA-nAc neuronal circuitry for alcohol addiction.

Keywords: acamprosate, alcohol, dependence, dopamine, glycine, nicotinic acetylcholine receptor

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

I. Chau P, Stomberg R, Fagerberg A, Söderpalm B, Ericson M. (2010) **Glycine receptors involved in acamprosates' modulation of accumbal dopamine levels: an *in vivo* microdialysis study** Alcohol Clin Exp Res. 34(1):32-8.

II. Chau P, Höifödt-Lidö H, Löf E, Söderpalm B, Ericson M. (2010) **Glycine receptors in the nucleus accumbens involved in the ethanol intake-reducing effect of acamprosate** Alcohol Clin Exp Res. 34(1):39-45.

III. Chau P, Söderpalm B, Ericson M. **Acamprosate-induced dopamine elevation is associated with its' ethanol intake-reducing effect** Submitted/Manuscript

IV. Chau P, Söderpalm B, Ericson M. **The mGluR5 antagonist MPEP elevates accumbal dopamine and glycine levels; interaction with strychnine-sensitive glycine receptors** Submitted/Manuscript

V. Ericson M, Chau P, Clarke RB, Adermark L, Söderpalm B. (2010) **Rising taurine and ethanol concentrations in nucleus accumbens interact to produce dopamine release after ethanol administration** Addiction Biology (E-pub ahead of print Aug 23 2010).

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LIST OF ABBREVATIONS

5- HT – Serotonin

AA/ANA – Alko alcohol/non-alcohol (rat strain)

ACh - Acetylcholine

ANOVA – Analysis of variance

CNS – Central nervous system

CRF – Corticotropin releasing factor

DSM-IV – Diagnostic and Statistical Manual of Mental Disorders 4th edition

EOS - Endogenous opioid system

GABA – γ-amino-butyric acid

GlyR – Glycine receptor

GLYT-1 – Glycine transporter -1

GLYT-2 – Glycine transporter – 2

HAS/LAS – High- low alcohol sensitivity (rat strain)

HPLC – High-pressure liquid chromotography

LDTg – Laterodorsal tegmental nucleus

mGluR5- Metabotropic glutamate receptor group 5

MPEP - 2-methyl-6-(phenyl-ethynyl)-pyridine

nAc – Nucleus accumbens

nAChR – Nicotinic acetylcholine receptor

NMDAR – *N*-methyl-*D*-aspartatic acid receptor

PFC – Prefrontal cortex

PPTg – Pedunculopontine tegmental nucleus

PLSD - Protected least significant difference

SEM – Standard error of the mean

sP/sNP – Sardinian ethanol Preferring/Nonpreferring (rat strain)

VTA – Ventral tegmental area

PREFACE

Since ancient time, alcoholic beverages have been used by people all around the world for medical reasons, in religious ceremonies, as a part of the standard diet and for its euphoric and relaxant effect. Today in most (Western) countries, alcohol consumption in moderate quantities is acceptable and often used at social occasions.

Depending on various factors such as genetic predisposition, provocative environmental experiences, social context and others, alcohol consumption can become compulsive and eventually an addictive behavior may evolve. What are the mechanisms of action underlying development of alcohol dependence? For years scientists have been trying to resolve this issue in an attempt to find a treatment. But the road to a disclosure has been obstructed by the multiple mechanisms of the small alcohol molecule in the human body and brain, and the involvement of genetic components and personality traits. With the investigation of the neurobiological mechanism of alcohol dependence, various pharmacological substances have been examined for their potential to decrease the risk of relapse in alcohol-dependent patients but only two (naltrexone and acamprosate) have been approved as pharmaceutical treatments. Although approved, the effect sizes of these agents are only moderate and this drives the continued search for new remedies.

Modern psychopharmacology has evolved from a close interaction between clinical and preclinical researchers, where one of the most important driving forces has been to unravel the often initially unknown mechanisms of action of compounds used in the clinic. The aim of the present thesis is to evaluate the hitherto largely unknown mechanism of action of the evidenced-based anti-relapse drug acamprosate and how it relates to a recently revealed mechanism of action of ethanol in the brain reward system.

INTRODUCTION

Alcohol Use in Sweden from a Sociocultural and Historical View

Since prehistory, beer was consumed in extreme quantities to balance the salty, pickled food in Sweden. In the 19th century, Sweden was industrialized and urbanized and the distilled alcoholic beverage called "brännvin" became more available causing increasing health and social problems. A social movement, the temperance movement, against the use of alcoholic beverages, rose and resulted in the creation of (and still existing) the governmental monopoly on sales of liquor and, for a few years, the establishment of a rationing system called Brattsystemet or motbok, which limited the buying of alcohol (used until 1955). The outcome of these initiatives was a continuous decline in consumption during the first half of the 20th century. But since Sweden entered the European union in 1995, the regulation and the alcohol consumption habits have been more continental and relaxed, resulting in a gradual increase in alcohol consumption. Today, the yearly consumption in Sweden is estimated to approximately 10 litres of pure alcohol (corresponding to approximately 100 (75 cl) bottles of wine) per person, 15 years and older, per year [2]. In addition, alcohol abuse and addiction causes enormous costs to society with an estimated annual total of 100 billion SEK per year [2-3].

Addiction

The best definition of addiction is loss of control over drug use, or compulsive seeking and intake of drugs despite adverse consequences. When the drug intake is discontinued, it results in psychological and physical withdrawal symptoms (see Table 1).

Addictive drugs are both rewarding and reinforcing. A reward is a stimulus that the brain interprets as intrinsically positive. A reinforcing stimulus is one that increases the probability that behaviors paired with it will be repeated. Notably, not all reinforcers are rewarding. A punishing or negative stimulus might reinforce avoidance [4].

Table 1. In the DSM (IV), substance dependence is defined as the occurrence of three or more of these criteria over a 12-month period [5].

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

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

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

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

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

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

7) The substance use is continued despite knowledge of having a persistent physical or psychological problem that is likely to have been caused or exacerbated by the substance.

Drug-taking behavior progresses from impulsivity to compulsivity in a three-stage cycle: binge/intoxication, withdrawal/negative affect and preoccupation/anticipation [6]. At the binge/intoxication phase, the individual consumes the drug for the positive reinforcing (euphoria, relaxant, "high") effects. The actions of this phase are primarily mediated by the mesolimbic dopamine system. As the individual is moving towards the compulsive state, the drive of consumption is rather removal of the aversive state (negative reinforcement) produced by dysfunctional hypo-dopaminergic and hyper-glutamatergic neurotransmission. During this phase, symptoms like chronic irritability, emotional pain, dysphoria and loss of motivation for natural rewards will appear. The third stage, preoccupation/anticipation, accounts for the chronic relapse

problem in addiction, in which the addict returns to compulsive drug intake after a long period of abstinence.

These three stages are conceptualized as interacting with each other, becoming more intense, and ultimately leading to the pathological state known as addiction [7]. Multiple brain regions and circuits are disrupted in drug addiction and are likely to contribute to the variety and complex phenotype observed in addicted individuals [6]. The divergence of mechanisms and the multiple sites of action complicate the understanding of the pathology underlying addiction. Although drugs of abuse are chemically divergent molecules with very different primary mechanism of actions, they converge in the production of some common actions. The most prominent action is the activation of the brain reward system. Thus, evaluation of the interaction between the reward system and drugs of abuse may be an excellent access point towards the search of an effective pharmacological treatment for addiction, especially alcoholism.

The Brain Reward System

In the 1950's, James Olds and Peter Milner [8] discovered that rats with electrodes implanted in their brains would sometimes self-stimulate or avoid stimulation at various specific regions. They named the sites where the rats self-stimulated as the "reinforcing structures". The reinforcing structures were later anatomically mapped and redefined as the brain reward system [9-10].

The brain reward system is also tightly connected with neurocircuitries involving learning and memory [11] since it is essential for survival to remember important events. For example, it is important for elephants to remember the location of the water supply in an otherwise dry and non-vital habitat. From an evolutionary biology point of view, the brain reward system is essential for the survival of species, as it motivates the individual for natural rewards such as intake of food and water and for copulation [12-13]. The system is therefore well-conserved among species. Unfortunately, the same system is also activated by non-vital stimuli/rewards, like drugs of abuse and compulsive behaviors (e.g., shopping,

gambling and over-eating), which may potentially lead to addictive behaviors. The tight connection between memory and learning processes that are essential in natural rewards can also have a devastating role in drug addiction. Drug-related events (called cues or stimuli) that occur during repeated drug intake will eventually be memorized and associated with the drug reward. These cues often become powerful primary triggers for relapse [14].

The Mesolimbic Dopamine System

Several neurocircuitries and neurotransmitters are implicated in the rewarding effects of drugs of abuse, but the major neurochemical pathway of the reward system involves the mesolimbic dopamine system [15-16].

The dopamine system has two modes of firing: tonic and phasic transmission. There are no distinct definitions but generally phasic dopamine transmission is considered a brief increase (up to 2 seconds) in dopamine concentration in the terminal regions [17-18], and characterized by an irregular pacemaker activity. A tonic signal is defined as a slow change in dopamine concentration, lasting from seconds up to days. Since there are two distinct firing patterns, it is also reasonable to hypothesize that there are two different functional roles. Indeed, phasic changes may play an important role in reward mechanisms since the burst would correspond to the reward signal. In contrast, the function of tonic firing is to maintain the baseline steady state levels of dopamine and the overall responsiveness of the dopamine system and enable a wide variety of motor, cognitive and motivational function [19].

Neuronal Pathways in the Mesolimbic Dopamine System

The mesolimbic dopamine system consists mainly of A10 dopaminergic neurons projecting from the ventral tegmental area (VTA) to the limbic areas such as the nucleus accumbens (nAc) and amygdala. Neurons of the VTA also project to cortical areas, referred to as the mesocortical dopamine system; the prefrontal cortex (PFC) and to the dorsal striatum and ventral pallidum [20-22]. The

mesolimbic dopamine system is regulated by various neurotransmitter systems. The excitatory input to the VTA consists mainly of glutamatergic afferents from the PFC, bed nucleus of the stria terminalis, laterodorsal tegmental nucleus (LDTg) and lateral hypothalamus [23]. Glutamatergic afferents to the nAc also exist, but with different origins depending on which nAc region (shell or core) they project to. The core region of the nAc receives glutamatergic neurons from the PFC and the thalamus whilst the shell region is innervated by the amygdala, hippocampus and PFC. The inhibitory control of the VTA is maintained by local GABAergic interneurons within the VTA and of descending GABAergic feedback neurons from the nAc and the ventral pallidum [24]. In addition, cholinergic afferents project from LDTg and pedunculopontine tegmental nucleus (PPTg), and activate primarily phasic firing of the VTA dopamine neurons via activation of the nicotinic acetylcholine receptor (nAChR) [25]. Here, only a small portion of the possible connections, regulations and functions are described. Besides communication to and from VTA, there are numerous ways for the structures in the brain reward system to communicate with each other. Figure 1 is a simplified schematic illustration of the different afferents and efferents to and from the VTA.

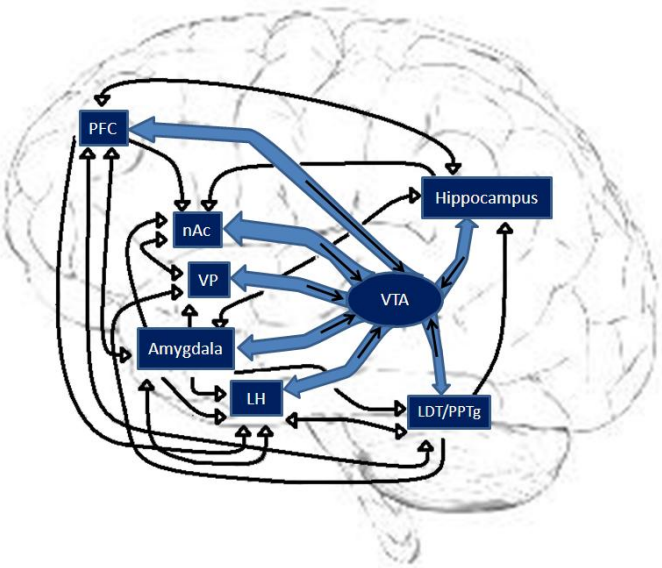


Figure 1. Schematic illustration of the neuronal connections between various brain regions involved in the brain reward system, with the origin from the ventral tegmental area (VTA). Abbrevations: LDTg/PPTg: laterodorsal and pedunculpontine tegmental nucleus LH: lateral hypothalamus, nAc: nucleus accumbens, PFC: prefrontal cortex, VP: ventral pallidum.

Dopamine

Dopamine is a catecholamine neurotransmitter which is synthesized in several areas of the brain. In the synthesis of dopamine, the amino acid precursor tyrosine, is transported across the blood-brain barrier and into dopamine neurons, thereafter hydroxylation and decarboxylation processes lead to the end-product dopamine [4]. In most brain regions, the catecholamine is inactivated by reuptake via the dopamine transporter. The reuptake is then followed by enzymatic metabolism mainly via two pathways, monoamine oxidase or catechol-O-methyltransferase, which both have the same end-metabolite, namely homovanillic acid [4].

Two types of dopamine receptors have been described and classified based on their pharmacological and protein sequence similarities, the D_1-like (D_1 and D_5) and the D_2-like (D_2, D_3, D_4) receptors. Both receptor types are coupled to G-protein signaling systems, the D_1-like to stimulatory G-proteins whilst the D_2-like receptors are coupled to inhibitory G-proteins [4]. Both types of dopamine receptors are located post-synaptically, whereas D_2-like receptors also exist pre-synaptically where they can act as autoreceptors [26].

Ethanol and Dopamine

As previously mentioned, the mesolimbic dopamine system, but particularly the VTA-nAc pathway, has been implicated as a major mediator of the rewarding effects for drugs of abuse, including alcohol [15-16]. In addition, the VTA-nAc pathway also plays an important role in reinforcement and motivation for reward-oriented behaviors [13, 27]. Stimulation by both artificial and natural rewards releases the neurotransmitter dopamine in various brain regions, but most pronounced in the nAc [28-29]. The release of dopamine is associated with the subjective feelings of pleasure [30] but is also important in learning and memory processes [31-34]. The shell portion of the nAc appears to be more important than the core for drug reward [35]. Pharmacological and physical (lesion) manipulations of the nAc shell may disrupt the rewarding effects of several drugs of abuse. For example, rats learn to self-administer psychomotor

stimulants (such as cocaine or amphetamine) into the shell but not the core [36-37]. Lesion of dopaminergic terminals in the shell region attenuates the conditioned place preference induced by systemic administration of cocaine or amphetamine [38-40]. The core region of the nAc is considered to be a motor region with associations to the dorsal striatum. Results obtained from our research group have also postulated that the nAc is the primary target site for ethanol in its' rewarding and reinforcing effects (which will be further discussed below).

Extensive numbers of studies demonstrate that voluntary consumption, systemic injection and local accumbal perfusion of ethanol increases dopamine levels in the nAc in rats [41-45]. Continuous long-term use of alcohol subsequently *decreases* the function of the mesolimbic dopamine system, therefore an increase in alcohol intake is usually needed to obtain the same effect. There is also evidence that accumbal dopamine levels increase also in the anticipation of ethanol consumption [46]. During cessation from long-term alcohol use, the suppressed function of the mesolimbic dopamine system is proposed to induce craving [7, 47].

Neuroimaging studies in humans reveal that ethanol-induced dopamine enhancement is correlated with the subjective feeling of euphoria, stimulation etc [48]. It has also been demonstrated that the dopamine D_2 receptor, which is one of the receptors transmitting the reinforcing effects of ethanol [49], is decreased in alcoholics [50-52].

Despite the fact that ethanol-induced dopamine release within the nAc is critically involved in the initiation of alcohol reinforcement processes [34, 53], 6-hydroxy dopamine-induced lesions of the mesolimbic tract failed to alter voluntary self-administration in rats, suggesting a less important role of dopamine in maintaining alcohol consumption [54]. However, postsynaptic alterations in the dopamine receptor signaling appears to rather be involved in the maintenance of voluntary ethanol consumption since dopamine D_1 and D_2

receptor knock-out mice demonstrated reduced alcohol intake [55]. In addition, D_1, D_2 and D_3 receptor agonists and antagonists were capable of modulating alcohol consumption in outbred and alcohol-preferring rats [56-60]. Recent studies also indicate that activation of dopamine transmission may function as a learning signal since dopamine is enhanced, not only by reward, but also during expectation of reward [34].

Ligand-Gated Ion Channels

Ligand-gated ion channels (LGICs) are a group of transmembrane ion channels characterized by the opening and closing in response to binding of a ligand, such as a neurotransmitter. LGICs are usually very selective to one or more ions such as Na^+, K^+, Ca^{2+} or Cl^-. LGICs are composed of multiple protein subunits. Subunit heterogeneity creates an extensive diversity among the receptors. LGICs are located at synapses and convert the chemical signal of pre-synaptically-released neurotransmitter directly and very quickly into a post-synaptic electrical signal. Many LGICs are additionally modulated by allosteric ligands, channel blockers, ions or the membrane potential. Another common characteristic of LGICs is desensitization which is defined as a decline in the response to repeated or sustained application of an agonist [4].

Ethanol and Ligand-Gated Ion Channels

The LGIC receptors are sensitive to pharmacologically-relevant concentrations of ethanol (10-100 mM) and have received considerable attention as putative targets underlying ethanol's behavioral effects (review; [61]). Alcohol research has been focused on two superfamilies of LGICs: 1) The cysteine-loop LGICs including the nicotinic acetylcholine (nACh), 5-hydroxytryptamine 3 (5-HT_3), γ-amino-butyric acid A ($GABA_A$) and glycine (Gly) receptors [62], 2) the ionotropic glutamate superfamily of LGICs including *N*-methyl-*D*-aspartatic acid (NMDA), α-amino-3-hydroxyisoxazolepropionic acid (AMPA) and kainate receptors and lastly the ATP-channels (P2X) [4], which have received less attention in alcohol

addiction research [63]. Alcohol can directly and indirectly interfere with nACh, 5-HT_3, NMDA, $GABA_A$ and Gly receptors [64-68].

Ethanol and Neurotransmission

Alcohol has a complex pharmacology and acts by disrupting distinct receptors or effector proteins via direct or indirect interactions. In the following sections, a selection of these will be briefly described.

Ethanol and GABA

GABA is the main inhibitory neurotransmitter in the mammalian brain. The GABA receptors are divided into three types, $GABA_A$ receptors (LGICs), $GABA_B$ and $GABA_C$ receptors (G-protein coupled receptors) [4]. $GABA_A$ receptors are Cl^--sensitive channels which are blocked competitively by bicuculline and non-competitively by picrotoxin and Zn^{2+} [69].

It is documented that acute ethanol directly and indirectly (via GABA release) can potentiate the activity of the $GABA_A$ receptors [67, 70]. This interaction accounts for at least part of alcohol's anxiolytic, sedative and psychotropic effects [71-72]. Electrophysiological and biochemical studies have revealed that chronic ethanol exposure reduces $GABA_A$ receptor-mediated chloride channel function in rodents [73-77] and differentially alters $GABA_A$ receptor subunit expression in the brain [78-80]. In other words, in contrast to effects of acute alcohol administration, chronic alcohol exposure results in *decreased* $GABA_A$ receptor function. Most likely such down-regulation represents a mechanism for tolerance development to ethanol and contributes to ethanol withdrawal symptoms (e.g. hyperexcitability, seizures and tremors). In fact, gradual tapering of positive modulators of the $GABA_A$ receptor is a treatment of choice for the alcohol abstinence syndrome [81]. Both genetic and pharmacological manipulation of the $GABA_A$ receptor has been more successful in reducing alcohol intake than glutamatergic manipulations (see "Ethanol and Glutamate"). Knockout mice lacking different $GABA_A$ subunits (α1, α2, α5 and δ) displayed low alcohol

consumption [82-85].. In clinical studies, polymorphisms in the $GABA_A$ receptor subunit have been associated with different alcohol response in humans [86] but, notably the same authors had, in a previous study, not detected any consistent evidence of association between $GABA_A$ receptors and alcohol dependence [87]. The knock-out mouse models, together with clinical polymorphism studies, suggests that alcohol sensitivity is not only affected by subunit composition but also polymorphisms at the subunit level

Also the $GABA_B$ receptor has received attention in the field of alcohol research. $GABA_B$ receptors are functionally coupled to K^+ and Ca^{2+} channels via G-proteins and are specifically activated by baclofen and antagonized by phaclofen [4]. Activation of $GABA_B$ receptors has been demonstrated to suppress acquisition of ethanol-drinking behavior in rats [88-89]. Baclofen suppressed voluntary alcohol intake in ethanol-preferring sP rats, however repeated use of baclofen appeared to decrease the ethanol-reducing effects [89]. Furthermore, this phenomenon could be reduced by co-administration of a positive allosteric modulator of the $GABA_B$ receptor [90].

Ethanol and Glutamate

Glutamate is the major excitatory neurotransmitter in the brain and acts on two categories of receptors; the LGICs (the kainate, AMPA and the NMDA receptor) which mediates fast excitatory glutamate transmission, and the G-protein coupled receptors, i.e., the metabotropic glutamate receptors (mGluRs), which in contrast to the LGICs use second messengers to open or close the receptor [4]. The most studied receptor in relation to alcohol is the NMDAR, which is coupled to voltage-sensitive ion channels permeable to Ca^{2+}, Na^+ and K^+. NMDARs have a role in several effects of alcohol, including synaptic plasticity, learning and memory [91] as well as neurotoxicity [92]

Several electrophysiological and neurochemical studies indicate that acute administration of ethanol inhibits or antagonizes the action of agonists at the NMDAR [65, 93-97]. Ethanol appears to have a biphasic response on glutamate

release in both the hippocampus and the nAc where low (0.5 g/kg) and high (2 g/kg) doses of ethanol increased and decreased glutamate release. Acute ethanol administration alters NMDAR function and potentially results in severe brain dysfunction [98]. In addition, ethanol has been demonstrated to prevent long-term potentiation, a process involved in learning and memory [99], via action on the NMDAR channel complex [100].

Chronic alcohol intake increases glutamatergic activity (i.e. up-regulation of NMDAR number and function), probably due to adaptive responses to ethanol's initial antagonistic effect on the NMDARs [18, 101-104]. Discontinued exposure of ethanol after chronic treatment often leads to withdrawal-related symptoms like seizures and hyperexcitability. Over-activation of glutamate receptors, due to ethanol exposure, is suggested to contribute to the generation of these symptoms [105-106]. Investigations of ethanol withdrawal by means of microdialysis demonstrated that ethanol withdrawal is associated with increases in extracellular levels of glutamate in several brain regions, including the nAc [107].

Numerous animal studies have demonstrated that NMDAR antagonists attenutate the rewarding and reinforcing effects of virtually all drugs of abuse, including alcohol, and efficaciously attenuate various forms of relapse-like behavior (for review see [108]. But, in contrast to studies with $GABA_A$ receptors, knock-out of NMDA subunits (NR2) in mice had no effect on alcohol intake [109]. Although several studies have demonstrated the positive effects on ethanol (and other drugs of abuse) by using antagonists of the NMDARs, few of these agents are without serious side effects in humans (memory loss, disorientation, hallucinations etc). Therefore, during recent years, the focus of pharmacological manipulations of the glutamatergic system has turned to the mGluRs (briefly described in a subsequent section) [108].

While there are numerous studies investigating the ethanol/NMDAR interaction, few have studied AMPA or kainate receptors. Ethanol appears to inhibit the function of these receptors, but they appear to be less sensitive to inhibition by

ethanol than NMDARs, requiring higher concentrations (>50 mM) (reviewed in [108]). AMPARs do not seem to have a critical role in any aspects of alcohol dependence [110-111], whilst a study by Sanchis-Segura and colleagues suggests that AMPARs are involved in the neuroplastic changes underlying alcohol seeking behavior and relapse [112].

In contrast to the fast excitatory neurotransmission mediated by the NMDARs, the mGluRs mediate a slower, modulatory neurotransmission. They are located either in the peri-synaptic annulus or on pre-synaptic terminals. To date, two families of receptors (group I and II), with a total of eight receptors are identified. These receptors seem to have diverse neuroanatomical distribution as well as unique pharmacological and intracellular signaling properties [113-114]. Group I mGluRs, particularly mGluR5, are positively coupled to NMDAR function and are also structurally linked to these receptors [115]. Group I mGluRs are rarely found pre-synaptically, in contrast the Group II (mGluR2 and 3) and III (mGluR4, 6, 7, 8) are localized pre-synaptically, and in particular, mGluR2 and mGluR3 are thought to act as inhibitory autoreceptors that suppress excess glutamate release from the pre-synaptic terminal [116].

Of all the antagonists or, more correctly termed, negative allosteric modulators, of the different mGluRs, the most promising "anti-addictive" receptor modulation involves the mGluR5 (i.e. MPEP). A key publication by Chiamulera et al used mice with targeted deletion of the mGluR5 gene. These mice failed to acquire intravenous self-administration of cocaine and did not demonstrate a hyperlocomotion response to the drug [117]. Mice lacking the mGluR5 gene have also been demonstrated to exhibit reduced ethanol consumption [118].

Pharmacological manipulations did not change alcohol self-administration in studies involving mGluR1 antagonists [119], mGluR2/3 agonists or antagonists [120], and no difference was seen in mGluR4 knockout mice [121]. More successful data was obtained with modulation of the mGluR5. Antagonism at this receptor clearly reduced alcohol-reinforced responding [119, 122-123].

Finally, the glutamatergic system is tightly linked to the nitric oxide (NO) pathway. Stimulation of NMDARs leads to a Ca^{2+} influx, and the binding of Ca^{2+} to calmodulin activates neuronal NO synthase, which results in the production of NO. This close link between NMDA/NO is very interesting since many pharmacological studies and studies with NO synthase knockout mice demonstrate that NO signaling can also modulate alcohol reinforcement [124-126].

Ethanol and Acetylcholine

The neurotransmitter acetylcholine (ACh) produces its effects on the central and peripheral nervous system via two distinct types of receptors: the muscarinic and nicotinic ACh receptors (nAChRs). nAChRs are formed as pentamers with functional diversity depending on the subunit composition. Of the 17 subunits identified, only α_{2-10} and β_{2-4} can be found in neuronal nAChRs [127]. Among the numerous nAChR subtypes that exist, the homomeric α_7 and heteromeric $\alpha_4\beta_2$ nAChR subtypes are the two most prevalent in the brain [128-129]. There are numerous subtypes of the nAChR, located at post- and pre-synaptic sites in cholinergic neurons throughout the CNS, where they are involved in processes connected to cognitive functions such as, learning, memory and reward [128]. At pre-synaptic and pre-terminal sites nAChRs act as autoreceptors and heteroreceptors regulating the synaptic release of ACh and other neurotransmitters, like dopamine, glutamate and GABA. Because of these regulatory inputs, nAChRs are proposed as potential therapeutic targets for treatment of several neurodegenerative and psychiatric disorders including alcohol addiction [130].

It is well established that smoking is a risk factor for alcoholism and alcohol use is a risk factor to become a smoker. Nicotine acts as agonist at the nAChR but can also quickly cause receptor desensitization [131]. Interestingly, ethanol is able to interfere with nicotine-induced desensitization of the $\alpha_4\beta_2$ nAChRs [132]. Whether this contributes to the high prevalence of co-use of alcohol and nicotine is not clear. Substantial evidence (both *in vivo* and *in vitro*) has indicated a direct

interaction between ethanol and nAChR [133-139]. The outcome (potentiation, antagonism or no effect) of the interaction between alcohol and nAChR is dependent on subunit composition, agonist (nicotine, ACh), alcohol type and concentration. The ethanol/nAChR interaction on a neurochemical and functional level has been extensively studied in our research group. There are several findings indicating that the ethanol-induced dopamine enhancement involves the nAChR. [42, 140-143]. Inhibition of the nAChR was also able to prevent a cue-induced dopamine increase, resulting in the avoidance of ethanol seeking [144]. Further studies for a more specific localization of the nAChRs revealed that only nAChRs located in the anterior but not the posterior part of the VTA are able to mediate the effects of ethanol [142, 145] (reviewed in [146]).

On a behavioral level, voluntary ethanol consumption in rats increases extracellular ACh levels implicating an indirect action of ethanol on nAChR [147]. In addition, mecamylamine (unselective nAChR antagonist) treatment reduces ethanol intake in ethanol-preferring Wistar rats [41-42, 148]. Interestingly, clinical studies demonstrate that mecamylamine reduces the euphoric and stimulant subjective effects of acute alcohol and decreases the subjects' desire to consume more alcohol [149-151]. Unfortunately, the use of mecamylamine as an anti-alcohol treatment is limited since the compound has many peripheral side effects, e.g., dizziness, fainting, tremors and dysphoria [152]. In addition, chronic mecamylamine treatment has surprisingly been demonstrated to *increase* ethanol intake in the rat, probably due to intermittent peripheral blockade of the nAChRs [153]. Recently, the $\alpha_4\beta_2$ nAChR partial agonist varenicline has received much attention. Varenicline has been demonstrated to be an efficacious smoking cessation aid in the clinic [154], and has previously been shown to prevent ethanol-induced dopamine elevation and reduce ethanol consumption and self-administration in rodents [155-156], as well to decrease alcohol consumption in heavy drinking smokers [157]. Human genetic association studies have identified a genetic locus, encoding for the α_3 (CHRNA3), α_5 (CHRNA5), and β_4 (CHRNB4) nAChR subunits in nicotine and alcohol-dependent subjects [158-160]. Recently, it was found that administration of a

partial agonist of another nAChR subtype, $\alpha_3\beta_4$*, decreases ethanol-seeking and consumption in rats [161].

Needless to say, the interaction of ethanol and nAChRs is complicated but ethanol is considered as a nAChR co-agonist, potentiating the acetylcholine effect rather than activating the receptor by itself. Modulation of the nAChR, especially with partial agonists or subtype-specific antagonists, has great potential as a therapeutic target for the treatment of alcohol addiction.

Ethanol and Serotonin

There are several subtypes of serotonin (5-HT) receptors, each receptor has its own specific influence on behavior related to alcohol consumption revealed by knockout models [162]. 5-HT_{1A} may control alcohol consumption [163], 5-HT_{1B} influences the development of tolerance to alcohol and contributes to alcohol's intoxicating effects [164]. A third subtype, 5-HT_2, modulates the rewarding effects of alcohol and influences the development of alcohol withdrawal symptoms [165]. And finally, the 5-HT_3 has a part in regulating alcohol consumption [162]. The activity of this receptor is positively altered by ethanol [166].

5-HT plays a role in the regulation of many behaviors, for example mood, eating, arousal, pain and sleep. There are two "serotonin hypotheses" that strongly implicate an important role for 5-HT in alcohol addiction. First, the relationship of low levels of a 5-HT metabolite in the CSF of alcoholic patients compared with non-alcoholics [167-168]. Secondly, treatment with selective serotonin reuptake inhibitors in both humans and rodents may reduce alcohol consumption [167-169]. Selective serotonin reuptake inhibitors (fluoxetine and dexfenfluramine) and 5-HT_3 receptor antagonists (MDL 72222, ICS 205-930, ondansetron and tropisetron) either reduce the alcohol deprivation effect or decrease ethanol reinstatement [170-171].

Ethanol and Glycine

Glycine, along with GABA, is the primary fast inhibitory neurotransmitter in the central nervous system. In addition, glycine may exert positive modulatory action on glutamate via its co-agonist site on the NMDAR [172].

The GlyR, also known as the strychnine-sensitive GlyR, consists of α(1-4) homomeric- and αß(1) heteromeric subunit composition. According to the literature, a structural switch, from homomeric to heteromeric receptor composition occurs during the development [173]. Until recently, it was generally believed that GlyRs were almost exclusively found in the spinal cord and brainstem of adult rats [174]. However, recent findings suggest that GlyRs are expressed in upper brainstem [175] and in forebrain structures [176]. Electrophysiological, *in situ* hybridization and immunohistochemical studies have demonstrated the existence of GlyR or GlyR subunits in the nAc [44, 177-179].

Besides glycine, also other amino acids, namely taurine and ß-alanine have affinity for the GlyR [180]. Taurine has been demonstrated to influence ethanol-induced dopamine effects which will be described in a later section. An important feature in glycinergic transmission is the reuptake process. This constitutes an effective mechanism by which the post-synaptic action can be terminated [181]. To date, there are two identified glycine transporters, the GLYT-1 and GLYT-2. The GLYT-1 is found in glial cell plasma membranes and is responsible for the tonic homeostatic glycine reuptake, whilst the GLYT-2 is located in the pre-synaptic neuronal terminal and maintains the phasic synaptic uptake [182]. Inhibition of the transporters has been suggested to be a potential treatment target in diseases such as schizophrenia, pain and epilepsy [183] and recently also in alcohol addiction [184-186].

The ethanol-induced potentiation or facilitation of the function of the GlyR has repeatedly been demonstrated using various methodologies. For example, in isolated cell preparations from the spinal cord, GlyR-mediated currents are

consistently facilitated by acute ethanol administration [187]. In Xenopus oocytes, low concentrations of glycine in combination with ethanol enhance GlyR function [188]. Similar potentiation has been detected in cultured hippocampal and spinal cord neurons [187, 189-190], as well as in synaptoneurosomes prepared from limbic brain regions [191].

Our research group has extensively studied the interaction between ethanol and GlyRs. The results implicate the ethanol-GlyR interaction in association with the dopaminergic system (this will be described in "the nAc-VTA-nAc circuitry theory" and is also reviewed in [146]). Findings from our research group have demonstrated that administration of GlyR agonists or antagonist in the nAc, enhance or reduce basal dopamine levels in the same area [44, 184]. However, after glycine administration, inconsistent dopamine responses were detected in the animals, which were therefore classified as glycine responders or non-responders [44]. Besides the differential dopamine response to glycine, these two subgroups also had distinct ethanol-intake responses (discussed later). The diversity is still unexplained in terms of neurobiology, but probably involves e.g. individual difference in desensitization of GlyRs, receptor subtypes or set-ups etc. Regardless of the responder or non-responder classification, local perfusion of glycine or strychnine prevents ethanol-induced dopamine elevation, probably due to receptor desensitization and receptor blockade, respectively, thus suggesting an interaction between ethanol and the GlyR [146, 192]. In addition to preventing ethanol-induced dopamine effects, glycine has been proposed to be involved in the anticipation of ethanol reward, since increased extracellular glycine levels have been detected in the nAc during this phase [193].

When the ethanol-GlyR interaction in the nAc (in terms of dopamine modulation) was established, the functional relevance in terms of ethanol consumption was investigated. Briefly, GlyR activation and inactivation decreased and increased ethanol intake in ethanol high-preferring rats respectively [43]. The findings, which will be further discussed in "the nAc-VTA-nAc theory", indicated that GlyR modulation has promising anti-alcohol properties.

Ethanol and Taurine

Taurine is a sulfonated ß-amino acid that shares structural similarities with GABA and glutamate. It is highly abundant in excitable tissue, including the heart and brain [194]. Besides agonistic effects on the GlyRs [195-198], taurine has been demonstrated to activate the $GABA_A$ receptor [196-199], antagonize the NMDAR [200-201] and bind to $GABA_B$ receptors [202-203], although no function of this binding has been explained. A few studies also suggest that a taurine receptor (still undefined) exists [204-205].

Taurine appears to have multiple functions in the brain. Some of these include neuroprotectant, antioxidant, osmoregulatory and Ca^{2+} modulatory effects [194, 206-207] and it may act as a neurotransmitter [208]. Taurine potentiates GlyR function but, unlike the full agonist glycine, taurine may act as partial [209] or full [210] agonist at the GlyR, depending on the brain region. In the nAc of young rats, taurine is a full agonist [211]. In contrast to the negligible quantity of glycine-immunoreactive cells [175], taurine-containing cells are abundant in the nAc [212] and striatal neurons express high levels of taurine transporter [213-214] which accumulate taurine in millimolar concentrations [215-216]. Therefore, it is likely that taurine, rather than glycine, is the potential regulator for tonic activation of GlyR [217] and plays an important role in the development and functional modulation of nAc neurons [211].

In vivo microdialysis studies have revealed that ethanol elevates extracellular levels of taurine in nAc [218-219], amygdala [220-221], hippocampus and PFC [222]. There is also evidence for genetic influences on ethanol-stimulated taurine release in the CNS. Two different genetically-bred rat strains, (high- and low-alcohol sensitivity (HAS/LAS) and the Sardinian ethanol-preferring and non-preferring (sP/sNP), show either higher or delayed elevation in accumbal taurine levels after an acute injection of ethanol in the alcohol-preferring rats compared with their non-alcohol preferring counterparts [223-224]. Another genetically-modified animal model, the epsilon isoform of protein kinase C knockout mice,

which are sensitive to ethanol, display spontaneously elevated accumbal taurine levels and an absence of ethanol-induced taurine increase [225].

Finally, taurine and several related molecules including homotaurine and the homotaurine derivate acamprosate have both been demonstrated to reduce ethanol self-administration and relapse to drinking in both animals and humans [226]. All these findings indicate that the taurine system, possibly via the glycine receptor system, is an important modulator of the effects of ethanol.

How Does Alcohol Produce its Positive Reinforcing Effect?

Alcohol and its interaction with the mesolimbic dopamine system, is without a doubt, an important feature in several aspects of alcohol addiction (i.e. reinforcement, development of addiction, maintenance etc). A massive number of publications have generated numerous hypotheses of how ethanol may activate this system. Here a few of them will be mentioned.

Historic Perspective

Due to its lipophilic and hydrophilic properties, alcohol easily passes over membranes, changes the environment of the protein-molecules embedded therein and interacts with several intra and extracellular sites. The first theory of the mechanism of action of alcohol was the "lipid theory". This theory was based on the observation that alcohol disordered membrane proteins [227]. However, the membranes were only affected at doses well above the normal pharmacological range and the same effect could be obtained by increasing the temperature by only half a degree Celsius [228]. After a publication by Lovinger et al, the "lipid theory" shifted towards the "protein theory"; direct interference with ion channels and receptors [65]. The "protein theory" is not only a more convincing theory but also offers a higher possibility to develop a successful pharmacotherapy. Treatments aimed at lipids are likely to have non-specific actions throughout the body, whereas many of the proteins which could be targeted are brain-specific, some even moderately site-specific within the brain. Several agents targeting

LGICs or other specific neurotransmitters like the endogenous opioid system or ghrelin (see below), produce one common action; modulation of the mesolimbic dopamine system. In fact, many clinical and experimental trials are already using or investigating these kinds of agents in the search of a new, effective treatment for alcohol-related disorders.

The Direct Interaction Theory

Since Gessa and co-workers discovered that ethanol *in vivo* could increase the firing rate of dopaminergic neurons in the VTA [229], other laboratories adopted the theory that ethanol had a local effect in the VTA. Findings indicating that ethanol is able to directly activate dopaminergic neurons in the VTA [230-231] and that rats self-administer ethanol in the VTA [232-233] support this theory. Notably, several of the electrophysiological studies that followed were performed *in vitro,* thus disregarding neuronal networks participating in the general outcome. In addition, the *in vivo* electrophysiological study merely demonstrated dopamine neuronal activation [229], an effect that theoretically could have been a secondary response. In a publication by Kohl et al, systemic administration of ethanol increased dopamine levels both in the nAc and in the VTA. The dopamine increase in the nAc was sustained for at least two hours after injection, whereas only a transient increase was observed in the VTA. The authors concluded that ethanol activates dopaminergic neurons in the VTA, resulting in increased accumbal dopamine release which, in turn, activates a negative feedback system regulating dopamine transmission in the VTA [234].

The 5-HT Theory

Among the LGICs, the interaction between the 5-HT system and the dopamine system is one of the most studied. 5-HT can alter dopaminergic signal transmission in several ways. For example, 5-HT *per se* can stimulate the activity of dopaminergic neurons in the VTA [235] and activation of the 5-HT_3 receptors in the nAc enhances dopamine release [236-237]. Findings from animal studies also indicate that 5-HT_3 receptor antagonists interfere with the 5-HT

induced dopamine release, implicating a role for this receptor in ethanol's reinforcing and rewarding effects [162, 236-238].

The Acetaldehyde Theory

Acetaldehyde is the first product of ethanol metabolism and is traditionally considered the mediator of alcohol's aversive and toxic effects [239]. Recently, the influence of acetaldehyde on different neurotransmitter systems has been thought to contribute to the behavioral effects of ethanol. Studies have indicated that acetaldehyde *per se* induces a range of behavioral (including reinforcing) effects similar to ethanol [240]. Rats will self-administer acetaldehyde intravenously into the cerebral ventricles and into the posterior VTA [241-243]. Acetaldehyde was demonstrated to increase firing of dopaminergic neurons and, when locally applied in the VTA, was also able to produce an elevation of nAc dopamine. In addition, inhibition of peripheral ethanol metabolism was demonstrated to prevent ethanol-induced dopamine elevation [244]. However, the question of whether brain acetaldehyde levels produced by physiologically relevant concentrations of ethanol are sufficient to produce any pharmacological or behavioral effects relevant to reward and addiction, remains controversial.

The Endogenous Opioid System Theory

The different components of the endogenous opioid system (EOS) are highly expressed in the brain reward system [245], and participate in the modulation of the reward circuits [246]. To this date, three opioid receptors have been identified; μ, δ, and κ, each receptor has an endogenous ligand. These ligands are β-endorphin, met- and leu-enkephalin and dynorphins, respectively [246]. The EOS has been demonstrated to play an important role in alcohol addiction. In fact, one of the three available anti-alcohol pharmaceutics naltrexone is a μ-receptor antagonist. Modulation of the EOS (μ- and δ-receptor antagonists and β-endorphin knockout mouse models) was found to alter the ethanol-induced dopamine elevation [247-249] and reduce ethanol intake [246]. Acute ethanol exposure increases brain enkephalin [250] and β-endorphin [251] content, and a correlation has been observed between increased β-endorphin level and the risk

of alcoholism in humans [252]. Chronic ethanol exposure leads to an imbalance in the EOS, which is suggested to participate in the development of alcohol addiction [253]. The interaction between EOS and the dopamine system is believed to be mediated via release of enkephalins in the VTA. This augmentation could then activate μ-opioid receptors located on presynaptic GABAergic interneurons in the nAc. By inhibiting GABAergic transmission, a facilitation of dopamine release in the same brain region will appear [246].

The Ghrelin Theory

Ghrelin is a stomach-derived hormone which interacts with CNS circuits where it regulates the energy balance and body weight. Recently, it has been demonstrated that the ghrelin signaling system may be required for alcohol reward [254]. For instance, central ghrelin administration in the VTA or LDTg, induced an increase in dopamine overflow in the nAc [255]. In addition, peripheral injection with mecamylamine inhibited the ghrelin-induced dopamine enhancement, suggesting that ghrelin activates the dopamine system via the acetylcholine-dopamine link (discussed in a previous section) thus interfering with the ability of ethanol to produce its dopamine-elevating effect [256].

The nAc-VTA-nAc Neuronal Circuitry Theory

The working hypothesis of our research group with respect to the dopamine-elevating and reinforcing properties of ethanol has evolved over the last 15 years. Briefly, ethanol acts primarily in the nAc by activating GlyRs. The GlyRs directly or indirectly inhibit GABAergic neurons projecting to the VTA, decreasing the GABAergic tone, allowing increased ACh to activate nAChRs located on dopaminergic cell bodies, resulting in elevated dopamine levels in the nAc. Figure 2 is a representative and simplified illustration of this tentative neuronal circuitry and its components. In a little more detail, the hypothesis was formed based on a number of *in vivo* microdialysis studies and voluntary ethanol consumption studies targeting ventral tegmental nAChRs and nAc GlyRs which resulted in the following findings:

1. *Ethanol-induced accumbal dopamine elevation involves indirect activation of ventral tegmental nAChRs.*

This was based on studies demonstrating that local perfusion of the nAChR antagonist mecamylamine in the VTA, but not in the nAc prevented systemic administered ethanol-induced elevation of dopamine [145]. Local administration of ethanol in the nAc was able to induce an elevation of dopamine in the same brain region, whilst ethanol perfusion in the VTA failed to cause a dopamine elevation [142-143, 145, 257]. This dopamine elevation, produced by ethanol perfused in the nAc, could be blocked by mecamylamine administration in the VTA [143]. Taken together with findings that ethanol consumption in the rat concomitantly increases acetylcholine levels in the VTA and dopamine in the nAc [147], these results strongly indicate a cascade where ethanol acts primarily in the nAc, that secondarily enhances acetylcholine release in the VTA which in turn stimulates dopamine-activating nAChRs [258].

2. *Glycine receptors are involved in ethanol-induced dopamine elevation.*

The simplest mechanism by which ethanol in the nAc could increase dopamine neuronal activity would be by interfering with backward-projecting inhibitory GABAergic neurons [259] which project to the terminals of cholinergic afferents in the VTA (see figure 2). The hypothesis was that inhibition of the GABAergic neurons in the nAc, via activation of either of the inhibitory ion-channels $GABA_A$ or GlyRs would release the inhibition of cholinergic afferents, which would result in acetylcholine release in the VTA and subsequently dopamine release in the nAc. It was demonstrated that GlyRs rather than $GABA_A$ receptors underlie the ethanol-induced effect, since local strychnine administration (nAc) antagonized the ethanol-induced dopamine output [44, 192]. In addition, blockade of $GABA_A$ receptors failed to inhibit the ethanol-induced dopamine elevation. On the contrary, local picrotoxin prolonged the dopamine elevation [192, 257].

3. Modulation of either ventral tegmental nAChRs or accumbal GlyRs modulates ethanol intake.

Administration of either mecamylamine in the VTA or glycine or strychnine in the nAc alters voluntary ethanol intake in the rat [42-43]. The GLYT-1 inhibitor ORG-25935, elevating extracellular levels of glycine by 70%, also demonstrates a robust ethanol intake-reducing effect [185].

In conclusion: Several studies from our research group have demonstrated that ethanol-induced stimulation of the mesolimbic dopamine system involves indirect activation of ventral tegmental nAChR as well as activation of the nAc GlyR (reviewed in [146]). Modulation of these receptors, by enhancing GlyR and inhibiting nAChR activity, also reduce ethanol intake, strongly implicating involvement of these receptors in a nAc-VTA-nAc dopamine-controlling neuronal circuit.

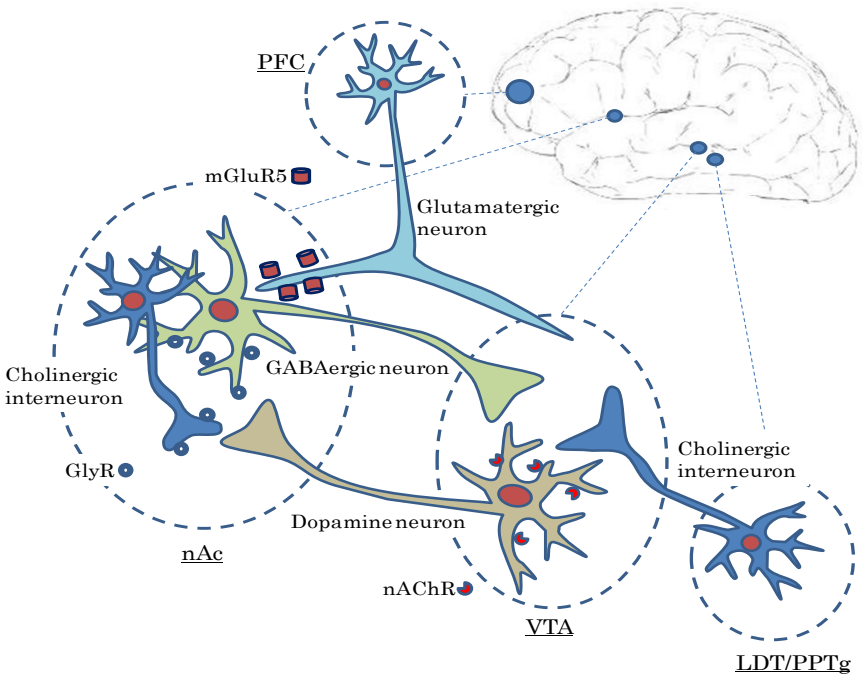


Figure 2. Schematic illustration of the components involved in the hypothetical nAc-VTA-nAc neuronal circuitry. Inhibitory GABAergic neurons are modulated by glycine receptors (GlyRs) in the nucleus accumbens (nAc), this disinhibition results in increased acetylcholine output from the laterodorsal /pedunculopontince tegmental nucleus (LDTg/PPTg) and subsequently elevated dopamine release in the nAc via activation of the nicotinic acetylcholine receptors (nAChRs) in the ventral tegmental area (VTA). In Paper IV, a tentative interaction between the metabotropic glutamate receptor 5 (mGluR5) and this circuitry has been proposed.

Alcohol Addiction

Alcohol addiction is a complex disorder. To understand it, one must comprehend how the effects of alcohol during an initial exposure lead progressively to stable molecular and cellular changes in the brain after repeated exposure. The transfer from initial alcohol consumption to the development of addiction is a downward spiral involving various neurotransmitter and neuropeptide systems during the different steps in the progress [260]. The first step (alcohol consumption) is dependent on variables such as the level of response to an acute first alcohol challenge (subjective response) and how fast the individual becomes intoxicated (objective response), where both responses are genetically. And in addition to environmental factors, these variables are strongly correlated to an elevated risk of developing alcohol addiction [260]. Once an alcohol-drinking behavior is established, further alcohol intake alters the balance of inhibitory (GABA) and excitatory (glutamate) neurotransmission. Besides alterations of the activity of GABAergic and glutamatergic transmission, chronic alcohol exposure affects several other neurotransmitter systems as well, such as downregulating the mesolimbic dopamine system, and dysregulating the endogenous opioid systems. These changes in neurotransmission are thought to promote and maintain further alcohol consumption.

The third phase in addiction is craving/alcohol-seeking behavior (reviewed in [261]). According to an expert committee gathered by the United Nations International Drug Control Programme and the World Health Organization, the definition of craving is: "*the desire to experience the effect(s) of a previously experienced psychoactive substance*". Craving can occur even after long-term abstinence and is typically provoked by i.e. stress or conditioned alcohol-associated cues (reviewed in [262]). Studies have demonstrated that stressors can facilitate alcohol consumption by increasing the activity of several neurobiological systems, such as the hypothalamic-pituary-adrenal axis and extra-hypothalamic corticotrophin-releasing factor (CRF) signaling [263]. In addition, cue- and stress-induced alcohol reinstatement can be blocked by administration of CRF_1 receptor antagonists [264-265].

Furthermore, alcohol addiction has also been found to have profound negative effects on the cerebrum and the cerebellum, with significant physical alterations on several brain regions, including the PFC. Disruption of this region is suggested to be the principal neural mechanism underlying alcohol addiction's prominent and enduring deficits, i.e. ataxia and executive dysfunctions (reviewed in [266]).

Treatment for Alcohol Addiction

The complex interactions between ethanol and various neurotransmitters and neuropeptides in addition to gene/environment influences on the development of alcohol addiction, obstructs to the discovery of an optimal pharmacotherapy to aid alcoholic patients. Nevertheless, there are currently three pharmacological treatments approved in Sweden for the treatment of alcohol dependence. Disulfiram (Antabus®), naltrexone (Naltrexone Vitaflo®) and acamprosate (Campral®). Disulfiram was the first available pharmaceutic and this increases the metabolite acetaldehyde by inhibiting the degradation of ethanol. Excessive quantities of acetaldehyde leads to unpleasant symptoms, e.g. flushing, nausea and headache, which will deter from further alcohol consumption, but the patients will still endure craving. Disulfiram treatment is most effective when given under supervision of a physician [81]. Naltrexone and acamprosate are the "new generation" anti-craving substances and have some additive effect when given in combination [267-268]. Naltrexone is a non-selective opioid antagonist that reduces ethanol intake in rat models [269-270]. In humans, naltrexone has been demonstrated to attenuate cue-induced alcohol craving [271] and this might be the explanation for naltrexone's efficacy.

Acamprosate

The third and last anti-craving substance is acamprosate. Acamprosate is a synthetic molecule with a chemical structure similar to that of the endogenous amino acid N-acetyl homotaurine [272], a small, highly flexible molecule with analogy to many amino acids, most notably glutamate, GABA, aspartate, glycine and taurine [273-274]. The analogy with so many amino acids gives acamprosate

a potentially complex pharmacodynamic profile. At the time when the first clinical report appeared, the mechanism of action was proposed to be via a GABA agonist action [275]. The prevailing view of its mechanism of action today is mainly as a glutamatergic modulator [118, 273, 276-283].

Clinical findings

The efficacy of acamprosate in modulating alcohol consumption in humans has been evaluated in more than 20 double-blind randomized controlled trials and more than 6000 patients, performed all around the world. In a recently published Cochrane review, by Rösner et al, the conclusion was that acamprosate is an effective treatment compared to placebo [284]. The number needed to treat (NNT), which refers to the number of patients who need to be treated in order to prevent one additional bad outcome (i.e. relapse), was estimated to be 7.8 by Mann [285] and 9.09 by Rösner [284]. This NNT is comparable to naltrexone treatment (NNT=12 [286]). In a meta-analysis by Mann et al (2004), which included 17 studies and more than 4000 patients, the result was that 36.1% of the acamprosate-treated patients, compared to 23.4% of placebo treated patients, remained abstinent [285].

Mechanisms of action; GABA, NMDA, mGluR5

Much has been debated about the mechanism of action of acamprosate. Since acamprosate shares structural similarities with GABA, the first suggested mechanism of action was solely by GABAergic transmission [287]. But a key paper by Dahchour and De Witte suggested that acamprosate normalizes the hyperglutamatergic state (during alcohol withdrawal) in the nAc that is caused by excessive alcohol consumption [278]. Ever since, the focus has been shifted towards glutamatergic mechanisms. Early electrophysiological studies demonstrated that acamprosate inhibits glutamate-mediated post-synaptic potentials on the NMDAR [288]. Exactly how acamprosate inhibits the NMDAR is still unknown, although it has been suggested that acamprosate binds to an allosteric site of the NMDAR [281]. A study by al Qatari et al, proposed that acamprosate acts as an antagonist when the receptor activity is high, e.g. when

the brain is undergoing alcohol withdrawal, but at low receptor activity, acamprosate has agonistic properties [276].

In a study by Harris et al, acamprosate was found to have binding and functional characteristics similar to group I mGluR antagonists. The functional similarities between acamprosate and the mGluR5 antagonist SIB-1893 supported an idea of interaction between acamprosate and the mGluR5, where the authors suggested that the alteration of the glutamatergic neurotransmission was a result from direct acamprosate-mGluR5 interactions [289].

Activation of the brain reward system by acamprosate

A few studies have attempted to investigate if acamprosate may modulate the rewarding and reinforcing, i.e. dopamine-elevating, effect of alcohol. Olive et al found that pre-treatment with acamprosate abolished the ethanol-induced elevation of accumbal dopamine in rats [290]. Cano-Cebrian et al came to the same conclusion and suggested that this effect was mediated by glutamate neurons co-localized with dopamine neurons in the nAc [291]. An indirect measure of an interaction between acamprosate and the mesolimbic dopamine system was demonstrated in a study by Cowen et al, in which they monitored the dopamine system (binding studies measuring densities of the dopamine transporter and dopamine D_2–like receptor) and the ethanol intake behavior in alcohol-preferring rats. An acute injection of acamprosate increased the dopamine transporter but decreased the D_2-like receptor density (i.e. diminished dopamine-mediated responses). With repeated injections of acamprosate, the markers of the dopamine system returned to steady state levels. Interestingly, the alteration of the dopamine system mirrored the ethanol intake behavior, after the acute injection ethanol intake was reduced but after repeated injections, tolerance against acamprosate's ethanol intake-reducing effect developed [292].

Other mechanisms of action suggested for acamprosate

Both preclinical and clinical studies have reported that acamprosate counterbalances alterations in ß-endorphin plasma concentrations [293-294]

which are associated with alcohol addiction. There is also evidence that acamprosate has a mild effect on plasma concentration of the appetite-regulating peptide leptin, that is associated with craving and relapse during abstinence [295]. It remains to be elucidated whether these changes in plasma hormones underlie the beneficial effect of acamprosate in relapse or if it might be an indirect effect due to alterations in other CNS neurotransmitter systems.

One interesting feature of acamprosate is its' taurine-elevating effect. Acamprosate, when administered acutely, induced a dramatic increase in taurine microdialysate content in the nAc [222, 296]. The same research group revealed a significantly higher basal level of taurine in chronically ethanol-exposed rats treated with acamprosate [296].

AIMS OF THE THESIS

The general aim of this thesis was to further investigate the role of the nAc-VTA-nAc neuronal circuitry for regulating ethanol-induced dopamine elevation and for ethanol consumption. This was primarily evaluated by usage of the approved anti-relapse agent acamprosate.

Specific aims

- To evaluate the effect of acamprosate on nAc dopamine transmission and the involvement of the nAc-VTA-nAc neuronal circuitry on this effect (Paper I).

- To investigate the role of nAc GlyRs in the ethanol intake-reducing effect of acamprosate (Paper II).

- To study the interactions between ethanol and acamprosate with regards to mesolimbic dopamine transmission, and its possible association with the development of tolerance to the ethanol intake-reducing effects of acamprosate (Paper III).

- To investigate a possible link between GlyRs and mGluR5 in their ability to modulate mesolimbic dopamine transmission (Paper IV).

- To explore whether the ethanol-induced elevation of nAc dopamine depends on concomitant release of taurine in the same brain area (Paper V).

MATERIALS & METHODS

Experimental Design

Paper I

Drug-naïve male Wistar rats were implanted with one or two microdialysis probes locally unilaterally in the nAc and VTA or solely in the nAc. The nAc dopamine response to acute local (0.5 or 5 mM in the nAc) and systemic (200 or 400 mg/kg) administration of acamprosate was measured by means of *in vivo* microdialysis. The involvement of accumbal GlyRs and ventral tegmental nAChRs were investigated by pre- and co-perfusion with the respective receptor antagonists, strychnine and mecamylamine.

Paper II

Male Wistar rats on a voluntary ethanol paradigm (water or 6% ethanol) that consumed more than 30% of their daily total fluid intake from the ethanol bottle were placed on a limited access schedule where they had access to the bottles for 2.5 h/day. The rats were surgically implanted with microinjection guides bilaterally in the nAc and treated with either 200 mg/kg acamprosate or vehicle before access to the bottles for two days. On the third day, microinjection needles were inserted into the guides and injection with either 5 μg strychnine or Ringer preceded the systemic injection and access to the bottles. Ethanol and water intake was monitored throughout the entire experiment.

Paper III

Three separate microdialysis experiments were conducted in this paper; 1) drug-naïve rats received acute, local perfusion (nAc) with 0.5 mM acamprosate and/or 300 mM ethanol, 2) rats treated with 200 mg/kg acamprosate or vehicle for three days were acutely administered with ethanol (oral injection of 1 g/kg ethanol) 3) rats in a voluntary ethanol paradigm (water or 6% ethanol) were treated with

200 mg/kg acamprosate or vehicle for 12 days and were perfused with ethanol (300 mM) on the day of microdialysis. Extracellular levels of dopamine were monitored in each experiment.

Paper IV

Naïve rats were implanted with a microdialysis probe in the nAc. The nAc dopamine response to MPEP (100 or 500 μM) alone or after pretreatment with strychnine (10 or 20 μM) was analyzed by means of *in vivo* microdialysis. The extracellular levels of glycine, taurine and ß-alanine were also analyzed in a subset of animals.

Paper V

In vivo microdialysis was used in freely-moving Wistar rats to measure the effects of ethanol diluted in an isotonic (0.9% NaCl) or hypertonic saline solution (3.6% NaCl), with or without the addition of 50 μM taurine in the perfusate, on extracellular levels of taurine and dopamine in the nAc. In a separate microdialysis study, rats received ß-alanine in the drinking water for five weeks, in order to deplete the endogenous stores of taurine, and were perfused with ethanol (300 mM). Extracellular levels of taurine, glycine, ß-alanine and dopamine were monitored.

Animals

All studies are performed using male Wistar rats weighing approximately 250-350 g (supplied by B & K Universal AB, Sollentuna, Sweden or Taconic, Denmark). The animals were housed in groups of four in a temperature (22ºC) and humidity (65%) controlled room. The animals were allowed to adapt for one week to the novel environment before any experiment was performed. In all papers, except for Paper II and partly in Paper III, the animals were kept under regular light-dark conditions (lights on at 7:00 am and off at 7:00 pm). In the ethanol consumption studies in Papers II and III, ethanol high- and medium-preferring Wistar rats were kept under reversed light-dark conditions (light off at 8:00 am and on at 8:00 pm). All rats had free access to standard rodent chow (Lantmännen, Sweden) and, if not stated elsewhere, free access to tap water.

In Paper I, III, IV and V, the rats were housed in groups of four but were kept in individual cages after surgery. In Paper II and III, the animals were housed in groups during the ethanol adaptation period (gradual increase of ethanol concentration over two weeks), and then separated during the screening period (six weeks).

Ethics

All studies were performed according to the Declaration of Helsinki and were approved by the Ethics committee for animal experiments (Gothenburg, Sweden) with the diary number 5/04 (Paper I and II) or 337/06 (Paper III, IV, V).

Experimental Techniques

In vivo microdialysis

In vivo microdialysis is one of the major tools for sampling endogenous and exogenous substances in the extracellular space. A microdialysis probe has a semi-permeable membrane which is inserted into tissue to act in a similar manner as a blood capillary. The membrane area is the active space area whilst

the shaft only has a supporting role. A dialysis buffer that is similar to the cerebral spinal fluid in ionic composition (Ringer) is perfused through the membrane via tubings attached to a syringe pump. This method allows sampling of the interstitial environment by process of diffusion along a logarithmic concentration gradient towards (illustrated as black arrows and dots in the figure 3) and away (white arrows and dots) from the probe [297].

The microdialysis probe

The majority of the microdialysis probes used in the experiments were a modified version of the I-shaped probe, custom-made in our laboratory. The inlet and outlet of the probes were made of 20 gauge polyethylene tubing, with outer/inner diameter of 1.09/0.38 mm (VWR, Sweden). The fused silica (Skandinaviska Genetec, Sweden) extended 9 mm for nAc probes and 10 mm for VTA, from the tip of the probe. During manufacturing and implantation of the probe a glass rod was used as a holder. The dialysis membrane was prepared from a copolymer of polyacrylonitrile and sodium methallyl sulfonate (Hospal-Gambro, Sweden) with an outer/inner diameter of 310/220 μm and a protein size of 20 kDa was the cutoff for what could pass through the pores. The length of the exposed tip (the active space) was 2.0 mm, and the remaining area was covered with silicone glue (CAF 3; Rhodorsil Silicones, Nils Eksandh AB, Sweden). Before implantation, the probes were washed by perfusion (2 μl/min) with 70% ethanol solution followed

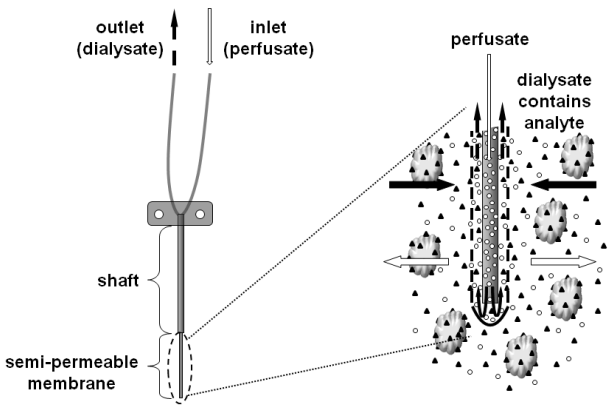


Figure 3. Schematic illustration of the principle of microdialysis. The extracellular fluid exchange is only possible at the tip of the probe, via the semi-permeable membrane. (Adapted with permission from www.wikimedia.org).

by approximately 120 µl of Ringer perfusion. The inlet and outlet tubes were then sealed by heating and the probes were stored in +4ºC until the day of implantation (maximal four days of storage).

In Paper III, pre-manufactured probes (6 kDa protein size cutoff) with guide cannulae, purchased from Agntho's (Lidingö, Sweden) were used. The guide cannulae were implanted at least seven days before the experimental day to minimize any possible disruption of the ethanol intake due to the surgical procedure. The microdialysis probe was then inserted on the day of the microdialysis experiment.

Surgery

Rats were anaesthetized by isoflurane (3.5-4.0% in air; Baxter, Apoteket AB, Sweden), mounted into a stereotaxic instrument (David Kopf Instruments, Agntho's AB, Lidingö, Sweden) and put on a heating pad to prevent hypothermia during the surgery. Holes were drilled superficially for placement of two anchoring screws and one or two I-shaped dialysis probes. The dialysis probes were lowered unilaterally into the nAc (A/P: +1.85, M/L: -1.4 relative to bregma and D/V: -7.8 relative to dura) and in the anterior VTA (A/P: -5.2, M/L: -0.7 relative to bregma, D/V: -8.4 relative to dura; [1]). Probes as well as anchoring screws were fixed to the scull with Harvard cement (DAB Dental AB, Sweden).

Microdialysis procedure

After surgery, the rats were allowed to recover for two days before the dialysis experiments were initiated. All *in vivo* brain microdialysis was performed in awake, freely-moving rats in order to measure extracellular concentrations of dopamine (Paper I, III, IV, V) and also glycine, taurine and β-alanine (Paper IV and V). On the experimental day, the sealed inlet and outlet of the probes were cut open and connected to a micro-perfusion pump (U-864 Syringe Pump, AgnTho's, Sweden) via a swivel which allowed the rat to move around. The probe was perfused with Ringer solution at a rate of 2 µl/min and dialysate samples (40

μl) were collected every 20 minutes. The rats were perfused with Ringer solution for at least 1 hour before baseline sampling began, in order to obtain a balanced fluid exchange. Drug administration was initiated once a stable (±10%) baseline had been obtained. The dopamine content of the collected samples was analyzed "on-line" by using a high-pressure liquid chromatography (HPLC) system with electrochemical detection. Briefly, the HPLC system consisted of a pump (Dionex P580, Kovalent AB, Västra Frölunda, Sweden), a stainless steel column 2 x 150 mm) packed with Nucleosil, 5 μM SA 100A (Phenomenex Skandinaviska Genetec, Västra Frölunda, Sweden) and an electrochemical detector (Decade, Kovalent AB) operated at 0.40 V versus the cell (Hy-REF). The time of analysis was 6 minutes, and an external standard containing 3.25 fmol/μl of dopamine was used to identify the dopamine peak.

To analyze the amino acids (glycine, taurine and ß-alanine), 5 μl of the dialysate sample was diluted in Ringer and sodium azide and were stored up to two weeks in -80°C. When analyzing the samples, a gradient HPLC system with a fluorescence detector was used. Here, briefly, a Waters 7179 percolumn derivatisation (Waters, Sollentuna, Sweden) allowed the amino acids to react for one minute in order to be able to separate them. Thereafter, they were separated in two Onyx columns (4.6 x 50 mm and 4.6 x 100 mm; Skandinaviska Genetec) and detected by a fluorescence detector (Perkin Elmer LC240, Perkin Elmer Sverige AB, Upplands Väsby, Sweden). The time of analysis was 13 minutes and external standards in three concentrations (0.1-1.0 μM) were used to identify the different amino acid peaks.

Verification of probe placement

Animals were sacrificed directly after the experiment and brains were removed and placed in Accustain (Sigma-Aldrich, Sweden) for fixation. Probe placement was verified using a vibroslice device (Campden instruments, Leicester, UK), and visual determination of the probe locations was performed. Only animals with correct probe placement and no visual defects (e.g. bleeding) were included in the analysis (see figure 4).

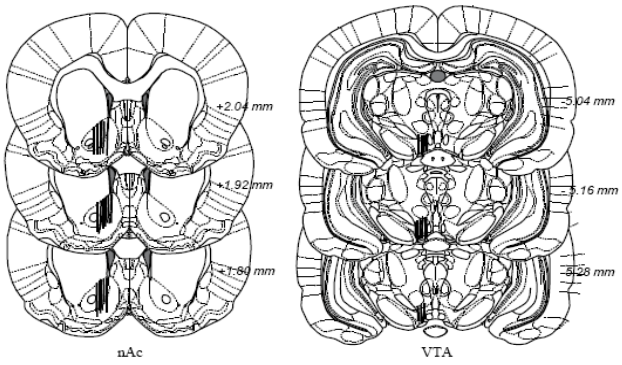


Figure 4. Coronal sections of the rat brain indicating the placements of a selection of the microdialysis probes in the nAc and the VTA. Adapted from Paxinos and Watson [1]

Voluntary Ethanol Consumption

Screening period

Outbred male Wistar rats arrived at the animal facility and were housed in groups of four. After one week of adaptation to the new environment, they received the addition of a bottle of ethanol solution in the home cage. The ethanol concentration was gradually increased (2-4-6%) over a two-week period. The choice of 6% ethanol was based on a study by Fahlke et al which indicated that this concentration results in an optimal ethanol intake in Wistar rats [298]. The animals were then subsequently housed individually in plastic cages with continuous access to ethanol and water. Over a six-week period, the bodyweight, and intake of ethanol and water was measured. The amount (g) of ethanol solution consumed, in percentage of total fluid intake (g), was used as an index of ethanol preference. Rats were classified as low (<30% ethanol), medium (30-60%) and high (>60%) ethanol-preferring according to the index. Only medium- and high ethanol-preferring rats were selected for further experiments (Paper II and Paper III).

Limited access

After establishing a steady ethanol intake, a limited access paradigm was used to ensure that drug treatment had optimal effect during ethanol consumption. To

this end, the rats were only allowed to access the bottles for 2.5 hours/day. The limited access period lasted for two weeks in both Papers II and III.

Microinjection

Surgery

Rats were anaesthetized by isoflurane (3.5-4.0% in air; Baxter, Apoteket AB, Sweden), mounted into a stereotaxic instrument (David Kopf Instruments) and put on a heating pad to prevent hypothermia during the surgery. Holes were drilled superficially for placement of two anchoring screws and guide cannulae (15 mm width and 7 mm long Plastic One, VA, USA; A/P: +1.85, M/L: ±1.5 relative to bregma and D/V: -6.2 relative to dura). An obturator (Plastic One, VA, USA) was inserted into the guide cannulae to prevent contamination. Guides as well as anchoring screws were fixed to the scull with Harvard cement (DAB Dental AB, Sweden).

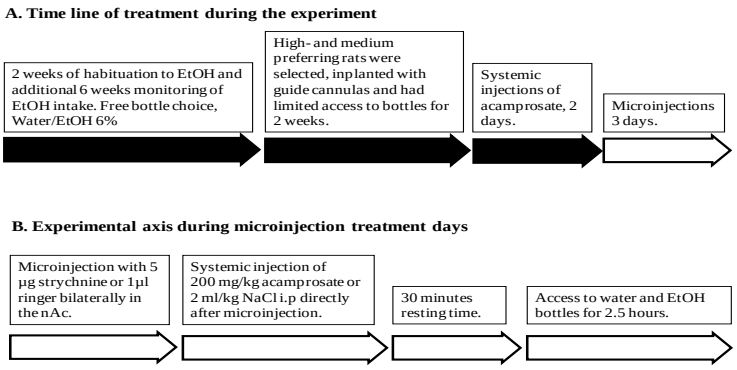


Figure 5. Treatment-protocol for Paper II, (A) illustrates the time line of the experiment. (B) is a detailed description of the microinjection procedure.

Microinjection procedure

Two weeks after surgery, each rat was handled 5-7 times in order to habituate the animal to the microinjection procedure. The animal was first held and then gently restrained in a towel and the obturator (inserted into the guide cannulae) was removed and replaced. After the habituation procedure, the rat was returned

to the home cage. On the microinjection days, extended syringes, with injection cannulae (Plastic One, VA, USA), were connected to a microperfusion pump (U-864 Syringe pump, AgnTho's Sweden) and were inserted into the guides and placed immediately above the nAc, approximately 1 mm below the tip of the guide cannulae. The pump was set to 0.5 µl/min and a total of 1 µl of either Ringer or strychnine (5 µg) was injected bilaterally. After the injections, the extended syringes were left in place for an additional minute to allow the solution to stabilize in the tissue. After the microinjection procedure in Paper II, the rats received a systemic injection and access to the water and ethanol bottles. Figure 5 is a schematic illustration of the whole experimental design.

Verification of injection site

After the third day of microinjections, the rats received a microinjection of dye (Chicago Sky Blue diluted in Ringer) for verification of the injection area. The animals were then sacrificed and the brains were removed and fixed in accustain and stored cold until the brains were sectioned using a vibroslicer and the injection sites were depicted (figure 6).

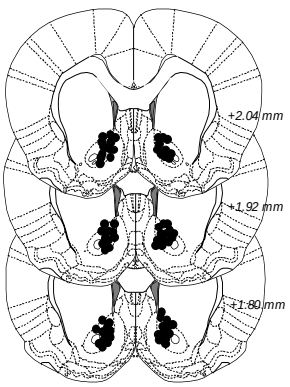


Figure 6. Schematic illustration of the microinjections sites dyed with Chicago Sky Blue.

Depletion of Endogenous Taurine Levels

There are two ways of depleting endogenous stores of taurine noted in the literature, either by means of the taurine transport inhibitor guanidinoethane sulfonate or by adding ß-alanine to the drinking water. ß-alanine and taurine use the same transporters so excess amounts of ß-alanine will deplete endogenous levels of taurine [299]. In Paper V we chose to add 5% of ß-alanine in the drinking water for five weeks since guanidinoethane sulfonate is known to act as an antagonist at the GlyR [300]. The ß-alanine treatment reduced the endogenous levels of taurine by 40%.

Drugs and Chemicals

Ethanol (purchased from Svensk Sprit AB, Sweden) was either dissolved in Ringer when perfused via reversed microdialysis, in 0.9% NaCl when injected intraperitoneally, or diluted in tap water when administered orally by gavage or as an ethanol solution for voluntary ethanol intake. The ethanol concentration used for perfusion in the nAc (300 mM) is based on a concentration-response study performed in our laboratory [143] and was chosen since it is a high but pharmacologically-relevant concentration that elevates extracellular dopamine levels in the nAc by approximately 30%, i.e. to the same extent as observed after 2.5 g/kg i.p. In Paper III, when ethanol was administered by gavage at a concentration of 1 g/kg, the dose was chosen based on similar studies in the literature, and by the fact that voluntary oral intake of this amount of alcohol also increases dopamine levels by approximately 30% [42]. Finally, as previously mentioned, the choice of 6% ethanol in the voluntary ethanol intake studies was based on a study by Fahlke et al which demonstrated that this concentration was optimal [298].

Acamprosate (Calcium acamprosate, kindly provided by Merck Serono, Lyon, France) was either dissolved in Ringer solution for perfusion in the nAc or in 0.9% NaCl for i.p. injection. The systemic concentration used (200 or 400 mg/kg), is based on a previous study performed by Cano-Cebrián et al [291]. For local perfusion we chose 5 or 0.5 mM acamprosate and focused on the lower concentration due to its ability to elevate nAc dopamine to a similar extent as ethanol.

Strychnine (strychnine hydrochloride, purchased from Sigma) was dissolved in Ringer solution and perfused in the nAc (10 and 20 μM) or microinjected into the nAc (Paper II). The perfusate concentrations of strychnine used were based on results obtained from previous studies [44]. These concentrations do not affect accumbal dopamine output *per se*.

Mecamylamine (2-(methylamino) isocamphane hydrochloride) purchased from Sigma) was dissolved in Ringer solution and perfused in the VTA in Paper I. The chosen concentration (100 μM) was based on previous studies by Blomqvist et al and Ericson et al [42, 145].

MPEP (2-methyl-6-(phenyl-ethynyl)-pyridine purchased from Sigma) was dissolved in Ringer solution and locally administered in the nAc by perfusion via the dialysis probe. Previous studies of MPEP are almost solely via i.p. administration. Therefore, the concentrations (100 and 500 μM) were based on a pilot study in our laboratory. The concentrations used were based on their dopamine-modulatory effects (20-60% increase in nAc dopamine output).

Taurine (purchased from Sigma) was dissolved in Ringer solution and administered via local administration in the nAc. The rather small concentration of 50 μM was chosen to simulate the actual *in vivo* concentration under normal conditions [301] and did not influence nAc dopamine *per se*.

β-alanine (purchased from Sigma) was dissolved in tap water and administered via the home cage fluid bottle.

Ringer solution The contents of the Ringer were (in mmol/l): 140 NaCl, 1.2 CaCl2, 3.0 KCl, and 1.0 MgCl2.

Statistical Analysis

In the microdialysis studies (Paper I, III, IV, V), the baseline level of dopamine was determined by the average dopamine content of the last two dialysis samples before local administration of drug. Data from the dialysis experiments were analyzed using ANOVA (analysis of variance) for repeated measures followed by post-hoc analysis by means of Protected Least Significant Difference (PLSD) test or, when appropriate, the paired t-test was used for dependent comparisons between values obtained at different time points (in the same group) and the

unpaired t-test for independent comparisons between values obtained at a certain time point (between groups).

Methodological Considerations

Microdialysis – Recovery and Excovery of the Probe

In microdialysis studies, it is a great challenge to choose concentrations of drugs to administer via the dialysis probe since it is almost impossible to know the exact concentration (excovery) that will reach the target/tissue. Besides the excovery, the recovery, which is the amount of neurotransmitter/substance that is diffusing from the tissue into the probe, is another factor to take into consideration. Both these factors are dependent on numerous variables, for example the substances' temperature, pH, molecular weight, shape and charge. They are also dependent on the surface area of the dialysis membrane, the flow of the perfusion liquid, the speed of diffusion of the substance through the extracellular fluid and the properties of the membrane. In addition, implantation of the probe causes mechanical damage, e.g oedema and minor hemorrhages', resulting in gliosis around the probe that will likely deteriorate the recovery [302]. One should also take into account that there is a concentration-gradient in the extracellular space surrounding the probe, where the highest excovery concentration will naturally be found in the space immediately outside of the probe. The *in vivo* excovery has been determined for a few substances. For example, nicotine has an estimated excovery of 0.1-0.5% [303-304].

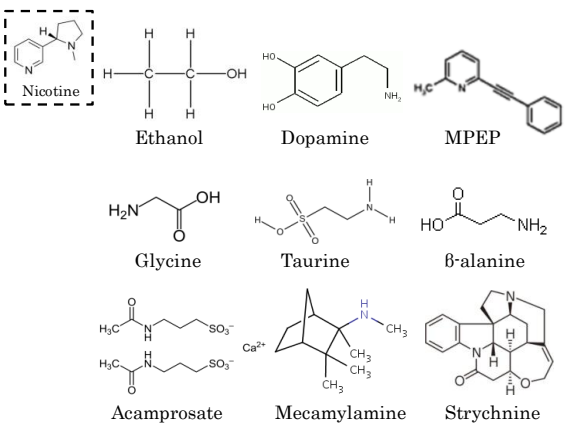


Figure 7. Comparison of the nicotine molecule (with a known excovery) with the different substances that are used in the studies.

Although, these studies do not include observations that nicotine can bind to the tubing material, thus reducing the amount passing over the membrane. Furthermore, it could be generalized that substances larger than nicotine are likely to have an excovery of 0.1-0.2% or below, while smaller molecules are likely to have up to 1% excovery. Since the excovery has been determined for nicotine, Figure 7 is an illustration of the molecular structure of the different substances used in this thesis, to allow for comparison between these and nicotine.

According to Ericson et al perfusion of 300 mM ethanol is expected to result in approximately 45-60 mM ethanol immediately outside of the probe [143]. 10 and 20 μM strychnine would probably result in approximately 0.01-0.1 μM (in situ), concentrations that are considered to be rather selective for the GlyR [210].

Microinjection procedure

According to Ikemoto, microinjected substances are only active for a few minutes after the injection and will probably rather rapidly diffuse into other regions of the brain [37]. In Paper II, there was a thirty minute lag time between the microinjection and access to the bottles in order to ensure a full effect of acamprosate. And, based on the outcome of the study, i.e. that strychnine clearly reversed the ethanol intake-reducing effect of acamprosate, it is obvious that strychnine maintained its pharmacological effect throughout the experimental period. However, it can-not be excluded that strychnine might have diffused into nearby brain regions of the nAc. Although the relevance of this action is unclear, we strongly believe that the outcome of this study was specific to actions at the nAc.

Shell vs. core

Studies have demonstrated that addictive drugs preferentially increase dopamine in the nAc shell region, rather than in the core [305-307]. These findings suggest that the nAc shell is the primary dopamine terminal area affected by acute exposure to addictive drugs [35]. On the contrary, the precise significance of dopaminergic responses in the nAc core is unclear. The nAc core region appears

to be associated with instrumental performance and motor-related behaviors, because of its neuronal connections with the dorsal striatum.. In our studies (microdialysis and microinjection) the dialysis probes and injection cannulaes were placed in the core/shell region, suggesting that we are sampling and/or injecting in both the core and shell of the nAc.

Animal models for studying human alcoholism

To be able to study the biological mechanisms involved in different psychiatric disorders, animal models are invaluable. Unfortunately, there is no single experimental rodent model that reflects every aspect of alcohol addiction. Instead, various animal models for alcohol consumption have been described in the literature which study the separate aspects, such as reward, withdrawal, relapse and cue-induced craving [308].

A valid animal model should fulfill the criteria of face validity, construct validity and/or have predictive validity. Face validity is the evaluation if the testing parameters in the animal are applicable in the patient, for instance, “drinking a certain amount of ethanol per day”. The predictive validity is whether the results obtained from the study would appear similarly in the patient, e.g., treatment response. The third value, construct validity, is the evaluation of whether the model involves the same pathophysiological mechanisms in the animal and man.
The home cage voluntary ethanol consumption paradigm used here is considered to represent the initiation and maintenance of alcohol consumption [309]. Our model fulfills the criteria of face validity and predictive validity since both rats and humans voluntarily consume a certain amount of alcohol and acamprosate and naltrexone treatment reduces ethanol intake in this models and in humans.

RESULTS AND DISCUSSION

Paper I.

Elevation of accumbal extracellular dopamine levels by local and systemic administration of acamprosate is mediated via glycine receptors in the nAc and nicotinic acetylcholine receptors in the VTA.

The first study of acamprosate was designed to evaluate, and to some extent, reconfirm results of Cano-Cebrian that local accumbal administration of acamprosate elevates dopamine output in the same brain region in a dose-dependent and reversible manner [291]. To mimic the clinical situation, we also investigated systemically-administered acamprosate in the same experimental design. By means of *in vivo* microdialysis, elevated accumbal dopamine levels were detected after both routes of administration (figures 8 and 9).

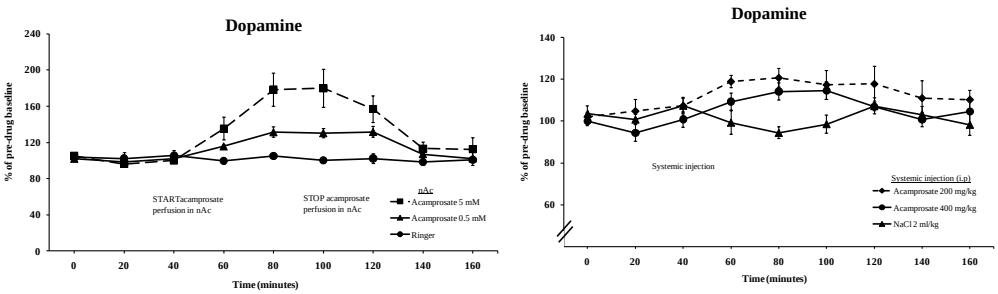
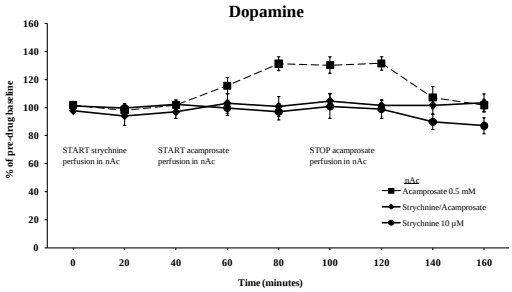
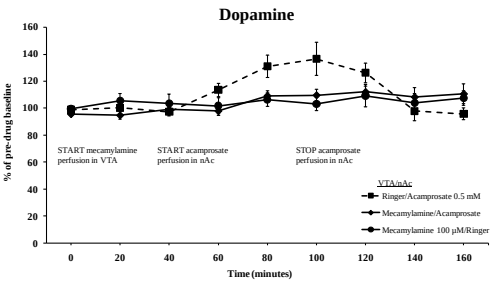


Figure 8 and 9: Local accumbal perfusion with acamprosate dose-dependently increased extracellular dopamine levels. After systemic administration of acamprosate, both 200 and 400 mg/kg were able to elevate accumbal dopamine levels.

Since our previous studies trying to elucidate the mechanism underlying the dopamine-elevating and reinforcing effects of ethanol led us to hypothesize that the primary target site for ethanol is in the nAc, more specifically via GlyRs and secondarily via nAChRs in the VTA, we wanted to investigate whether the acamprosate-induced dopamine increase was mediated via the same neuronal pathway. So after establishing that acamprosate elevates accumbal dopamine levels, we pretreated animals with the GlyR antagonist strychnine (10 μM) in the nAc and in another set of animals with the nAChR antagonist mecamylamine (100 μM) in the VTA. The pretreatments were followed by co-perfusion of

acamprosate (0.5 mM) in the nAc. Both antagonists completely abolished the acamprosate-induced dopamine elevation in the nAc (figures 10 and 11). This demonstrates that the dopamine modulatory effect of acamprosate is, like ethanol, mediated via the GlyRs and the nAChRs.





Figures 10 and 11. Pre-treatment and co-perfusion with the GlyR and nAChR antagonists (strychnine and mecamylamine) both completely abolished locally administered acamprosate-induced dopamine elevation.

These results are also similar to those observed with taurine, ß-alanine and glycine, suggesting that acamprosate may act as a ligand at the GlyR. However, contradictory to this suggestion, a study by Reilly and colleagues found no direct effect of acamprosate on several ionotropic and ligand-gated receptors, among them the glycine α_1 homomeric and $\alpha_1\beta_1$ heteromeric receptors [310]. Two important features could explain the discrepancy between our study and the study by Reilly. First, in the genetically-selected alcohol-preferring and non-preferring rat strain AA and ANA (Alko alcohol/non-alcohol), the most pronounced glycine receptor subunit expression was the ß subunit and the α_2 subunit in most forebrain regions [177]. Although, it has been proclaimed that the $\alpha_1\beta$ GlyR is the standard composition in adult animals, the results from the study by Jonsson et al. suggest that the α_2 subunit exists in the adult brain and that the $\alpha_2\beta$ subunit composition is the most common in the forebrain [177]. Secondly, there are studies demonstrating that both ethanol and acamprosate enhance extracellular levels of taurine in the nAc [107, 222, 311-312]. Augmented levels of nAc taurine have also been detected during operant self-administration of ethanol [193]. Since taurine is an endogenous ligand at the GlyR, the taurine-like substance acamprosate could either activate the GlyR *per se* or could secondarily activate the GlyR by an initial release of taurine.

In conclusion: Local perfusion and systemic administration of acamprosate increase levels of accumbal dopamine. This effect is mediated via GlyRs in the nAc and nAChRs in the VTA.

The results from Paper I are in conjunction with the suggested mechanism underlying the reinforcing effects of ethanol, via the hypothesized mesolimbic neuronal circuitry, indicating that acamprosate might exert its anti-relapse and anti-alcohol effect via the same or a similar mechanism.

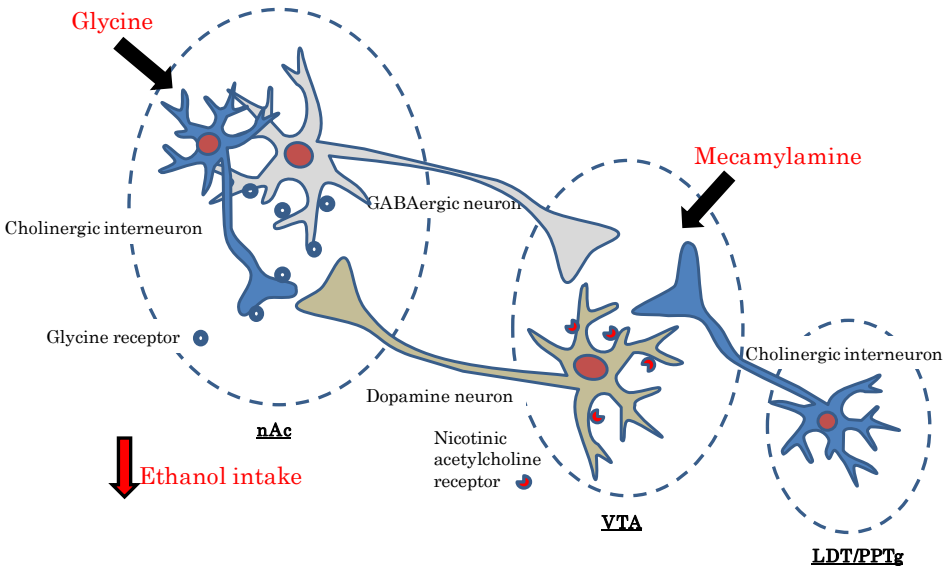


Figure 12. Schematic and simplified illustration of the hypothetical neuronal circuitry. Local perfusion of glycine or mecamylamine reduces ethanol intake in high- and medium- ethanol-preferring animals.

Previous studies have demonstrated that the hypothesized neuronal nAc-VTA-nAc feedback circuitry also is involved on a functional level influencing ethanol intake. This was suggested since bilateral perfusion of glycine in the nAc as well as ventral tegmental perfusion of mecamylamine both reduced ethanol intake in ethanol-preferring rats (figure 12). In the following manuscript, we tested the hypothesis that the ethanol intake-reducing effect of acamprosate also is mediated via this nAc-VTA-nAc neuronal loop.

Paper II

Antagonism of the accumbal glycine receptors is sufficient to inhibit the ethanol intake-reducing effect of acamprosate

In male Wistar ethanol high- and medium-preferring rats, acamprosate treatment (200 mg/kg i.p) significantly reduced ethanol intake, while vehicle-treated animals maintained their ethanol intake. In the group of vehicle-treated animals, a microinjection with strychnine (5 μg) did not affect the ethanol drinking behavior. In contrast, the acamprosate-treated animals receiving a microinjection of strychnine before their systemic acamprosate injection and access to the bottles normalized their ethanol intake to baseline levels (strychnine *reversed* the effects of acamprosate) (figure 13). This finding suggests that the ethanol intake-reducing effect likely is mediated via accumbal GlyRs since blockade of these altered the ethanol intake-reducing effect of acamprosate. Due to technical limitations, extracellular dopamine levels were not measured in this study. But, taken together with previous results where the same receptor family also mediated the acamprosate-induced dopamine elevation, it may be suggested that GlyR activation is important both for the ethanol-induced increase of accumbal dopamine levels as well as for the ethanol consumption behavior. The effects of pharmacological modulation of the nAc-VTA-nAc neuronal loop on voluntary ethanol intake have been investigated in a previous study.

Ethanol high-preferring rats were administered with either glycine or strychnine, bilaterally in the nAc, during which both ethanol intake and nAc dopamine levels were monitored. Activation of GlyRs by glycine increased the dopamine output and decreased the ethanol intake significantly [43]. In line with these results, acute acamprosate was found to elevate accumbal dopamine levels via GlyRs in the same brain region (Paper I), and in the present paper (Paper II) acamprosate was able to reduce ethanol intake, an effect that was reversed after GlyR antagonism.

Other studies have reported acamprosate's ethanol intake-reducing effect to be associated with an increase in dopamine transporter activity and a decrease in

dopamine D_2-like receptor density, i.e. a decrease of activity in the dopamine system [292]. These effects could tentatively be secondary to the dopamine-activating effects of acamprosate observed in Paper I. The ethanol intake-reducing effect of acamprosate has also been associated with reduction of extracellular glutamate overflow [278]. It is possible that the reduction of glutamate is an outcome of taurine-related mechanisms, since taurine *per se* has been found to inhibit release of glutamate [313]. In addition, both ethanol and acamprosate have been demonstrated to increase accumbal levels of taurine [107, 218, 222-223, 278, 314]. Thus, the reduction of glutamate could theoretically derive from GlyR activation via acamprosate-induced augmented taurine levels. Further discussions of the possible involvement of taurine-related mechanisms will re-appear in Paper V.

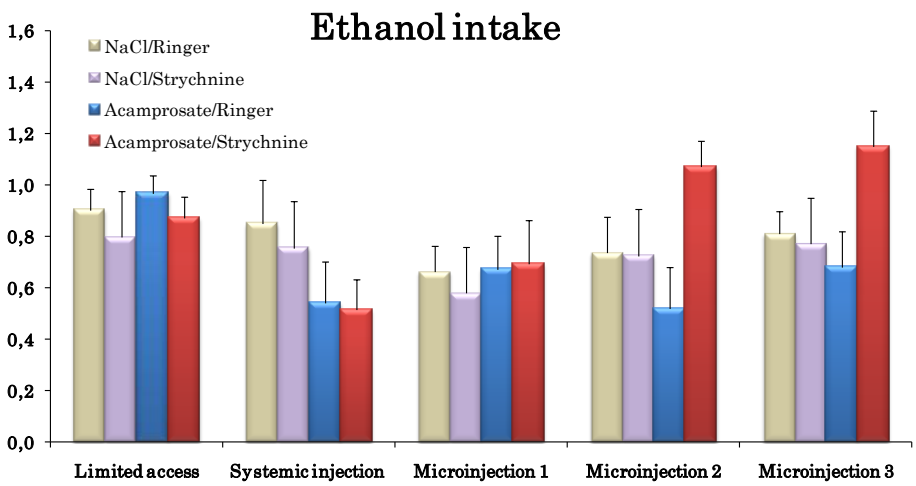


Figure 13. Ethanol intake in all four treatment groups during limited access, systemic treatment and the three microinjection days. In acamprosate treated animals, the ethanol intake was markedly reduced, this effect was prolonged in the animals receiving ringer and acamprosate, whereas strychnine microinjection reversed the ethanol intake reducing effect of acamprosate.

In conclusion: The results from Paper II reconfirmed the important role of the nAc-VTA-nAc neuronal pathway involved in the voluntary ethanol consumption. The inhibition of acamprosate's ethanol intake-reducing effect with strychnine is, to our knowledge, the first study demonstrating a reversal of the acamprosate-induced behavior. We have now demonstrated that GlyRs located in the nAc mediate both the acamprosate-induced elevation of dopamine and reduction in

voluntary ethanol intake. We hypothesize that the two phenomena might be strongly associated with each other, i.e. acamprosate's dopamine-elevating effect is the underlying mechanism for its ethanol intake-reducing effect, in other words, acamprosate may (partly) act as a substitution for ethanol. To further explore this hypothesis, we designed a series of studies investigating co-administration of acamprosate and ethanol.

Paper III

Acamprosate-induced dopamine increase is associated with its ethanol intake-reducing effect

This paper consists of three separate experiments, which differ in route of drug administration and length of acamprosate treatment. In the first study, drug-naïve male Wistar rats increased their dopamine output after acute local (nAc) perfusion with acamprosate (0.5 mM). When ethanol (300 mM) was added to the perfusion medium, the accumbal dopamine levels remained increased but did not display a further elevation and could not be statistically separated from the group treated with acamprosate alone (figure 14).

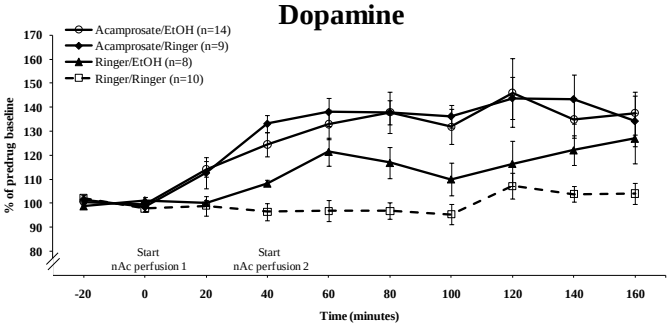


Figure 14. The modulation of accumbal dopamine levels by local adminstration of acamprosate and ethanol may derive from acamprosate and not ethanol, treatment.

This suggests that the dopamine elevation observed in the acamprosate/ethanol-group is only acamprosate-induced and did not derive from ethanol. In an attempt to mimic the clinical situation (i.e. where the patients are treated with acamprosate and consume alcohol orally) the laboratory animals were treated

with acamprosate (200 mg/kg i.p) for two days and then on the microdialysis day, a third injection preceded 1 g/kg ethanol administered orally via gavage. The results from this study were similar to the previous one in naïve rats and demonstrated an increase in nAc dopamine after acamprosate administration but no further elevation after ethanol.

In the third study, we wanted to investigate whether the observed tolerance development to the ethanol intake-reducing effect of acamprosate after repeated administration, is related to its effect on basal- and ethanol-induced dopamine elevation. To this end, *in vivo* microdialysis on ethanol medium- and high-preferring rats was used. After the establishment of a stable ethanol intake behavior, the rats were placed on a restricted access to the bottles, and were treated with acamprosate (200 mg/kg) or vehicle for eleven days. During the first five treatment days (SI1-5), acamprosate reduced the ethanol intake. Notably, the ethanol intake was never totally abolished which may be explained by findings that acamprosate does not decrease the motivation to initiate ethanol intake in rats (figure 15). In other words, the animals still wanted to taste ethanol, but would not continue to consume due to interference with its post-ingestive pharmacological stimuli [315]. Acamprosate appears to have the same behavioral profile in animals as in humans. A clinical study by Hammarberg and colleges challenged acamprosate-treated patients with a priming dose of alcohol. The patients reported that they still wanted to consume alcohol, but chose not to ingest more alcohol [316].

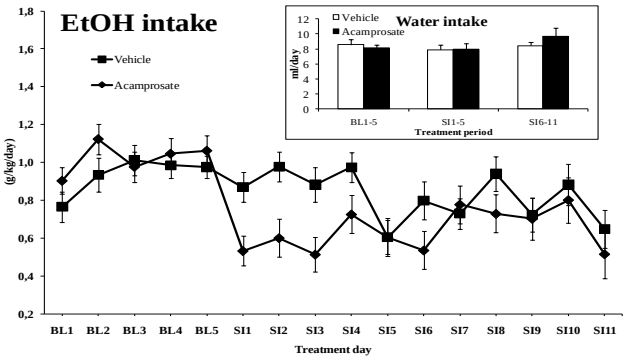


Figure 15. Overview of the ethanol intake in acamprosate vs. control group. Acamprosate administration clearly reduces the ethanol intake during the first five days, this effects diminishes and does not differ from the control group after day six. Inset demonstrates no difference in the water intake in the two groups.

The ethanol intake-reducing effect of acamprosate was diminished on the fifth treatment day, and no significant difference regarding ethanol intake could be detected between the acamprosate- and vehicle-treated groups of rats. On the day of the microdialysis experiment, all animals received an acamprosate injection during continuous monitoring of accumbal dopamine levels. Acamprosate was only able to induce a significant dopamine increase in the acamprosate-naïve animals, where the increase was to the same extent as observed in previous studies. One hour and forty minutes later, all animals were perfused with ethanol (300 mM) in the nAc (figure 16). Interestingly, the group that was treated with long-term acamprosate, responded with a pronounced elevation of dopamine (compared to pre-ethanol level) as compared to the long-term vehicle-treated group (inset figure 16). Although the ethanol intake was not monitored during the day of the microdialysis experiment, it is plausible that the acute acamprosate injection would have reduced the ethanol consumption in the long-term vehicle-treated animals.

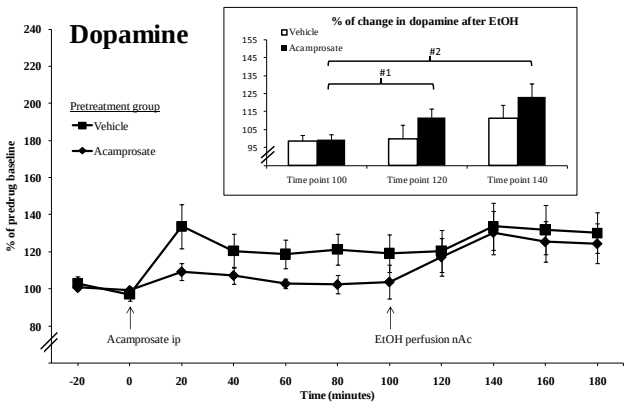


Figure 16. Long-term acamprosate treated animals, in comparison with the acamprosate-naïve, did not respond to acamprosate injection with an increase in dopamine. In contrast, these animals had a higher percentage of change in accumbal dopamine output after ethanol perfusion (inset).

This finding suggests that there is a divergence of response in nAc dopamine depending on the length of acamprosate treatment, i.e. a different outcome of activation of the dopamine system depending on the status of acamprosate susceptibility. Acute administration of acamprosate is able to induce increased dopamine levels and prevents further dopamine elevation by ethanol. But, after five days of treatment, the dopamine system is not responding to acamprosate and ethanol is again able to induce a dopamine increase. In a study by Dahchour and De Witte, the authors observed that chronic ethanol exposed rats treated

with acamprosate for four weeks (400 mg/kg/day), was the only group, compared with ethanol- exposed and naïve rats, that had a higher basal level of taurine [296]. Taking the previously mentioned taurine-releasing mechanism of acamprosate into consideration, the abolished dopamine-enhancing effect after long-term acamprosate administration may be hypothesized to derive from an altered ability to increase extracellular levels of taurine. If the basal level of taurine already is high, it is plausible that no additional taurine elevation can be achieved or detected. Indeed, this was recently demonstrated in a study by Lidö et al, where administration of acamprosate in long-term acamprosate-treated ethanol high-preferring rats, was not able to induce an increase of neither taurine nor dopamine. In the same study, the ethanol intake-reducing effect of acamprosate had been lost [317].

A study by Cowen et al, also concluded that the tolerance development of acamprosate is dependent on an alteration of the activity of the dopaminergic system. They found that in the acute phase, acamprosate increased the density of the dopamine transporter and decreased density of the dopamine D_2-like receptor (an acute decrease of the dopaminergic system). This phenomenon was returned to baseline after repeated injections with acamprosate [292]. The mechanism underlying this effect was suggested by the authors to be mediated via interaction with the mGluR5 receptor, since acamprosate tentatively acts as an mGluR5 antagonist [289] and antagonism at the mGluR5 had been observed to increase dopamine transporter density (unpublished observations by Cowen). The acamprosate/mGluR5 interaction has received increasing focus in recent years. Several studies have revealed that mGluR5 antagonists reduce alcohol self-administration as well as alcohol seeking, relapse and reward in rodents [122-123, 318-319]. In a study by Blednov and Harris, comparison of acamprosate and the selective mGluR5 antagonist MPEP showed similar dose-dependent changes in behavioral effects of ethanol in wild-type mice but both substances failed to produce these effects in mice lacking mGluR5 [118]. Interestingly, it has also been demonstrated that acamprosate failed to interact directly with the mGluR5 [310]. This fact leads us to the aim of paper IV, elucidation of MPEP's

neurochemical profile in relation to our suggested dopamine-controlling nAc-VTA-nAc neuronal circuitry.

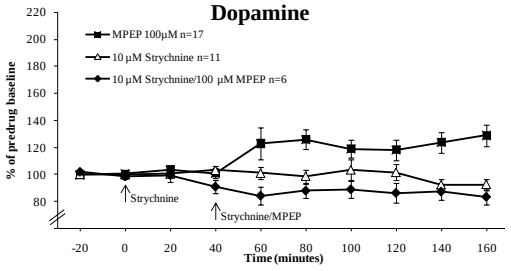
In conclusion: Acamprosate's dopamine modulatory effect, together with the observed alteration of ethanol intake after long-term treatment and the previously-suggested behavioral profile (i.e. no effect on the initial "wanting" but rather interference with the discontinuation of further consumption) suggest that acamprosate, to some extent, might act as a substitution for ethanol's rewarding and/or reinforcing effects. Both ethanol and acamprosate share the dopamine-elevating mechanism, namely acting via accumbal GlyRs and ventral tegmental nAChRs. Another interesting result from this paper is that acamprosate's ability to elevate accumbal dopamine output was lost after approximately five consecutive treatment days, an effect that was paralleled with the loss of the ability to reduce ethanol intake. This suggests that the ethanol intake-reducing effect of acamprosate is tightly linked with its dopamine modulatory properties. This finding should be taken into consideration when evaluating a clinical responder profile to acamprosate, since only 20-30% of the patients respond to acamprosate treatment [273, 285]. Noticeably, the general view of acamprosate's mechanism of action is that it normalizes a hyperglutamatergic state, but clinical studies that associate hyperglutamatergic stages with increased efficacy of acamprosate are still missing.

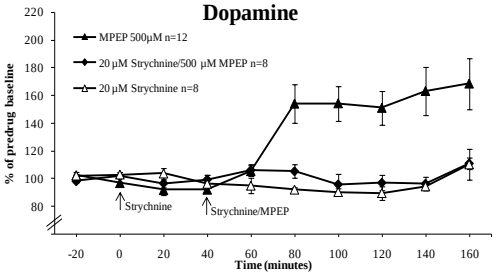
Paper IV

The mGluR5 and GlyR may jointly modulate accumbal dopamine output

A number of studies suggest that the underlying mechanism of acamprosate's ethanol intake-reducing effect is by a reduction of the excessive glutamate levels observed during ethanol withdrawal ([171, 311]. Acamprosate interacts with the glutamatergic receptor system and has been demonstrated to antagonize the mGluR5 [118, 171, 289]. Treatment with the specific mGluR5 antagonist MPEP reduces ethanol-intake in ethanol high-preferring animals [319-320]. At the same time, our findings indicate that the ethanol intake-reducing effect of acamprosate

is mediated via interaction with GlyRs in the nAc. Needless to say, the scientific challenge is to investigate whether these separate findings regarding the mechanism of acamprosate are due to two parallel mechanisms or whether they convergence into one joint mechanism. To start to investigate a possible link between the dopamine modulatory effects of mGluR5 and GlyR we used *in vivo* microdialysis in freely-moving male Wistar drug-naïve rats. First the effect of MPEP on accumbal dopamine output was established. The mGluR5 antagonist (100 µM and 500 µM in the nAc perfusate) significantly increased accumbal dopamine levels. Furthermore, pre-treatment and co-perfusion with strychnine (10 µM and 20 µM) totally abolished the MPEP-induced dopamine increase, suggesting that the MPEP-induced dopamine elevation is linked to GlyR activation (figures 17 and 18). This is in concordance with the previous findings of acamprosate.





Figures 17 and 18. Local (nAc) perfusion of mGluR5 antagonist MPEP induces elevation in accumbal dopamine output in a dose-dependent manner. This effect was totally abolished by pre-treatment and co-perfusion with strychnine.

In the same study (in a subset of animals) we analyzed the amino acids glycine, taurine and ß-alanine in the dialysate samples. Th e highest dose of MPEP significantly elevated extracellular levels of glycine in the nAc (figure 19), whereas no alteration in taurine or ß-alanine was detected. This result suggests that the dopamine-elevating effect of MPEP could involve activation of the GlyRs in the nAc. However, perfusion with the lower dose of MPEP (100 µM) demonstrated a significant elevation of dopamine but failed to increase glycine levels. This may derive from a methodological disadvantage, since the system used for amino acid analysis is less sensitive than the dopamine analysis system, therefore a minor change in the amino acid level might be undetected.

Although we did not explore whether the dopamine-elevating effect of MPEP involves ventral tegmental nAChRs, a recently-published paper demonstrated that systemically-administered MPEP totally inhibits nicotine-induced dopamine increase in the nAc [321]. The authors concluded that the dopamine-dependent reinforcing properties of nicotine depend upon co-stimulation of mGluR5, whilst we would like to interpret the finding as an interaction with GlyRs. The finding is in line with our hypothesis since we have repeatedly demonstrated interplay between accumbal GlyRs, tegmental nAChRs and accumbal dopamine output.

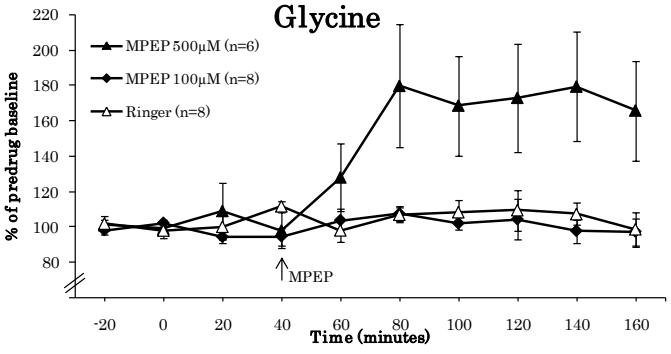


Figure 19. Local (nAc) perfusion with 500 µM but not 100 µM MPEP increased accumbal glycine levels.

In conclusion: The major finding of this study is that there is a link between the mGluR5 and GlyR in modulating accumbal dopamine levels. Since the experimental design of the MPEP study and the studies in Paper I are similar, we can compare the two studies. Both acamprosate and MPEP have been reported to antagonize mGluR5, whereas the present and our previous study also propose that both substances also share the ability to influence GlyRs. Modulation of accumbal GlyRs have been demonstrated to play an important role in the ethanol intake-reducing effect of acamprosate, therefore it is not far-fetched to suggest that the same interaction underlies the ethanol intake-reducing effect observed (by others) after MPEP administration.

Paper V

Augmentation of extracellular taurine levels in the nAc is required for the ethanol-induced elevation of dopamine

The final paper (V) continued to explore the importance of the endogenous GlyR agonist taurine for the dopamine-elevating properties of ethanol. There are several studies suggesting that both ethanol- and acamprosate-induced dopamine elevation involves taurine-related mechanisms. 1) acute ethanol administration increases extracellular levels of taurine [218, 314]. 2) acamprosate administration elevates accumbal levels of taurine [311], 3) and in a previous study from our laboratory it was demonstrated that accumbal perfusion with taurine had the similar dopamine-modulating profile as ethanol, glycine and acamprosate, namely via activation of the GlyRs in the nAc as well as ventral tegmental nAChRs [322].

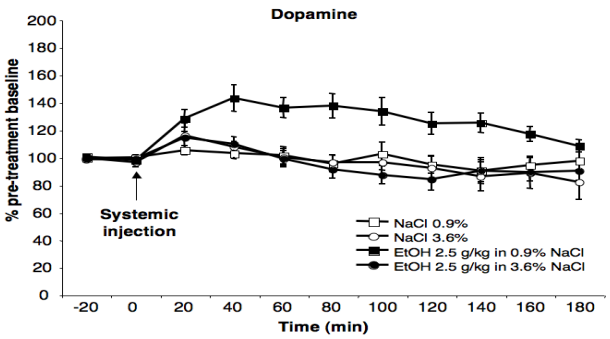


Figure 20. Effect on accumbal dopamine levels after systemic injection with 2.5 g/kg ethanol diluted in an isotonic (0.9 % NaCl) or hypertonic (3.6 % NaCl) solution. The isotonic ethanol-solution significantly increased dopamine levels, while the hypertonic ethanol-solution failed to induce elevation.

Since studies from our research group imply that there is a strong connection between ethanol intake and acamprosate-induced dopamine activation mediated via GlyRs, it is plausible that the endogenous amino acid taurine is a key participant. Taurine has been reported to have osmoregulatory properties and ethanol administration with different osmolarity (0.9%, 1.8%, 3.6%) has been shown to gradually lower the ethanol-induced elevation of taurine [323] however, there is no report of whether the ethanol-induced alteration of taurine also alters the ethanol-induced dopamine response. Thus, the aim of this paper was to investigate whether the ethanol-induced dopamine increase is influenced by ethanol-induced elevation of taurine. To this end, two *in vivo* microdialysis studies with monitoring of extracellular levels of dopamine and taurine were

used. Administration of ethanol diluted in an isotonic or hypertonic saline solution confirmed previous findings of Quertemont et al where ethanol diluted in a hypertonic (3.6%) saline solution completely prevented the ethanol-induced increase of taurine and ethanol diluted in an isotonic solution (0.9%) elevated the extracellular taurine levels by approximately 60% [323]. Furthermore, analysis of dopamine content in the dialysates revealed an elevation of dopamine only after administration of ethanol in the isotonic solution. Systemic administration of ethanol in the hypertonic solution did not influence the dopamine output significantly (figure 20). However, the addition of a small concentration of taurine (50μM perfused in the nAc) restored the dopamine-elevating properties of ethanol, even when administered in a hypertonic saline solution. In a second set of experiments, depletion of endogenous stores of taurine (by approximately 40% after the addition of β-alanine in the drinking water for five weeks) did not prevent ethanol from increasing taurine or dopamine (figure 21). This could be due to the fact that the endogenous level *per se* is not the key factor, but rather the elevation of taurine that initiates a dopamine response. In addition, the nAc-VTA-nAc dopamine regulatory circuitry appears to be a very robust system so it is not unlikely that there are compensatory mechanisms initiated to maintain the dopamine tone.

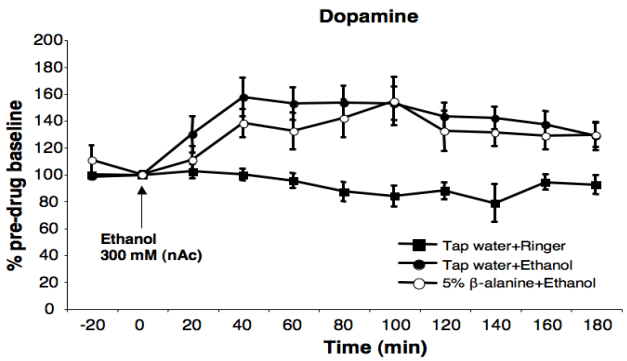


Figure 21. Local perfusion with 300 mM ethanol in the nAc is able to induce dopamine elevation in taurine-depleted animals.

In conclusion: We here demonstrate that the ethanol-induced extracellular elevation of both taurine and dopamine in the nAc appears to be closely related. We suggest that ethanol induces an elevation of dopamine by first initiating an increase of extracellular levels of taurine. This could be a response to a disrupted osmotic milieu after ethanol administration or by other mechanisms. This would

thus again suggest that activation of nAc GlyRs are important for the dopamine elevating and reinforcing properties of ethanol. In addition, taken together with previous findings, it provides further support for the idea that agonism of the GlyR, at least in part, substitutes for the effects mediated by ethanol. This would also imply that targeting the GlyR with pharmacological agents could provide new efficient pharmacotherapies for alcohol addiction.

SUMMARY OF RESULTS

1. Elevation of extracellular nAc dopamine levels by acamprosate is mediated via GlyRs in the nAc and nAChRs in the VTA.
2. The ethanol intake-reducing effect of acamprosate is mediated via accumbal GlyRs.
3. The acamprosate-induced dopamine increase inhibits further elevation of dopamine after ethanol administration. Once the dopamine-elevating property of acamprosate is lost the ethanol intake-reducing effect is also lost.
4. Elevation of extracellular nAc dopamine by the selective mGluR5 receptor antagonist MPEP is mediated via GlyRs in the same brain region.
5. Ethanol-induced dopamine elevation requires a joint action of an initial increase of extracellular levels of taurine.

GENERAL DISCUSSION

More than a decade ago, in 1995, John Littleton published a paper entitled ”Acamprosate, how does it work?”, summarizing the knowledge about a drug that was shown to be efficacious in relapse prevention treatment of alcoholism, but the mechanism of action of that substance was unclear [272]. In the conclusion it was written “It is possible that acamprosate will provide a similar (the advanced understanding of pathology by using an agent with unknown mechanism, authors comment) stimulus for research into alcohol dependence, and that in itself is sufficient reason for wanting to understand how it works”. This perspective summarizes this thesis perfectly. When the work for this thesis began, the mode of action of acamprosate was still unknown and evidence supporting our nAc-VTA-nAc neuronal hypothesis was scarce. An irresistible curiosity to investigate whether an approved anti-relapse compound could interact with the proposed nAc-VTA-nAc neuronal circuitry arose. Findings could hopefully add to the understanding of how excessive alcohol consumption can be controlled.

The working theory of this thesis has mainly been based on previous work from our research group, initialized in the early 1990s’ by Professor Bo Söderpalm. This has resulted in several theses, in chronological order: Ola Blomqvist (1996) [324], Mia Ericson (2000) [325], Anna Molander (2005) [326], Elin Löf (2006) [327] and Helga Höifödt Lidö (2011) [328]. At the beginning, the majority of studies were investigating the role of the nAChRs in alcohol reinforcement (Blomqvist, Ericson and Löf), whereas the latter had focus on the accumbal GlyRs (Molander, Lidö). The findings are all consistent: ethanol’s dopamine-elevating effect as well as ethanol intake is mediated primarily by interaction with GlyRs in the nAc and secondarily via nAChRs in the VTA. The latter receptor family was demonstrated to also be of importance in ethanol cue-induced dopamine elevations and the conditioned reinforcing properties of the cues [327].

The findings of this thesis have confirmed the essential role of accumbal GlyRs in modulating mesolimbic dopamine activity and ethanol consumption.

Acamprosate-induced elevation of dopamine, as well as the ethanol intake-reducing effect, was efficaciously blocked, and reversed, by pretreatment with the GlyR antagonist strychnine in nAc (Paper I and II). These effects are possibly mediated via acamprosate's ability to elevate the endogenous GlyR agonist taurine, since 1) evidence of a direct interaction between acamprosate and GlyR are lacking (notably the α_2 subunit has not yet been investigated), 2) the dopamine-enhancing effect of acamprosate resembles the one observed with both taurine and ethanol, and finally, 3) the ethanol-induced dopamine enhancement is dependent on an initial increase of taurine (Paper V). The fact that there is a resemblance between acamprosate's and ethanol's neurochemical profiles, i.e. the extracellular dopamine and taurine-enhancing effects, raises the question of whether acamprosate *per se* also has addictive properties. There are no reported clinical or preclinical studies indicating this. Furthermore, how can acamprosate increase accumbal dopamine but not evoke a rewarding sensation? To this date, this is still unclear as no studies have been designed to evaluate this issue. However, it is an interesting question, since, in a way, acamprosate may act as a substitution agent for ethanol in the nAc (i.e. it elevates dopamine and taurine levels and inhibits further dopamine elevation by ethanol), but, treatment with acamprosate (in our animal models) never totally reduced the ethanol intake. This finding suits the prevailing view of the mechanism of action of acamprosate, that the substance does not interfere with the initial drinking pattern ("wanting" to drink) but rather with the post-ingestive stimuli in rodents [315] and in humans [316]. One important feature is that acamprosate specifically targets ethanol-related behavior and has no altering effects on sucrose-, water- or food-related behaviors [315].

In a study by Li et al, glycine and taurine elevation had two distinct roles in the ethanol reward processes. They discovered that extracellular glycine levels in the nAc were significantly increased before the experiments were initiated and at the time out periods during operant self-administration. The authors concluded that glycine may be more involved with the anticipation of the reward [193]. The same study also confirmed the findings of Dahchour and De Witte, that extracellular

taurine levels were elevated during the first 15 minutes of the operant sessions. They could also detect that the magnitude of the increase was correlated to the amount of ethanol consumed [222]. In other words, taurine elevation, rather than glycine, seems to be more involved in the reward itself. Extrapolating these findings to the action of acamprosate, which is a taurine-elevating compound, it is plausible that acamprosate only interferes with the ethanol-induced taurine mechanism and not the initial glycine mechanism. This could explain why acamprosate is not effective in reducing the initial "wanting". Rather, its' important ethanol intake-reducing effect is mainly mediated via inhibition of ethanol's taurinergic mechanisms, i.e. the reward itself.

Several studies point towards an important interaction between ethanol and taurine. Studies have indicated that administration of ethanol causes elevated extracellular taurine levels in various brain regions, among them the nAc [218, 314]. Since taurine is an osmoregulator and ethanol causes swelling of neuronal cells [329], the taurine-elevation may derive from the ethanol-induced swelling of astrocytes. In addition, our research group also demonstrated that taurine *per se* elevates dopamine via the proposed neuronal circuitry and that ethanol-induced dopamine elevation may be mediated via an interaction between an initial release of taurine and ethanol ([322], Paper V). Thus, there might be an important interconnection between these events (ethanol-induced cell swelling, taurine elevation, GlyR activation and dopamine enhancement). In fact, this hypothesis was tested in a combined *in vivo* microdialysis and electrophysiology study by Adermark et al. Ethanol induced concentration- and ion-dependent cell swelling of astrocytes in primary cultures from rat, which was blocked by application of the $Na^+/K^+/2Cl^-$ co-transporter inhibitor furosemide (preventing cell swelling). Furthermore, local (nAc) application of furosemide prevented ethanol-induced increases in microdialysate concentrations of both taurine and dopamine. The authors suggested that swelling of astrocytes, taurine release and GlyR activation might be processes upstream from the ethanol-induced dopamine elevation [312]. Taking all these findings into consideration, there is strong evidence that taurine could be important for the acute intoxicating effects of

ethanol. This further implicates that the acamprosate-taurine-GlyR interaction may be a major influential mechanism underlying the ethanol intake-reducing effect of acamprosate.

The prevailing view of acamprosate's anti-relapse mechanism, via mGluR5 antagonism, was also evaluated in relation to our proposed neuronal circuitry. It was revealed that MPEP induced a dopamine elevation which was totally abolished by pretreatment with strychnine, indicating that both acamprosate and MPEP elevate accumbal dopamine levels either by one common target, or by different pathways but with the same outcome (activation of the GlyR). The latter option is most possible since acamprosate activates the GlyR most probably via elevated taurine levels, and MPEP by enhanced glycine levels. MPEP and acamprosate share many functional and neurochemical mechanisms but the GlyR system is rarely mentioned in this aspect. Discussions rather exclusively focus on glutamatergic transmission. Thus, the results of this study represent an initial finding of a link between these two receptor systems.

As we now believe that we have largely elucidated the mechanism of action of acamprosate, another important factor needs to be addressed, namely, the lack of effect in a majority of alcoholic patients. Non-compliance is reported as the most common reason. In one small clinical study by Reid et al, medical management combined with compliance therapy was compared to only medical management in conjunction with 16 weeks of acamprosate treatment. The authors found no differences between the groups with regard to medical compliance or the alcohol measurements (time to first drink, time to first relapse etc) [330]. This could simply reflect the difficulty to treat a patient-population where the motivation (to achieve sobriety) is a main factor to success. But it could also implicate an actual tolerance development to the anti-relapse effects of acamprosate. We and others have repeatedly observed the occurrence of tolerance development to acamprosate in animal models, so it has to be considered possible that patients also develop tolerance and therefore discontinue the treatment. As previous results implied, acamprosate's dopamine-enhancing effect is tentatively

associated with its ethanol intake-reducing effect (Paper I, II), tolerance development in the dopaminergic system would thus have serious consequences for the anti-relapse effect of acamprosate. In fact, our findings strengthen this prediction since acamprosate's ethanol intake-reducing effect was associated with its ability to elevate dopamine by itself and inhibit further alcohol-induced dopamine activation (Paper III). In addition, Lidö et al observed that long-term acamprosate-treated ethanol high-preferring rats failed to elevate extracellular levels of neither dopamine nor taurine after acamprosate administration [317]. As discussed above, enhanced extracellular levels of taurine tentatively mediates the dopamine-enhancing effect of acamprosate (Paper 1). Thus, the finding by Lidö et al is coherent with the taurine-derived dopamine-elevating mechanism of acamprosate, and suggests that the ethanol intake-reducing effect may also be dependent on taurine-related mechanisms. Presuming that the dopamine-elevating effect underlies acamprosate's ethanol intake-reducing property, the taurine modulation must stay intact. In fact, it has been observed that rats receiving simultaneous, long-term exposure of ethanol and acamprosate had higher basal levels of taurine [296]. The enhanced levels of taurine in these animals could mean that acamprosate has lost the ability to further elevate taurine which may lead to signs of tolerance once the rat does not experience the rapid rise in taurine.

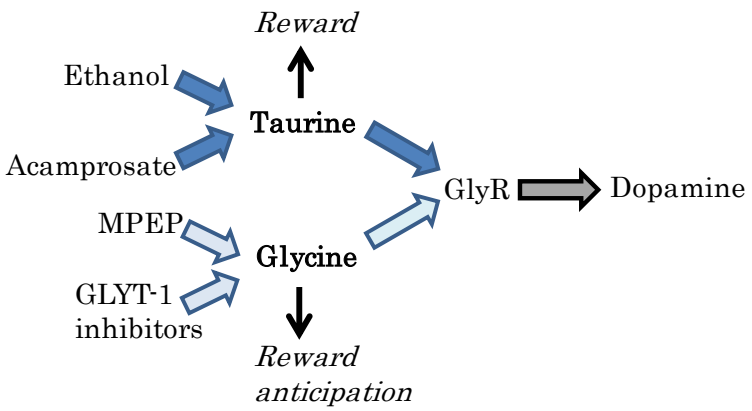


Figure 22. Schematic, simplified illustration of possible pathways to activation of the glycine receptor (GlyR) which results in enhanced dopamine output in the nucleus accumbens. Ethanol and acamprosate may interact with GlyRs via elevation of extracellular taurine levels, while MPEP and GLYT-1 inhibitors enhance extracellular glycine levels. The two different amino acids (taurine and glycine) are also suggested to have two distinct roles in the ethanol reward process (reward or anticipation of reward).

In our animal models, tolerance development was detected after approximately five treatment days in a majority of the animals, but, notably, there were in fact some animals which maintained low ethanol-intake during the whole experimental period, most likely as a result of acamprosate-treatment. It might appear contradictory that acamprosate shares or has a similar neurochemical profile as ethanol; both increase extracellular taurine and dopamine and have the GlyR as a common target. Other substances with a robust and effective ethanol intake-reducing property but with no signs of tolerance development are the glycine transporter (GLYT-1) inhibitors. These GLYT-1 inhibitors elevate extracellular glycine levels, and slightly also dopamine, prevent ethanol-induced dopamine elevations and efficiently reduce ethanol-intake in rats, probably via modulation of the GlyRs [328]. Of the two ethanol intake-reducing substances (GLYT-1 inhibitors and acamprosate), only administration of acamprosate results in rapid tolerance development towards the ethanol intake-reducing effect. One can only speculate on the reasons for this. It may be that the different substances act on receptors with different subunit composition, or have divergent activation-routes upstream of the GlyR, or tolerance development may depend on which endogenous ligand for the GlyR is elevated. Since the major difference between acamprosate and GLYT-1 inhibitors is that they elevate extracellular taurine and glycine levels (figure 22), respectively, one should also have in mind that both ethanol and acamprosate interfere with other receptor systems which may have some additional impact. To this day, the knowledge regarding the mechanisms underlying acamprosate- and ethanol-induced dopamine elevations and tolerance development to the former is not enough. Such knowledge can only be obtained through further innovative experimentation.

CONCLUDING REMARKS

Although the anti-relapse profile of acamprosate appears weak, and in a majority of patients, non-effective, the compound does relieve and aid craving in 20-30% of the patients. And, as professor John Littleton stated, by unraveling the anti-relapse substance acamprosate's mechanism of action, the success rate of future compounds may be improved in a similar vein as has occurred in other areas of psychopharmacology [272]. The present preclinical studies have generated an entirely new hypothesis as regards the mechanism of action of acamprosate in its ethanol intake-reducing effect and thus ideas for the development of more effective compounds may be inspired by acamprosate. In this thesis, acamprosate has been the core, inspiring every experimental design, perforating every experiment. The major findings of this thesis are however not limited to the increased knowledge of the actions of acamprosate *per se* but are also of high relevance for, and in line with, our general hypothesis on the neuronal circuitry involved in alcohol reinforcement as such.

Glycinreceptorer i centrala nervsystemet är en viktig angreppspunkt för akamprosat; ett läkemedel som minskar alkoholintag och risk för återfall

Alkoholberoende är geografiskt och socialt utbrett världen över och klassificeras som en folksjukdom, det vill säga man uppskattar att mer än 1% av befolkningen är drabbad. Problemets omfattning belyses av beräkningar som visar att var fjärde sjukhussäng upptas av en patient med alkoholrelaterade problem, att ca 5000-7000 personer dör i Sverige varje år av alkohol eller alkoholrelaterade sjukdomar och statliga utredningar har bedömt att ca 300 000 svenskar lever med alkoholmissbruk. En hög alkoholkonsumtion medför inte bara stora konsekvenser för den drabbade och dess anhöriga. Den innebär också enorma belopp, uppskattningsvis 100 miljarder kronor per år, i alkoholrelaterade kostnader.

Beroende är tätt sammankopplat med hjärnans belöningssystem. Detta system består av ett nätverk av nervceller som kan frisätta olika signalsubstanser där receptorer på närliggande celler tar emot och förmedlar signalen. På så sätt skapas en kedjereaktion som gör att signaler förs vidare. Hjärnans belöningssystem styrs huvudsakligen av signalsubstansen dopamin, och är ursprungligen till för att belöna oss för att vi gör sådant som är nödvändigt för vår arts överlevnad, såsom att äta, dricka, och fortplanta oss. Dessvärre finns det även icke-livsnödvändiga aktiviteter som kan frisätta dopamin och därmed upplevs som njutningsfulla, t ex att konsumera alkohol och andra droger. Beroende uppstår då belöningssystemets kommunikation ändras så att personen i fråga till sist endast upplever belöning/njutning av det de är beroende av och inget annat. En beroendesjukdom är alltså en förvärvad, kronisk förändring av hjärnans belöningssystem där även minnet är inblandat. Minnet har visat sig spela en stor roll inom beroende, t ex kan belöningssystemet hos en alkoholist aktiveras bara av åsynen av ett vinglas eller andra alkoholrelaterade saker, och alkoholisten/personen i fråga upplever då ett sug efter alkohol.

Idag finns det inga effektiva metoder att ”återställa” hjärnan när ett beroende väl har uppstått. Det är orsaken till att man benämner de personer som lyckats ta sig ur ett beroende med termer som ”nykter alkoholist”. Däremot finns det två läkemedel, naltrexon och akamprosat, som kan påverka det centrala nervsystemet och minska suget efter alkohol. Dessvärre hjälper dessa preparat endast 20-30 % av de patienter som behandlas, det finns alltså ett stort behov av mer effektiva läkemedel för behandling av alkoholsjukdomar. Trots att akamprosat har använts som läkemedel sedan 1989 har man inte lyckats förklara varför den minskar suget efter alkohol. Ett flertal studier har försökt komma underfund med exakt hur akamprosat fungerar, och även om dessa studier inte kunnat ge någon bra förklaring så har de bidragit till en ökad förståelse av hur alkohol påverkar hjärnan. Till exempel har studier med akamprosat visat att det under perioden då kroppen försöker vänja sig vid att vara utan alkohol finns ett överflöd av signalsubstansen glutamat. Överflöd av glutamat betyder delvis att hjärnan ”går på högvarv”. Vid behandling med akamprosat går glutamat ner till en normal nivå. Förmågan att minska glutamat hos akamprosat har föreslagits vara den huvudsakliga mekanismen för läkemedlets verkan på alkoholsug.

Än idag är det omdiskuterat och fortfarande inte helt klarlagt hur alkohol utövar sin effekt i hjärnan och i belöningssystemet. Under ledning av professor Bo Söderpalm, har denna forskningsgrupp lagt fram en hypotes om hur alkohol fungerar i hjärnans belöningssystem, att alkohol aktiverar en nervkrets som inkluderar glycinreceptorer (GlyR) och nikotinacetylkolinreceptorer (nAChR) i belöningssystemet. Denna hypotes är baserad på resultat från tidigare studier från forskningsgruppen, som visat att dessa två receptorklasser har en viktig roll i alkoholens belönande effekt. Genom att blockera antingen GlyR eller nAChR uteblir alkoholens dopaminökande effekt och ingen belöning upplevs. Vi har också visat att dessa receptorer påverkar alkoholintag hos råtta.

Avhandlingens första delarbeten visar att akamprosat ökar dopamin via samma nervkrets och receptorer som alkohol och att den alkoholsänkande förmågan hos medlet dessutom kan hävas genom blockad av GlyR. Det här är de första

studierna där läkemedlets anti-alkohol effekt har kunnat upphävas genom en specifik manipulation, vilket starkt talar för att medlets relevanta farmakologiska mekanism nu har identifierats.

Kliniska studier har visat att en majoritet av de patienter som behandlas med akamprosat tyvärr inte får önskad effekt av läkemedlet. Detta var också tydligt i våra djurmodeller; när vi studerade akamprosat och alkohol tillsammans fann vi att när akamprosat ges akut eller dagligen i upp till fyra dagar ger det upphov till ökade dopaminnivåer i belöningssystemet. När djuren sedan fick alkohol, gav det inte någon ytterligare frisättning av dopamin, med andra ord, akamprosat verkar hindra alkohol från att utöva sin belönande effekt. Detta kan tolkas som att akamprosat delvis fungerar som en ersättning för alkohol. Efter en längre tids behandling med akamprosat (totalt elva dagar) förlorade substansen både sin dopaminhöjande egenskap, och sin förmåga att sänka alkoholintaget hos försöksdjuren. Dessutom fann vi att när akamprosat förlorat sin dopaminökande effekt gav alkohol åter en ökning av dopamin och antagligen en känsla av belöning hos djuret.

Tidigare forskningsresultat har visat att akamprosat blockerar en typ av glutamatreceptor, mGluR5, och på så sätt normaliserar glutamatnivån. Vi undersökte därför om även mGluR5 antagonisten MPEP, en substans med liknande egenskaper som akamprosat, verkar genom samma nervkrets i belöningssystemet som alkohol och akamprosat, eller om substansen har en annan mekanism. Resultatet var att mGluR5 och GlyR samverkar för att öka dopamin i belöningssystemet.

Då akamprosat, likt alkohol, frisätter aminosyran taurin ger detta ytterligare en möjlighet att påverka GlyR i belöningssystemet. Taurin, liksom glycin, aktiverar nämligen GlyR och har tidigare självt visats öka dopamin i belöningssystemet via den nervkrets som alkohol också använder. I avhandlingen visar vi nu att för första gången, att alkohol ska kunna öka dopamin krävs att det samtidigt med alkoholtillförseln sker en frisättning av taurin.

Utgångspunkten för avhandlingsarbetet har varit att studera den mekanism genom vilken akamprosat sänker alkoholintag hos råtta. Studierna har bekräftat att den nervkrets som beskrivits ovan har stor betydelse för alkoholens dopaminfrisättande och positivt förstärkande egenskaper. Vi har också visat att läkemedlet akamprosat fungerar via samma nervkrets och då, i alla fall delvis, substituerar för alkohol. Genom dessa ökade kunskaper gällande alkohol och akamprosat kan vi nu fokusera på nämnda nervkrets som en angreppspunkt för nya farmakologiska preparat för behandling av alkoholism.

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