Thesis for the degree of Doctor of Medicine

PRECLINICAL INVESTIGATIONS OF GLYT-1 INHIBITION AS A NEW CONCEPT FOR TREATMENT OF ALCOHOL DEPENDENCE

Helga Höifödt Lidö

2011

UNIVERSITY OF GOTHENBURG

Addiction Biology Unit

Department of Psychiatry and Neurochemistry

Institute of Neuroscience and Physiology

The Sahlgrenska Academy at the University of Gothenburg

Gothenburg, Sweden

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Printed by Geson Hylte Tryck, Kungsbacka, Sweden

Contact: Helga.lido@neuro.gu.se

ISBN 978-91-628-8221-1

Abstract

Helga Höifödt Lidö, *Addiction Biology Unit, Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Sweden*

Alcohol addiction and abuse is a main contributor to the global burden of disease and is a high public health priority. Alcohol addiction is a chronically relapsing neurobiological disorder affecting multiple neurotransmitter systems. Considerable evidence suggests that the mesolimbic dopamine system is the primary substrate for the acute rewarding and reinforcing effects of alcohol. Over time, excessive alcohol intake causes chronic functional changes in this system that may trigger off the transition from controlled recreational alcohol use to the compulsive intake that characterizes true addiction. Pharmacotherapy is emerging as a valuable tool for treatment of alcohol addiction, yet the current agents approved for this condition are only modestly effective and there is a need for improved treatments. It was recently revealed that extracellular glycine levels are important for regulating alcohol consumption and that the glycine receptor (GlyR) in nucleus accumbens (nAc) is an access point for alcohol to the mesolimbic dopamine system. The glycine transporter-1 protein (GlyT-1) is the main regulator of extracellular glycine concentrations and thus a key substrate for pharmacological manipulation of brain glycine levels. The aim of this thesis was to investigate (1) how modulation of extracellular glycine levels by inhibition of GlyT-1 affects the mesolimbic dopamine system, (2) how it interacts with alcohol-induced activation of mesolimbic dopamine, and (3) how GlyT-1 inhibition influences voluntary ethanol consumption. Effects on ethanol drinking were studied by using a limited access free-choice model in out-bred Wistar rats. Effects on dopamine and glycine levels in nAc were examined by using *in vivo* brain microdialysis. First it was demonstrated that the GlyT-1 blocker Org25935 robustly and dose-dependently reduced voluntary ethanol intake and that the effect was reinstated after an alcohol withdrawal period. Next it was shown that Org25935 raised extracellular glycine levels by 87% in nAc, increased dopamine levels *per s*e and most importantly prevented an ethanol-induced dopamine increase in nAc. It was then shown that the GlyR in nAc rather than the NMDA receptor is involved in mediating the effect of Org25935 on dopamine levels in nAc. The last study investigated the anti-alcohol drinking profile of another selective GlyT-1 inhibitor Org24598, and compared the effect to that of acamprosate. In summary, the results propose that the GlyT-1 blocker Org25935 increases and stabilizes extracellular glycine levels which, via the GlyR, elevate and preserve a steady dopamine level, which in turn prevents additional ethanol-mediated GlyR activation and dopamine elevation. This adds to the growing evidence for the GlyR as an important player in the dopamine reward circuitry and in ethanol's effects within this system. Two different GlyT-1 inhibitors demonstrated an excellent ability to decrease ethanol consumption in experimental animals. This thesis proposes that GlyT-1 inhibition may represent a new concept for treatment of alcohol addiction.

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List of publications

The thesis is based on the following research papers, referred to in the text by their Roman numerals:

I. Molander A, **Höifödt H**, Löf E, Ericson M, Söderpalm (2007) The Glycine Reuptake Inhibitor Org25935 Decreases Ethanol Intake and Preference in Male Wistar rats. *Alcohol and Alcoholism, 42:11-18*

II. **Höifödt Lidö H**, Stomberg R, Fagerberg A, Ericson M, Söderpalm B (2009) The Glycine Reuptake Inhibitor Org25935 Interacts with Basal and Ethanol-induced Dopamine Release in Rat Nucleus Accumbens. *Alcohol Clin Exp Res 33:1151-1157*

III. **Höifödt Lidö H**, Ericson M, Marston H, Söderpalm B. A Role for Accumbal Glycine Receptors in Modulation of Dopamine Release by the Glycine Transporter-1 Inhibitor Org25935. *Under revision, Frontiers in Phsychopharmacology, Jan 2011*

IV. **Höifödt Lidö H**, Marston H, Ericson M, Söderpalm B. The Glycine Reuptake Inhibitor Org24598 and Acamprosate Reduce Ethanol Intake in the Rat; Tolerance Development to Acamprosate but not to Org24598. *Under revision, Addiction Biology, Jan 2011*

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List of abbreviations

AA	Alko Alcohol rat line
AD	Alcohol deprivation
ANOVA	Analysis of variance
BL	Baseline
BNST	Bed nucleus of the stria terminalis
ED	Electrochemical detection
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders 4th edition
GABA	Gamma-amino-butyric acid
GlyB	NMDA receptor glycine site
GlyR	Glycine receptor
GlyT-1	Glycine transporter-1
GlyT-2	Glycine transporter-2
HPLC	High-pressure liquid chromatography
5-HT	5-Hydroxytryptamine (serotonin)
ICD-10	International Classification of Disease-10th edition
LA	Limited access
LDTg	Laterodorsal tegmental nucleus
mGluR5	Metabotropic glutamate receptor subtype 5
nAc	Nucleus accumbens
nACh	Nicotinic acetylcholine
NMDA	*N*-methyl-*D*-aspartate
PLSD	Protected least significant difference
PPTg	Pedunculopontine tegmental nucleus
SEM	Standard error of the mean
VIAAT	Vesicular inhibitory amino-acid transporter
VGAT	Vesicular GABA transporter
VTA	Ventral tegmental area
WHO	World Health Organization

Preface

As long as we have recorded human history alcohol has been used and misused by mankind. Alcohol intake behavior has been shaped with human development and is a part of our normal behavior. As in ancient times as today, alcohol plays a prominent role in numerous social settings and is often used as a daily incentive. Social drinking, defined as the occasional but limited use of alcohol without intent to get drunk, produces a general feeling of well-being that is familiar to many. Alcohol's profile is highly dependent on dose; whereas alcohol in low doses produces positive rewarding and relaxing effects, having too much leads to drunkenness with loss of judgment and desire for more, and may trigger aggression and negative mood states. Alcohol drinking is deeply embedded in our lives and a hangover, that in reality is an alarming sign of serious intoxication, can easily pass without further notice.

Yet the real dark side of alcohol is when an escalated alcohol use leads to compulsive addictive behavior. What are the neurochemical underpinnings for the transition to the desperate desire for alcohol destroying lives and families, that is experienced by far too many people? How does the brain adapt to chronic alcohol intake and when is the point of no return?

Alcohol addiction is now recognized as a neurobiological brain disorder where pharmacotherapeutic treatment can be of great help, and is consequently receiving increasing attention in medical research. As alcohol interacts with most neuronal networks in the brain, the pursuit of improved medication for alcohol dependence is a challenge. The present thesis is an attempt to unravel a small piece of alcohol's actions and how this can be manipulated in order to reduce alcohol consumption. The thesis thus explores the role of glycinergic signaling in relation to alcohol's rewarding effects, a neurotransmitter system to date scantily explored in the brain. The work aims to investigate whether modulation of brain glycine level, by inhibition of the glycine transporter-1 protein, may offer a new pharmacological treatment principle for alcohol dependence.

Alcohol addiction

The socio-economic impact of alcohol

Alcohol abuse and alcohol dependence, with all their serious medical, economic and social consequences, contribute significantly to the global burden of disease. Harmful use of alcohol is by The World Health Organization (WHO) listed as the third leading risk factor for premature death and disabilities in the world, which is in the same order as tobacco and hypertension (2). Huge amounts of alcohol are consumed in many parts of the world and the Swedish citizen consumes on average 10 liters of pure ethanol per year (3). As toxic effects of alcohol damage all organs of the body, excessive alcohol use has serious health consequences to the individual and may lead to liver cirrhosis, neuropsychiatric diseases, cancer and cardiovascular diseases among other things (2, 4). Intentional and non-intentional injuries provoked by drunkenness are common problems for emergency wards. Moreover, chronic alcohol intake may lead to alcohol addiction, which, with a prevalence of 4-6%, ranks among the dominating psychiatric disorders in Western countries and is a common comorbid disorder to the other mental disorders (5). Besides devastating medical and psychiatric consequences for the alcoholic, alcohol addiction is a heavy burden to family, friends and social services and is a significant component in crime and traffic accidents (6). In total, alcohol misuse and addiction cause enormous costs to society and in Sweden alone the annual total costs are estimated to 100 billion SEK (3, 7).

Risk factors

Alcohol addiction has many risk factors and is a result of a complicated interplay of biological vulnerability, metabolic capacity and social and environmental exposure. Stress, mental health, age, sex and ethnicity are among the well-known risk factors. The prevalence is higher among men but an emerging concern is the increasing consumption observed among women and the proportion of female alcoholics has increased (8). Alcohol dependence runs in families and the inheritance is explained partly by the family background and partly by the individual's genetic predisposition. In both men and women, alcoholism is 50-60 % genetically determined leaving 40-50 % to environmental influences (9, 10). The individual's phenotype can affect physiological and neurobiological processes or it can interact with life experiences, by either causing protection or susceptibility of developing addictive behavior.

Due to the large genetic influence on alcohol addiction there has been great effort towards identification of alcohol dependence-related genes. Genetic polymorphism of genes encoding neurotransmitter signaling molecules in dopamine, gamma aminobutyric acid (GABA), opioid and serotonin systems have been identified, however, results are often inconsistent and the mechanisms of action of these genetic aberrations remain to the elucidated (11). The dopamine D_2 receptor gene is among the stronger candidate genes implicated in alcoholism, probably acting via incentive salience and craving mechanisms (12) and there are also reports on D_4 receptor gene involvement (13). In fact, six genes on chromosomes 4, 7, 8, 11, 15 and 20, which are involved in dopamine signal transfer and generation of dopamine receptors have been associated to alcoholism (14). Also an important component of the dopaminergic reward pathway, variants in nicotinc acetylcholine (nACh) receptor genes are associated with alcohol-related phenotypes (15, 16). Moreover, a polymorphism in the gene encoding the opioid mu-receptor is associated with increased sensation of the intoxicating effect of alcohol (13). This polymorphism is linked to treatment response to the opiate receptor antagonist naltrexone, yet clinical studies do not clearly report an association to alcohol dependence (17). Lastly, the most consistent genetic risk factors are found in genes coding for enzymes involved in alcohol metabolism. In fact, the low alcoholism prevalence in East Asia is probably mainly explained by a common polymorphism of a gene coding for an enzyme involved in alcohol degradation, i.e. acetaldehyde dehydrogenase, leading to accumulation of acetaldehyde after alcohol intake (14). It is obvious that the genetics of alcoholism are complex and a next challenge will be to characterize the risk associated with identified genes. The hope is that such advances will increase our ability to treat alcoholic patients.

Symptoms and diagnostic criteria

'Alcohol dependence is a chronically relapsing disorder characterized by compulsion to seek and take the drug, loss of control in limiting intake and emergence of a negative emotional state, e.g., dysphoria, anxiety, irritability, reflecting a motivational withdrawal syndrome when the drug is not on board' (18). When diagnosing alcohol dependence, clinicians are often obstructed by the social barriers connected to the disease. Stigmatization, moral attitudes and social stereotypes often lead to patterns of hiding and denial by the afflicted. There is no biological marker for alcohol addiction and criterion-based diagnostic

instruments are the standard tools. As an aid for clinicians, several questionnaire-based screening protocols to detect harmful drinking patterns and alcohol dependence are available for health care workers. Alcohol dependence is a psychiatric diagnosis described in the International Classification of Disease-10th edition, WHO (ICD-10) and in the Diagnostic and Statistical Manual of Mental Disorders – 4th edition, American Psychiatric Association, 1994 (DSM-IV). The DSM-IV is the common global standard in psychiatry and its criteria are displayed in Table 1.

Table 1. Diagnostic guidelines/criteria for alcohol dependence from DSM-IV
Washington, DC, American Psychiatric Assoication, 1994

'A maladaptive pattern of alcohol use, leading to clinically significant impairment or distress,
as manifested by at least three of the following, occurring at any time in the same 12-month period'

1 Tolerance, as defined by either of the following:
  a A need for markedly increased amounts of alcohol to achieve intoxication or desired effect
  b Markedly diminished effect with continued use of the same amount of alcohol
2 Withdrawal, as manifested by either of the following:
  a The characteristic withdrawal syndrome for alcohol
  b Alcohol (or a closely related substance) is taken to relieve or avoid withdrawal symptoms
3 Alcohol is often taken in larger amounts or over a longer period than was intended
4 There is a persistent desire or unsuccessful efforts to cut down or control alcohol use
5 A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects
6 Important social, occupational, or recreational activities are given up or reduced because of alcohol
7 Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol

Tolerance and withdrawal, criteria one and two, describe the physical dependence. Criterion three describes the loss of control, a striking feature of addiction reflecting failure to stop, cut down or control the use despite great harm. Criterion three and four may describe the state of 'craving', which is a strong desire and resistant urge to consume alcohol, as well as loss of control. Criterion five, six and seven refer to the compulsive state and reflect the social and medical consequences of alcohol consumption. Criterion seven may also reflect denial or the phenomenon of neglect, as the afflicted lacks insight to the consequences of

their drinking behavior. Besides these measures, another remarkable aspect of the disease is the chronic relapses that can occur after many years of abstinence, and rates of recurrence are high also in patients highly motivated to abstain.

The classification systems also differentiate between alcohol use, abuse and dependence. Alcohol use refers to social or recreational drinking, defined as the occasional but limited use of alcohol without intent to get drunk. According to DSM-IV alcohol abuse is defined as repeated use despite recurrent social and legal adverse consequences, and is not defined as an addictive state. Alcohol dependence or alcoholism (Table 1) is defined by physiological, behavioral and psychosocial symptoms and is classified as a 'drug addiction'. Thus alcohol dependence is described as alcohol abuse combined with tolerance, withdrawal and an uncontrollable drive to drink. Since alcohol dependence is such a clinically diverse condition, there is an ongoing discussion whether the new and 5th version of DSM, planned for 2011, will merge alcohol abuse and dependence into the new single disorder 'alcohol use disorders', with graded clinical severity. Here, the term 'dependence' covers physical dependence only, defined as a transient neuroadaptive process and thus a normal homeostatic response to repeated dosing, not only to addictive drugs but also to medications such as β-blockers and antidepressants. Accordingly, the presence of tolerance and withdrawal symptoms, criteria one and two, will not be regarded as symptoms of the new diagnosis 'substance use disorders'. Lastly, the term 'alcohol addiction', which defines the pathological condition with underlying persistent changes within specific neuronal systems, is clearly distinct to the above described 'dependence'. However due to the tradition of using the term 'alcohol dependence', this term and 'alcohol addiction' is used interchangeably in this thesis.

Available treatments

Alcoholism is medically defined as a treatable disease and the available treatment options are varied, reflecting its multifaceted appearance. When appropriate, the intervention may be set off by specialized treatments for alcohol overdose and/or the alcohol withdrawal syndrome ('detoxification'), the latter often by tapering with a cross-tolerant drug, preferably a benzodiazepine (19). Following this, a long-term treatment program with a combination of pharmacotherapy, psychosocial therapy or attendance at self-help groups is

often required in order to prevent relapse. Since the beginning of the 1990s, several behavioral and pharmacological treatment alternatives have been developed, though the access to these treatment alternatives varies considerably throughout the country. Along with the recently developed drugs naltrexone and acamprosate, drugs under discovery today are increasingly based on alcohol's neurobiological mechanisms of action (20). Promising drug candidates to reduce compulsive alcohol drinking and relapse have reached Phase II clinical trials, however several concepts have proven negative when tested in man (21-23). At present time, three pharmacology-based treatments are approved by the Swedish Medical products agency (as well as the US Food and Drug Administration) for the treatment of alcohol dependence: disulfiram (Antabuse®), acamprosate (Campral®) and naltrexone (Naltrexon Vitaflo®). Disulfiram has been used clinically since the late 1940s and exerts an aversive mechanism of action. By blocking the liver enzyme acetaldehyde dehydrogenase, the toxic metabolite acetaldehyde accumulates when alcohol is consumed, producing a profound aversive state that will deter alcohol consumption, characterized by flushing, shortness of breath, tachycardia, headache and nausea. Recent research has revealed that disulfiram also prevents the breakdown of dopamine probably by inhibition of dopamine β-hydroxylase, which in turn may restore a hypodopaminergic state in the brain reward system (24). Another recently proposed mechanism of inhibition of aldehyde dehydrogenase's interference with brain dopamine systems involves tetrahydropapaveroline, which inhibits tyrosine hydroxylase to reduce dopamine production (25). In systematic reviews, disulfiram lack efficacy as long-term treatment (26) and is by many clinicians regarded as an out-dated drug. Yet disulfiram was recently reported superior to acamprosate in patients with a long duration of alcoholism (27).

The mechanisms of action proposed for naltrexone and acamprosate are to decrease alcohol's dopaminergic reward signal and to stabilize a hyperglutamatergic state provoked by chronic alcohol intake, respectively. Research on the neurobiological actions of alcohol as well as the use of alcohol self-administration models have contributed in the development of these agents, pointing to a potential significance of the present work (28, 29, 30). Acamprosate was approved in Sweden in 1996 and in the US in 2007, and has been shown to be of special value in maintaining alcohol abstinence (41). As a GABA analogue, acamprosate was believed to be a GABAergic acting drug (30, 31), but was later demonstrated to rather

act as an NMDA receptor antagonist (32, 33) that may relieve the hyperglutamatergic state following alcohol withdrawal. Yet acamprosate is also reported to have no effect on the NMDA receptor (34), to potentiate the receptor (35) and to rather antagonize the metabotropic glutamate receptor subtype 5 (mGluR5) (36). The reported effects of acamprosate on the NMDA receptor are inconsistent and may depend on the brain region examined, the receptor subunit composition or possibly other factors. Other studies have pointed to the glycine receptor (GlyR) as involved in acamprosate´s alcohol intake reducing effect and in its interference with the brain reward system (37, 38). To date acamprosate is most often referred to as a functional NMDA receptor antagonist, possibly also acting through an interaction with calcium release (39, 40, 41), yet the mechanism of action is under debate.

The opiate antagonist naltrexone was first introduced clinically to terminate heroine abuse but is now rather approved for alcohol dependence, in the US in 1992 and in Sweden in 2000. Recently, a depot injectable formulation of naltrexone has become available in certain countries including the US, showing good evidence for clinical efficacy (23). Naltrexone interferes with the opioid system by antagonizing primarily μ-opioid receptors, but also κ- and δ-receptors, and thus blocks effects of endorphins set free by alcohol (42-45). The alcohol-opioid interaction is linked with activity in the mesolimbic dopamine pathway, as opioid agonists are self-administered into the ventral tegmental area (VTA) and produce conditioned place preference when applied in the VTA (46, 47). Naltrexone suppresses ethanol-induced dopamine release in the nucleus accumbens (nAc) which is associated with a decreased operant alcohol-reinforced behavior (48). Naltrexone reduces craving and relapse in heavy drinking and is suggested to produce a better treatment response in patients with the Asn40Asp polymorphism of the mu-opioid receptor gene (49). That this functional polymorphism might predict naltrexone response suggests that mu-opioid receptor genotyping might be useful for optimizing the treatment, and demonstrates that an individualized approach in the treatment of alcoholism may hold promise (50).

Also medications with other approved indications are being used off-label in the treatment of alcohol dependence. The antiepileptic drug topiramate (Topamax®) (51, 52, 55) and the spasmolytic $GABA_B$ agonist baclofen (Lioresal®)(53) as well as the serotonin (5-HT3) receptor antagonist ondansetron (Zofran®) (54) have been found effective for the long term-

treatment of alcoholics. Topiramate is suggested to antagonize excitatory glutamate receptors, inhibit dopamine release and to enhance GABAergic activity (56). Also other drugs acting on glutamate neurotransmission that indirectly affect mesolimbic dopamine such as modafinil (Midiodal®), lamotrigine (Lamictal®), gabapentine (Gabapentin®) and memantine (Ebixa®) have demonstrated effect in treatment of alcoholism (57, 58). The atypical antipsychotic drugs quetiapine (Seroquel®) and clozapine (Leponex®) and the partial dopamine agonist aripiprazole (Abilify®) have demonstrated some effect in reducing alcohol consumption (59), but overall, dopamine modulating agents only show modest effects on alcohol consumption. A depot-formula of a classical antipsychotic actually increased both relapse rate and alcohol consumption (60). Some of the drugs in the pipe-line are compounds that target the cannabinoid receptor 1, metabotropic glutamate receptors, nACh receptors well as neuropeptidergic drugs targeting the stress axis via corticotrophin releasing factor, neuropeptide Y and nociceptin (21, 23).

It is clear that development of effective treatments for alcohol dependence represents an important public health concern. Acamprosate and naltrexone have demonstrated some ability to reduce drinking and/or to increase the time spent abstinent, but the results are not consistent and reviews and meta-analyses reveal modest effects of these approaches, with a number needed to treat in the order of 7-9 or higher (61). The biggest challenge in treating alcohol-dependent patients is long-term relapse prevention and the limited efficacies of the available agents justify the search for more effective medications.

Pathophysiology

Alcohol addiction is today seen as a chronic relapsing condition but detailed etiology and pathophysiology remain to be established. The disease theory of alcoholism has often been popularized by different movements, that understand the disease as a matter of self-control, motivation and spiritual awakening, without recognizing the neurobiology component. The pathophysiology may relate to many factors, as genetic vulnerability, social influences and the degree of alcohol exposure are in constant interaction with brain neurobiology. It is therefore likely that the neuropathological pattern, observed as the course and the severity of the disorder, differs between subjects. Several trait theories have been proposed in order to explain addictive behavior and has influenced alcohol research, like sensation seeking,

harm avoidance and sensitivity to alcohol reinforcement (62-64), suggesting that a hedonistic personality may be predisposed to addictive behavior. Indeed alcoholics score higher on novelty seeking which involves impulsiveness and disinhibition (65) and alcohol-related problems are associated with specific behavioral features in at least some forms of alcohol addiction such as Cloninger type 2 alcoholism (66, 67). Moreover the use of alcohol and drugs of abuse can also be regarded as a form of self-medication for the relief of affective symptoms, such as depression, tension and anxiety (68). A personality trait may point to susceptibility factors but cannot by itself predict alcohol dependence and does not describe or explain the pathological changes underlying addictive behavior.

During the past decade there has been a shift in alcohol research towards identifying long-term neuroadaptive changes that may underlie relapse and the excessive consumption after periods of abstinence. There is increasing evidence for a common pathology for alcohol and other drugs of abuse (69, 70). The brain is a highly reactive organ, which rapidly responds and adapts to its surroundings. Chronic intoxication of drugs of abuse causes neuroadaptations in brain structure, plasticity and altered gene expression, leading to persistent changes in brain functions and transition from controlled to compulsive alcohol use. The VTA-nAc pathway mediates acute rewarding, reinforcing and motivational effects of alcohol and drugs of abuse and plays a crucial role in alcohol consumption behavior (71-73), as described in the following sections. A dysfunction of the reinforcement system and thus a change in the motivation for the drug is proposed to be a key component of addiction (74). The dopamine response in nAc provoked by alcohol will with long-term exposure lead to an allostatic downregulation of the system with a reduced dopamine set point (75, 76). A subsequent alcohol withdrawal will then leave the system severely impaired and trigger further alcohol intake, and a hypodopaminergic state is further developed when escalated alcohol drinking further reduces reward sensitivity (75, 77).

One current neurobiological theory of addiction conceptualizes addiction as a sequence of neuroadaptations within a cycle of three phases: ‘1) the binge/intoxication, 2) the withdrawal/negative affect stage and 3) preoccupation/anticipation (craving stage)’ (18, 78). 1) Alcohol’s positively reinforcing effect, primarily mediated by the mesolimbic dopamine system, is a critical starting point for the transition to addiction. Eventually, alcohol’s reward signal in ventral striatum will transform into habitual (stimulus-response) learning signals in

the dorsal striatum, manifested by a switch in dopamine activity from ventral to dorsal striatum. 2) Moreover, the dysfunctional hypodopaminergic state during drug withdrawal produces negative emotions by engaging activity in the extended amygdala, primarily via corticotropin-releasing factor, norepinephrine in the hypothalamic-pituitary-adrenal axis and dynorphin. The recruitment of antireward mechanisms is also linked to hyperfunctional glutamatergic transmission. 3) The preoccupation/craving stage involves a widely distributed network. The subjective effects called drug craving in humans involves activation of glutamate signaling from frontal regions to striatum, processing of conditioned reinforcement in the basolateral amygdala/orbitofrontal cortex/anterior cingulate gyrus, contextual information by the hippocampus and additional brain regions involved in disrupted inhibitory control. As full-blown addiction evolves, the frontal cortex control circuit is weakened with subsequent loss of executive control and the free will is turned into automatic behavior. Dopamine reward-driven learning activates forebrain regions and produces long-term associative memories with increased expectation sensitivity to alcohol and alcohol cues, as well as increased stress sensitivity (70, 78,79).

In summary, a wave of secondary effects that ultimately produces enduring pathology is brought about by the decreased sensitivity of the reward pathway provoked by alcohol. The system not only plays a crucial role for normal alcohol drinking behavior, but is implicated in development of both positive and negative reinforcing effects of alcohol, and thus in development of impulsivity and compulsivity as addictive behavior evolves. This sheds light on the importance of understanding the mechanisms for alcohol's interaction in the mesolimbic dopamine system and may justify development of pharmacotherapy that target alcohol's effects on mesolimbic dopamine.

The brain reward system

History

In 1953, James Olds and Peter Milner observed that rats with implanted brain electrodes would work by pressing a lever at high rates to obtain brief stimulation pulses into certain brain regions (80). The rats were not satiated and responded over 6000 times per hour,

taking only brief breaks from the lever pressing. In a classic experiment, subjects forced to make a choice preferred electrical brain stimulation over food and water until eventually dying from exhaustion (81). The study dramatically illustrated the two fundamental characteristics of direct stimulation of brain reward mechanisms, the super-potency and the lack of satiation. Soon after it was shown that animals self-administered drugs of abuse intracranially with high rates of responding, implying that drugs of abuse are powerful reinforcers in the same manner as electrical stimulation (82). The findings opened up for the understanding of the physiological underpinnings of reward and motivation, and the phenomenon of brain stimulation reward has since then been demonstrated in all species, including humans (83). The brain structures involved were later anatomically mapped and referred to as 'the brain reward system'. This system is essential from an evolutionary biology perspective. It benefits the organism and contributes to survival of the species by stimulating motivation for eating, drinking, fighting and breeding (84). The reward circuitry is highly conserved among species and is strikingly similar in rat and man. Also vital for survival is the ability to predict future events and to remember where food can be obtained, and the brain reward pathway is accordingly interconnected in a larger neurocircuitry of learning (85). Advanced forms of pleasure are rewarding in the highly developed human brain, such as romantic love (86), listening to music (87) as well as attractive faces and initiation of social interaction (88). All events that activate the brain reward system are strong driving forces and primary factors that govern normal behavior both in animals and humans.

The mesolimbic dopamine system

Several neuroanatomical elements and neurotransmitters are implied in reward, with the mesolimbic dopamine system being the most sensitive to electrical self-stimulation (84, 89). Converging evidence from self-administration, pharmacological, physiological and behavioral studies point to the mesolimbic dopamine system as the core substrate for reward and positive reinforcement (90-93). The VTA is located in the ventral midbrain medial to the substantia nigra (94) and comprises dopamine neurons that project via the medial forebrain bundle to the limbic structures nAc, amygdala and hippocampus (termed the mesolimbic pathway) and to the medial prefrontal cortex (termed the mesocortical pathway) (91, 95, 96). Additional regions such as dorsal striatum and ventral pallidum are innervated by VTA dopamine neurons. The distinct projections probably differ in neurobiology and function in

relation to dopamine's role in liking, wanting, motivation and learning (97, 98). However the VTA-nAc pathway is known to be central for mediating the actual pleasure of a reward stimulus as well as for reinforcement and motivation for reward-oriented behavior (90, 99, 100). The dopamine projections from VTA are the main afferents to nAc and since accumbal dopamine release in linked to hedonic feelings by natural rewards, i.e. food, sex, exercise and social interactions, it is popularly referred to as the 'brain pleasure centre' (84, 101, 134). The nAc consists of two sub-regions with different morphology and functions, the shell and the core region. The nAc shell, as part of the extended amygdala, is considered a limbic structure and is preferentially implicated in drug reinforcement, while nAc core is a motor region which is more associated with the dorsal striatum (94). 95% of the neurons in nAc are medium spiny GABAergic outward projecting neurons and the remaining population is comprised of GABAergic interneurons and large aspiny cholinergic interneurons (102). The GABAergic neurons largely connect with the VTA, thalamus, the prefrontal cortex and the striatum. The nAc core also sends projections to substantia nigra, promoting motor activation and motivated behavior by the 'direct' or 'indirect' pathway (103).

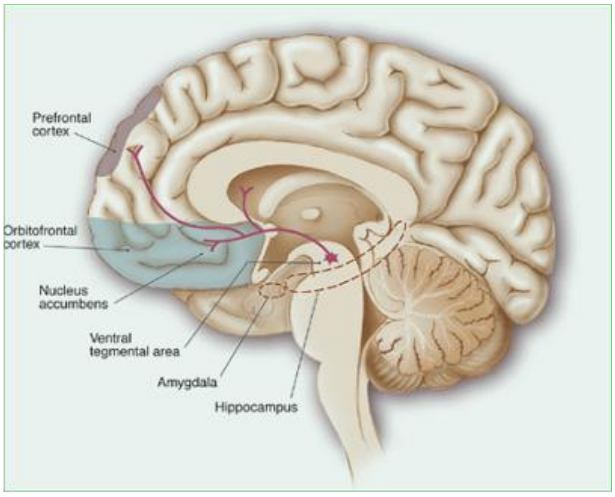

Figure 1. The mesolimbic dopamine pathway, here shown in the human brain**,** consists of dopaminergic cell bodies in the ventral tegmental area that project primarily to nucleus accumbens but also to hippocampus, amygdala and prefrontal cortex. Activation of this system promotes motivation and positive reinforcement and is associated with feelings of reward and pleasure. The mesolimbic dopamine system is regulated by various neurotransmitter systems, as described below. Holden, 2001 (1).

Neuronal connections of the VTA-nAc pathway

The VTA-nAc pathway is regulated by various neurotransmitter systems, and the GABA, glutamate serotonin and acetylcholine systems, as well as endogenous opioids and endocannabinoids, are all involved in the reinforcing effects of drugs of abuse, either by acting directly in nAc or by indirect actions in the VTA (69, 72, 78). Among these, glutamate activity in particular is shown to control the mesolimbic dopaminergic pathway and to interact with dopamine in drug reinforcement and addiction (58, 79). The excitatory input to VTA is mainly comprised of glutamatergic afferents from prefrontal cortex, bed nucleus of the stria terminalis (BNST), laterodorsal tegmental nucleus (LDTg) and lateral hypothalamus (104). Also the nAc is innervated by glutamatergic neurons; most afferents to nAc core arrive from the prefrontal cortex and thalamus, while the nAc shell receives glutamatergic innervation from amygdala and hippocampus but also from prefrontal cortex. The excitatory input to VTA and glutamate transmission in nAc, by acting on ionotropic glutamate receptors, can switch the firing mode of the dopamine neurons from single spikes to burst firing (105, 106).

VTA is further under tonic control of local GABAergic interneurons within the VTA and of descending GABAergic feed-back projections from the nAc and the ventral pallidum, the latter a connecting basal ganglia structure that receives dopaminergic inputs from the VTA and GABAergic inputs from nAc (102, 107, 108). The released GABA activates $GABA_A$ receptors on GABAergic interneurons and $GABA_A$ and $GABA_B$ receptors on dopaminergic cell bodies. The negative GABAergic feedback system to the VTA regulates the activity of the VTA neurons by providing a modulatory inhibitory tone onto the VTA dopaminergic cell bodies (109, 110). It is known that $GABA_A$ receptors in the VTA tonically inhibit dopamine release in nAc (111), while it remains more unclear whether $GABA_B$ receptors are involved in terminal dopamine release and/or somatodendritic dopamine release in the VTA (112).

In addition, cholinergic afferents that project from LDTg and pedunculopontine tegmental nucleus (PPTg) activate primarily phasic firing of the VTA dopamine neurons via nACh receptors (113, 114). Serotonergic projections from raphe nuclei also modulate the mesolimbic dopamine pathways both in the VTA and nAc (115) and the neuropeptide ghrelin enhances dopamine release in nAc, possibly via a cholinergic mechanism in the VTA (116,

117). Of high relevance for the present thesis, it has recently been demonstrated that glycinergic signaling and GlyRs modulate mesolimbic dopamine activity (118).

Function and activity of the VTA-nAc pathway

The mesolimbic dopamine system has two modes of firing pattern. Phasic transmission occurs when the VTA neurons fire in clusters, and contrasts to spontaneous and random single-spike firing. The latter random firing produces a slow (minutes) and lower elevation of extrasynaptic dopamine as compared to burst-induced elevation, that nevertheless can activate postsynaptic neurons or alternatively down-modulate spike-dependent phasic dopamine release via stimulation of presynaptic dopamine autoreceptors (119). The slow, irregular cell firing maintains base-line steady state dopamine levels and sets the overall responsiveness of the dopamine system. Tonic dopamine enables different behavioral processes, for instance maintaining alertness during learning and working memory functions (96). It has been shown that changes in spike firing can be detected by the *in vivo* microdialysis technique used in the present thesis (120, 121). A stimulus signaling reward is predominantly, but not exclusively linked to phasic dopamine evoked by burst firing of multiple neurons, producing a short (seconds) phasic increase of synaptic dopamine (122). The VTA dopamine neurons can be activated by reinforcers that are primary (the actual reward, alcohol) as well as conditioned (cues, the sight of a bottle) (123). The dopamine response provoked by alcohol can be a non-conditioned pharmacological effect and/or a conditioned effect provoked by the alcohol presentation alone (124-126). This ability is suggested to promote learning of the association between cues and rewards (127) and may be linked to a role for accumbal dopamine in craving and relapse processes (128, 129). Dopamine also serves an important role in reward-related aspects of learning. In a learning process dopamine transfers from primary rewards to reward prediction (92, 123) and can in addition display a short phasic signal, which marks the difference between actual and predicted reward, a 'prediction error signal'. Thus dopamine reward motivation and anticipation is believed to promote memory formation (130) and to connect motivation to cognitive control (131). Moreover, dopamine is shown to assign incentive salience to reward cues in stimulus-reward learning that will strongly control and motivate our behavior (132), thus implicating the role of mesolimbic dopamine in impulsivity towards rewards. Recently, midbrain dopamine neurons have been demonstrated to signal information about upcoming

rewards, suggesting that current theories of reward-seeking must be revised to include general information seeking (133). It is clear that dopamine in the reward pathway plays a prominent role and serves multiple functions.

Drugs of abuse converge on the VTA-nAc pathway

Stimulation of dopamine release in nAc is a fundamental property of addictive drugs (73, 74). Regardless of their distinct mechanisms of action there is overwhelming evidence that all classes of abused drugs converge on the VTA-nAc-pathway with common acute functional effects (69, 134, 135). Alcohol given systemically to rats as well as when injected locally in the nAc produces a dose-dependent release of dopamine in the nAc, preferentially in nAc shell (136-138). Similarly, when rats self-administer alcohol it produces a concurrent rise in dopamine levels in the nAc (139, 140), whereas withdrawal from alcohol decreases dopamine release in the nAc (77, 141).

By using modern brain imaging techniques it is also verified in the human brain that alcohol and other drugs of abuse promote dopamine release in nAc (142) and that long-term drug use is linked to decreased dopamine function (143), evidenced as a reduction in D_2 dopamine receptors and reduction of methylphenidate-induced dopamine elevations. Further it is shown that the larger and faster the dopamine release, the stronger the feeling of 'high' or 'rush' reported by drug abusers as well as non-drug abusers (144, 145). An equivalent increase in dopamine was experienced as reinforcing when injected intravenously (146) but not when administered orally in a slow-release formula (147). Further, the degree of dopamine release induced by the respective addictive substances varies considerably. Amphetamine and cocaine, which increase extracellular dopamine by displacing it from presynaptic sites and/or by blocking dopamine reuptake, typically provoke a 300-800% dopamine increment and the dopamine response is obligatory for promoting reinforcement by the above central stimulants. In contrast, dopamine-independent processes also may contribute significantly to the reinforcing effects of the opiates, ethanol and cannabinoids (148). For instance, in spite of extensive evidence favouring mesolimbic dopamine in drug reinforcement and reward, ethanol and also opioid self-administrations are unaffected by selective destruction of the mesolimbic dopamine system (149, 150). Although a matter of

debate, neuronal activity in nAc is truly central for reward regardless of whether provoked by a dopamine-dependent and/or non-dependent mechanism.

How ethanol produces dopamine release in the nAc is also a matter of debate. Both direct and indirect activation has been proposed. Brodie and co-workers have shown that isolated dopamine neurons can be stimulated by ethanol in vitro and have thus suggested a direct mode of action on dopamine neurons in the VTA (151, 152). Others have proposed that it is not ethanol per se but rather the metabolite acetaldehyde that activates VTA dopamine neurons (153, 154). However, perhaps the most prevailing theory is that ethanol indirectly, via endorphin release in the VTA, stimulates inhibitory opioid receptors located on GABAergic interneurons in the VTA and thereby disinhibits dopamine neurons, but that some local dopamine releasing effect in the nAc may also be involved (69, 155) and that the released dopamine in the nAc via feed-back mechanisms may modulate the response (109). The present research group has proposed yet another model which is described further below (see 'A loop hypothesis' for alcohol's access to the mesolimbic dopamine system).

Ligand-gated ion channel receptors - alcohol's primary targets

Despite the fact that a large body of evidence supports the role of mesolimbic dopamine in the positive and negative reinforcing effects of alcohol, the exact molecular and cellular mechanisms underlying alcohol's interference with this system are not clear. The difficult and "rich" pharmacology of alcohol and the small effect sizes provoked by this low potency drug offer an extra challenge to the neuropharmacologist searching for direct targets of alcohol's actions. However it is now established that ethanol exerts selective effects at ligand-gated ion-channel receptors (156-158). These ion channel receptors are composed of five protein subunits forming a pentameric arrangement around a central pore and a wealth of functional diversity can arise from the receptor heterogeneity provided by the different subunit types. The receptors are sensitive to pharmacologically relevant concentrations of ethanol (10-100 mM), and even as low a concentration as 1 mM may produce functional alterations of these receptors (159).

Ligand -gated ion-channel receptors consist of the cation-selective excitatory nACh, 5-HT_3 (serotonin) and NMDA receptors and the anion selective inhibitory $GABA_A$ receptor and glycine receptor (GlyR). Alcohol can directly interfere with all these receptors, and, in

addition, alcohol can interfere with voltage-gated and G protein-coupled Ca^{2+}-channels (160). Alcohol can both activate and inhibit nACh receptor activity, but pharmacological antagonism of nACh receptors prevents ethanol-induced dopamine release in nAc (162-165). The 5-HT_3 receptor activity is also potentiated by alcohol and blockade of this receptor also prevents ethanol-induced dopamine release in nAc (161, 166, 167). Alcohol both directly and indirectly (via GABA release) potentiates $GABA_A$ receptor activity, which accounts for at least parts of alcohol's sedative, anxiolytic and psychotropic effects (101, 157, 168, 169). Extrasynapic $GABA_A$ receptors have been shown to be especially sensitive to low ethanol concentrations (170). Alcohol is known to inhibit NMDA receptor function (159), and this action is implied in stimulant as well as intoxicating effects of alcohol (168). Yet there are apparent mixed and concentration-dependent effects of ethanol on glutamate release in nAc (58, 171), and it is not clear how effects of alcohol on glutamate are involved in ethanol's effects on mesolimbic dopamine (160). A cascade of secondary, long-term effects follow the direct action of alcohol on these receptors. The specific contribution of each receptor in alcohol's multiple actions is not fully characterized but the intriguing puzzle of understanding how the brain perceives alcohol is in full swing. To this end, the present thesis deals with the interaction between alcohol and glycine/GlyRs in alcohol's dopamine-stimulating, reinforcing effects and in alcohol drinking behavior.

The neurotransmitter glycine

Glycine, the smallest of the 20 amino acids, is a common precursor to proteins and a biosynthetic intermediate that fulfils important physiological functions in the body (172). Glycine's role as a neurotransmitter in the spinal cord was discovered in 1965, a decade after the discovery of GABA (173, 174). Today the functions of glycine in the spinal cord and brain stem are quite well characterized (175, 176). Whereas research on glycine as a neurotransmitter has lagged behind that of GABA, glycinergic signaling has lately received interest in fields of research such as schizophrenia (177, 178), neuropathic pain (179) and alcohol addiction (118, 180). GlyRs are emerging as pharmacological targets, yet to date, no glycinergic drug is available for clinical use, but several candidates and especially GlyT-1 inhibitors are under investigation. With the discovery of glycine as a neurotransmitter, the

presence of a specific receptor sensitive to glycine was revealed, and it was demonstrated that this glycine-receptor association was inhibited by the naturally occurring alkaloid strychnine (181). Strychnine is still held as the pharmacological diagnostic indicator for GlyR involvement, although at high concentrations it also inhibits the action of GABA (182). Recently it was shown that caffeine also inhibits the GlyR (possibly reflecting why coffee may produce a sense of sobering up after drinking alcohol) (183). The naturally occurring cation Zn^{2+} in the CNS is an allosteric modulator that can activate (in low nM conc) or inhibit (in μM conc) the GlyR (173). As a classical neurotransmitter, glycine is released after depolarization from synaptic vesicles in the nerve terminal by calcium-dependent exocytosis and binds to GlyRs on cellular elements opposed to these terminals. Certain GlyR subtypes may also be activated by the endogenous amino acids taurine and ß-alanine, though despite some controversy glycine is regarded as the endogenous GlyR ligand (173). This is at least true for GlyRs in the spinal cord, where glycine concentrations are high and GlyRs are especially prominent. The glycinergic transmission in spinal cord regulates the coordination of reflex responses and processes pain signals by forming inhibitory synapses onto pain sensory neurons (184, 185). The GlyR is also abundant throughout the auditory system (186) and in the retina (187), involved in processing auditive and visual information, respectively. The GlyR exhibits distribution in the entire mammalian CNS, including in forebrain regions (188-190) and is suggested to have a role in modulation of cholinergic (191), GABAergic (192, 193) and dopaminergic functions (194). It has been shown that dopamine release in the dorsal striatum is activated by application of glycine and blocked by strychnine application (195) and that glycine enhances the firing of VTA dopaminergic cells (196). Overall, glycinergic signaling is scantily explored in the forebrain and midbrain and there is clearly a need to increase the understanding of the role of glycine under normal and pathological conditions (175).

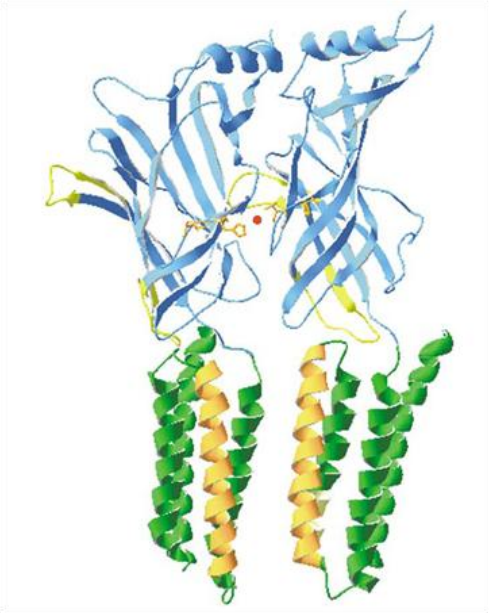

Figure 2. The glycine receptor is a prominent inhibitory receptor in the brain stem, spinal cord but also throughout the CNS including the forebrain. The receptor is a pentameric chloride-conducting channel composed of α1-α4 and ß subunits arranged to form a rosette with a central ion-conducting pore, either as heteromers or α-homomers. Glycine can facilitate phasic activation of postsynaptic receptors or facilitate tonic activity of extrasynaptic glycine receptors due to slow paracrine glycine release. Used with permission from Bowery and Smart , 2006 (173).

The glycine receptor

The GlyR, displayed in Figure 2, is a chloride channel composed of membrane-spanning subunits, and five functional GlyR subunits are known, α1-α4 and ß (185). These form heteromeric pentamers with a stoichiometry of 3α:2ß or 2α: 3ß or may exist as α-homomers (197). Binding of glycine to the glycine recognition site at α-subunits produces a conformational change, which under normal conditions causes an inhibitory postsynaptic potential by influx of chloride ions. The hyperpolarization decreases the probability that the postsynaptic neuron will fire an action potential. The level of synaptic glycine is returned to non-stimulating concentrations by specific glycine transporters which remove glycine from the synaptic cleft (see glycine transporter section below) (198). The cycle is completed when synaptic vesicles in the nerve terminals are reloaded with glycine from the cytoplasm through the action of the vesicular inhibitory amino-acid transporter (VIAAT) or the vesicular GABA transporter (VGAT) (199). The VGAT can store GABA and glycine in the same vesicle, allowing co-release of these neurotransmitters (200). GlyRs can also be co-localized with GABA receptors on GABAergic terminals, indicating the use of GABA and glycine by the same presynaptic terminal (175, 176). Contrary to the GABA receptor, the GlyR does not have a

counterpart in the metabotropic receptor family, yet it is remarkable that GlyRs can be modulated by G-protein betagamma subunits (201).

GlyRs can facilitate fast-response, inhibitory neurotransmission by phasic activation of postsynaptic GlyRs or facilitate tonic activity of extrasynaptic GlyRs that respond to slow paracrine release of glycine (202). The subunit composition determines functionality and location of the GlyR. The ß-subunit is a determinant for synaptic GlyRs as it binds to the anchoring protein gephyrin in the synapse, whereas α-homomeric GlyRs are localized extrasynaptically (176, 203). Synaptic glycinergic neurotransmission in the adult brain seems to be mediated primarily by heteromeric 1αβ GlyRs (203). GlyRs have been found presynaptically where they modulate release of other neurotransmitters, and they are recently also detected in non-neuronal cells (204). Immunohistochemical experiments suggest the presence of α1, α2 and α3 and β subunits at synapses in the adult rat retina, whereas the α3 subunit is found to be especially involved in downstream signaling of inflammatory pain in the spinal cord (203). The GlyR composition in forebrain and midbrain regions is to date not characterized, but in relation to glycine's involvement in the pharmacology of alcohol (118), α1-containing GlyRs are the most sensitive to low concentrations of alcohol (205) and their expression in nAc correlates positively with alcohol drinking behavior (206).

The α2-homomer is the abundant isoform in embryonic and neonatal spinal neurons and a consensus view is that during the second postnatal week, a developmental switch occurs where α2 subunits are replaced by the α1 subunit (207). This is at least true for the spinal cord, whereas the α2 subunit still is the most widely expressed subunit in the forebrain, despite being down-regulated during the postnatal time (206). It is suggested that α2-homomers serve a role in interneuron differentiation in the spinal cord during neuronal development, which in turn is reflected in decreased expression after birth, and further that they are only suited for non-synaptic paracrine-like release due to their slow kinetics (208). Moreover when the K^+/Cl^- co-transporter becomes functional after birth, it induces a decrease in intracellular Cl^- concentration in the mature neuron (209). The shift in the Cl^- equilibrium potential to more negative values converts the action of the GlyR from excitatory to inhibitory (210). The developmental switch in GlyR composition from α2 to α1 coincides with the switch in polarity but the implication of this co-event is not clear.

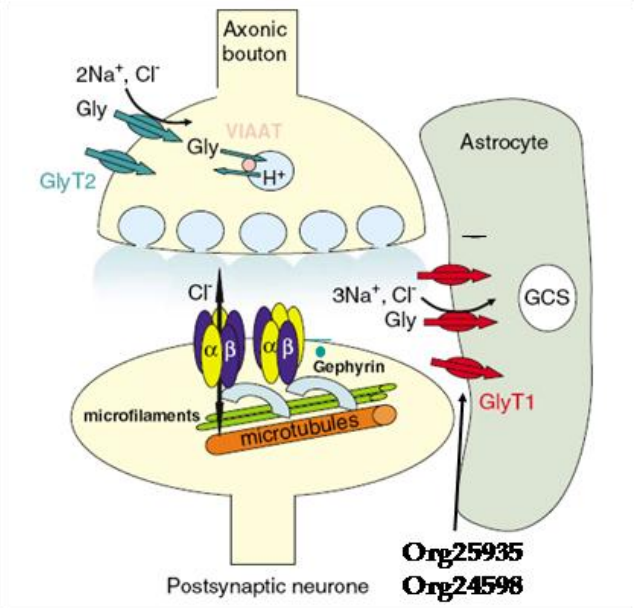

Figure 3. Schematic representation of a glycinergic synapse. Synaptic glycine receptors have a 3α:2β or 2α:3β stoichiometry and are anchored by the β-subunit to gephyrin on microtubules. Glycine is stored in vesicles by the vesicular inhibitory amino-acid transporter (VIAAT) or by the vesicular GABA transporter (VGAT). After release, neuronal and glial glycine transporters, GlyT-2 and GlyT-1, respectively, lower synaptic glycine levels by reuptake of glycine and thus terminate the glycine signal. Used with permission from Bowery and Smart , 2006 (173). NB: GlyT-2 uses $3Na^+$:1 Cl^-, whereas GlyT-1 uses $2Na^+$:1 Cl^-, i.e. opposite to what the figure depicts.

Glycine and the NMDA receptor

In addition to its role as an inhibitory neurotransmitter, glycine is engaged in excitatory neurotransmission by serving as a co-agonist of glutamate required for the activation of glutamatergic NMDA receptors (211, 212). NMDA receptors are ionotropic glutamate receptors conducting Na^+, K^- and Ca^{2+}, localized with other ionotropic and metabotropic glutamate receptors at glutamatergic synapses, or localized extrasynaptically (213). They have a binding site for glutamate/aspartate/NMDA and a second site that binds glycine or *D*-serine and occupancy of both sites are required for ion channel activation. The glycine binding site is often referred to as the strychnine-insensitive GlyB site. As glycine affinity on the NMDA receptor is higher (in the low μM range) than the glycine concentration in the synaptic cleft, the question whether the GlyB site is tonically saturated in vivo has been debated. However it has been demonstrated that NMDA receptor function is enhanced by an elevation of the surrounding glycine level (214) and thus that the receptor can be sensitive to manipulating extracellular glycine levels (178). Glutamatergic terminals do not contain vesicular transporters for glycine and do not release glycine themselves, yet recently the presence of vesicular glutamate transporters and VGAT in glutamatergic terminals in the dentate gyrus was identified (215), suggesting co-release of glutamate, GABA and, possibly

glycine. The proposed mechanism to provide glycine to glutamatergic synapses is by spillover from glycine released from nearby glycinergic terminals and/or by the reverse operation of glycine transporters (216, 217). The spillover theory is supportive of glycinergic and glutamatergic cross-talk and interestingly, it was recently shown that glutamate at higher concentrations than under normal physiological conditions is a positive allosteric modulator on the GlyR (218). A reciprocal allosteric enhancement of each other's receptor function, the potentiation of the NMDA receptors by glycine and the potentiation of the GlyR by glutamate, proposes a new model of functional cross-talk between two classical fast transmitters. This may represent an efficient mode of homeostatic regulation of neuronal excitability, at least under pathological conditions.

Glycine transporter proteins

Post-synaptic actions of glycine are terminated by specialized transporters that regulate the transmembrane gradient of glycine (219). The glycine transporters (GlyTs) belong to the SLC6A family of high-affinity Na^+/Cl^- dependent transporters comprised of the GABA, serotonin, dopamine, norepinephrine and the proline transporter in addition to some orphan transporters (220). Two mammalian glycine transporters are identified, termed GlyT-1 and GlyT-2, and these are pharmacologically discriminated since GlyT-1 is inhibited by sarcosine (*N*-methyl-glycine) and GlyT-2 is not (221). GlyT-1 and GlyT-2 differ in cellular distribution and are believed to have complimentary functions in neurotransmission. GlyT-2 is located presynaptically at glycinergic neurons and is most abundantly expressed in caudal CNS regions; the spinal cord, brain stem and cerebellum (222-224). GlyT-2 requires the binding of three sodium ions to transport one molecule of glycine while GlyT-1 requires only two. The steeper electrochemical glycine gradient maintained by GlyT-2 allows a stronger accumulation of glycine and thus maintains lower extracellular glycine levels, which may be related to GlyT-2's main function of replenishing the presynaptic glycine pool in order to refill the vesicles. However as inhibition of GlyT-2 also increases extracellular glycine levels the division of labor in not clear-cut (225).

GlyT-1 is widely distributed in the CNS including in forebrain regions such as nAc and at least six GlyT-1 subtypes exist, GlyT-1 a,b,c,d,e and f (224, 226). GlyT-1 is primarily expressed by astrocytes in the near vicinity of both GlyR and NMDA receptors, as glycinergic and

glutamatergic synapses are richly surrounded by GlyT-1 immunoreactive astrocytes (198). GlyT-1 terminates the post-synaptic response by lowering the glycine concentrations at inhibitory GlyRs and by preventing saturation of the glycine-binding site at excitatory NMDA receptors (198). Moreover, there is evidence for a neuronal GlyT-1 particularly located on pre-synaptic nerve endings of glutamatergic neurons (227, 228). This suggests that neuronal GlyT-1 regulates the binding of glycine to NMDA receptors, whereas astrocytic GlyT-1 serves a role in both glycinergic and glutamatergic neurotransmission. As the principal regulator of synaptic glycine levels, GlyT-1 is suggested to maintain physiologically correct extracellular glycine levels and the lower thermodynamic coupling of GlyT-1 enables it to operate in reverse direction (217). By reuptake it can either inactivate synaptically released glycine, or it may release glycine when cells are physiologically or pathologically depolarized. Potent pharmacological tools targeting GlyT-1 have been developed, yet GlyT-1 inhibitors with selectivity for various isoforms have not been identified (229). In behavioral terms, pharmacological GlyT-1 blockade has demonstrated anti-allodynic (179), procognitive and antipsychotic effects (178, 230), whereas the present thesis elucidates the tentative anti-alcohol properties of GlyT-1 blockade.

'A loop hypothesis' for alcohol's access to the mesolimbic dopamine system

The present research group has formulated a loop theory to explain how the mesolimbic dopamine system perceives alcohol. Both data and opinion are in agreement with suggestions that alcohol also after systemic administration may promote dopamine release via an action in nAc (69, 109, 155). Yet, the actual proof for this being the case, as well as the mechanisms for how ethanol produces this effect and that secondary events are involved in the VTA have been provided by the present research group:

The localization to the nAc of ethanol's primary interaction point in a chain of events eventually leading to dopamine release was based on studies of the involvement of nACh receptors in ethanol-induced dopamine release. Thus, a series of investigations from this group, reviewed in Söderpalm *et al*., 2000 (234), demonstrated that the dopamine releasing effect in the nAc after systemic ethanol is fully antagonized either by a systemic injection of the nACh receptor antagonist mecamylamine or by local application of this antagonist in the

VTA. However, and surprisingly, ethanol applied locally in the VTA does not affect accumbal dopamine levels (125, 162, 163) , whereas alcohol on site in nAc increases dopamine to the same extent as observed after systemic ethanol (162, 232-234) and, moreover, this latter effect is antagonized by mecamylamine given locally in the VTA but not in the nAc (162, 163). The research group has also demonstrated that voluntary ethanol intake/preference is markedly reduced after mecamylamine application in the VTA (139) and that the same type of nACh receptors appear to be involved also in ethanol cue-induced dopamine release and conditioned reinforcement to ethanol (125). Altogether these findings strongly indicate that nACh receptors in the VTA are involved in the dopamine activating and reinforcing effects of ethanol, but that the activation of nACh receptors is secondary to some primary action of ethanol produced in the nAc (163, 165, 235, 236).

The first candidate "primary" mechanism of ethanol in the nAc was an ethanol-induced potentiation of inhibitory $GABA_A$ receptors located on GABAergic neurons projecting to the VTA. However, local application of other positive modulators of $GABA_A$ receptors in the nAc decrease rather than increase dopamine output (237-239), and the dopamine elevating effect of accumbal ethanol is not prevented by the $GABA_A$ channel blocker picrotoxin (233), which instead prolongs the dopamine elevation produced by ethanol (236). In addition, picrotoxin by itself, similar to ethanol, increases dopamine levels in the nAc, indicating that accumbal $GABA_A$ receptors tonically reduce dopamine output.

The disfavoring of accumbal $GABA_A$ receptor involvement in alcohol's dopamine elevating effect, moved focus to another inhibitory receptor that alcohol interacts directly with - the GlyR (240). The GlyR is known to be involved in the effects of alcohol and general anaesthetics and ethanol has been demonstrated to increase the GlyR affinity for glycine (241) by binding to specific residues on the transmembrane domain and on the extracellular domain of α subunits, and also possibly via G- protein betagamma subunits (205, 242, 243). This shift of focal point was also facilitated by the clinical observation that clomethiazol (Heminevrin®) is an excellent substitution drug for alcohol during alcohol detoxification. Clomethiazol has an interesting pharmacological profile which is very similar to that of ethanol in that it is not only a positive modulator of $GABA_A$ receptors but also an NMDA antagonist, and, in addition, binds to the GlyR and potentiates inhibitory glycine currents

(244). This pushed forward the hypothesis that GlyRs in the nAc might be involved in alcohol's reinforcing and dopamine activating effects.

A series of studies using primarily *in vivo* microdialysis in rats thus investigated the role of the GlyR in nAc in modulation of the mesolimbic dopamine system (118). Here it was shown that there are functional GlyRs in the nAc that modulate accumbal dopamine release as such, and that these are involved in the positive reinforcing and dopamine-activating effects of ethanol (194, 233). This neurochemical event was next linked to ethanol preference, as bilateral glycine perfusion into nAc increased accumbal dopamine levels and decreased alcohol preference and intake (245), whereas the GlyR antagonist strychnine on site in nAc decreased accumbal dopamine levels and increased alcohol consumption (245). Accordingly, extracellular glycine appears to be involved in modulating alcohol consumption and this effect seems to be mediated via GlyRs. It was hypothesized that accumbal GlyRs provide tonic regulation of dopaminergic output via GABAergic feed-back to VTA. When activated by alcohol, GlyRs may hyperpolarize and thus inhibit the GABAergic neurons, which in turn reduces the inhibitory tone on VTA dopamine neurons and/or on incoming acetylcholinerigc neurons and ultimately allows dopamine neurons to fire in a nAChR dependent fashion (246), as Figure 4 illustrates.

The above findings imply that interference with extracellular glycine levels may offer a new pharmacotherapeutic approach for treatment of alcohol addiction. However, since synaptic glycine levels are kept low by efficient reuptake mechanisms adequate delivery of exogenous glycine to the GlyR, and especially to synaptically localized GlyRs, may be hindered. A more reliable pharmacotherapeutic approach to potentiate the time course of synaptic glycine is to target the principal regulator of synaptic glycine levels, the GlyT-1 uptake carrier for glycine. Org25935 and Org24598 are selective inhibitors of GlyT-1 that increase extracellular glycine levels by preventing the reuptake of glycine (see Figure 3). This indirect agonistic interference with GlyRs via GlyT-1 inhibition may elevate but also preserve more stable extracellular glycine levels, and in so doing possibly also protect the GlyR from further alcohol-induced activation.

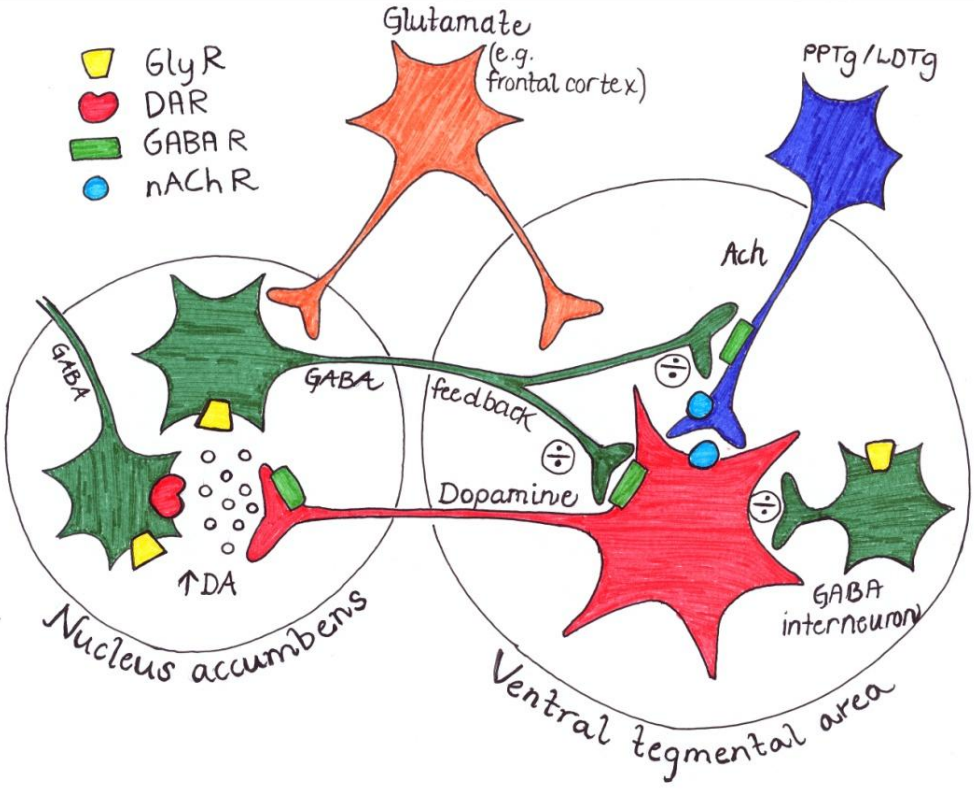

Figure 4. Schematic figure of the proposed mechanism for alcohol's access to the mesolimbic dopamine system via GlyRs. It is proposed that inhibitory GlyRs, localized on GABAergic nAc-VTA feedback projections, and/or possibly on outward projecting neurons in nAc (and/or cholinergic interneurons in nAc, not shown) and/or possibly on GABA interneurons in VTA, disinhibit GABAergic inhibitory control of the VTA-nAc dopamine neurons, which in turn allows dopamine neurons to fire. In addition to the GlyR, alcohol also accesses the mesolimbic dopamine pathway via nACh receptors and GABA receptors in the VTA and via presysnaptic GABA receptors in nAc. DAR: dopamine receptor, GlyR: Glycine receptor, GABAR: GABA receptor, nAc, nucleus accumbens, nAChR: nicotinic acetylcholine receptor; PPTg, pedunculopontine nucleus; LDTg, laterodorsal tegmental nucleus.

Aim of the thesis

The project concerns a number of preclinical studies of the anti-alcohol profile of GlyT-1 inhibitors as a possible new treatment concept for treating alcohol addiction or for reducing excessive alcohol consumption. The general aim of the thesis was to investigate how modulation of extracellular glycine levels by inhibition of GlyT-1 affects the mesolimbic dopamine system and how it affects voluntary ethanol consumption in the rat.

Specific aims

1. To examine the effect of subchronic, systemic administration of the GlyT-1 inhibitor Org25935 on alcohol intake in ethanol high- and medium-preferring rats in a limited access ethanol consumption model.

2. To examine the effect of acute systemic administration of the GlyT-1 inhibitor Org25935 on glycine and dopamine output in rat nAc, and to investigate a possible interaction between systemic administration of ethanol and Org25935 with respect to their effects on dopamine levels in nAc.

3. To investigate if Org25935 applied on site in nAc elevates accumbal dopamine levels, and, if so, to examine the possible contribution of GlyR versus NMDA receptor activation to Org25935-evoked accumbal dopamine release.

4. To probe whether Org25935 and ethanol applied locally in the nAc interact with dopamine levels in a similar manner as observed after systemic administration.

5. To examine whether the anti-alcohol effect of Org25935 is bound to this particular compound or to the mechanism of action by testing the effect of a different GlyT-1 blocker, Org24598, on voluntary ethanol consumption in rats.

6. To compare the tentative effect of Org24598 on alcohol consumption to that of acamprosate, a drug in clinical use for treatment of alcohol dependence.

7. To study the effects of acamprosate and Org24598 on dopamine output in nAc and on ethanol-induced dopamine increase in nAc, as well as on glycine, taurine and β-alanine levels in this brain region.

Animal models used for alcohol dependence

No single animal model can capture the complexity of nor manifest all symptoms of alcohol addiction. The approach is therefore to disassemble the disorder into distinct features where the animal model targets a certain well-defined aspect of alcohol drinking or addictive behavior. Rodent models have traditionally been used in research on alcohol addiction (247). Due to their shared ancestor there is a close similarity between rodent and human brain architecture and genomes. Rodents and humans use the same neurotransmitter systems and as they also share the same reward pathway, rodents consume alcohol and are susceptible to drug addiction (248). The strength of an animal model should be validated by applying the criteria of face validity, construct validity and predictive validity. The face value, the evaluation of whether the model resembles symptoms in the patient, may often be satisfying, for instance 'drinking a certain amount of alcohol per day' does not differ between rat and man. The predictive validity signifies whether the results obtained predict the response one would see in the patient, for example treatment response. The challenge in psychiatric models is often achieving content (construct) validity, which evaluates whether the model involves the same underlying etiology/physiological mechanisms in animal and patient. Various models are available that mimic the phases of alcohol drinking behavior in the transition to alcohol addiction; from acquisition of alcohol drinking to maintenance of controlled alcohol consumption (the present model) and to compulsive uncontrolled alcohol drinking (249). Relapse to alcohol are mimicked by alcohol deprivation (AD) models, which use prolonged periods of forced abstinence to promote alcohol drinking, and by reinstatement models, which use reinstatement of alcohol seeking after periods of training and extinction (250). Alcohol consumption can be monitored by using operant techniques or by home cage drinking models. Operant self-administration models use lever pressing that yields access to alcohol and can model alcohol-seeking behavior, alcohol reinforcement and craving (251, 253). For example, alcohol can be repeatedly paired with a cue or the animal can be tested on the choice it makes between a saline-paired or an alcohol-paired cue.

Traditionally, home cage drinking models have mimicked the 'liking phase' of alcohol drinking whereas a recent shift towards modeling the late stage of alcohol addiction is witnessed (22, 249, 252). Despite their clear limitations, animal models have been and

continue to be vital tools for gaining insight into neurobiological effects of alcohol, for research on alcohol addiction and for development of pharmacotherapies. Home cage drinking with voluntary alcohol intake is a simple but well-established way to study initiation, maintenance or, more recently, uncontrolled compulsive alcohol taking. Different voluntary ethanol drinking models exist that are used to study drug effects or neurobiological and genetic mechanisms underlying high ethanol preference. Most paradigms are based on the choice between a bottle of water and one or more bottles of ethanol solutions in different concentrations and the models are continuously being further developed and refined. A typical problem with these models is to obtain animals with high alcohol intake and different manipulations are being used in order to increase the ethanol consumption, such as intermittent drinking schedules, alcohol vapor chamber procedures, forced consumption and taste masking with sucrose fading procedures. In addition selective breeding to generate genetically high- and low- alcohol-consuming phenotypes is used and there are at least seven pairs of rat strains with genetically high versus low ethanol preference available (248, 254). In drug development, interpretation of data using genetically modified animals may be difficult, as inbred traits are not easily translated into the human scenario. To illustrate this, the Finish AA (Alko, Alcohol) rat line displays high preference for alcohol in home cage drinking via a bottle, but does not lever press for alcohol more than out-bred Wistar rats (255).

The present studies use free-choice ethanol consumption in out-bred Wistar rats screened for their innate ethanol preference in a choice between 6% ethanol solution and water. The paradigm is thought to model maintenance of an established controlled alcohol-intake behavior (alcohol liking) and/or an established drinking habit (alcohol wanting). In both rodents and humans, initial alcohol liking differs in the population and the trait is believed to contribute to continued and escalated alcohol use, and thus to susceptibility to development of alcohol addiction (256). The model has obvious advantages in the sense that Wistar rats, just like humans, display large individual differences in ethanol preference and the uncertainty of using forced or inbred procedures is eliminated. The tradition of using a 6% ethanol solution in the present laboratory derives from a study showing that alcohol consumption is maximal at this concentration in the Wistar rat strain (257). The model has generated false positives when used for identifying new candidate drugs, which may partly

be explained by an insufficient length of the test period or by the fact that also taste sensitivity, novelty seeking, sedation and negative affect may influence voluntary alcohol intake (258). The studies in this thesis combine the alcohol drinking parameter with neurochemical experiments on interactions with the dopaminergic reinforcement system. Adding information from such experiments may contribute to increase the predictive and construct validities of the model. A recent review favored the validity of home cage drinking models by reporting a positive relationship between home cage drinking and conditioned place preference, which in turn suggests a relationship between ethanol drinking in this model and ethanol-induced reinforcement (259). Also, that both the clinically active compounds acamprosate (30) and naltrexone (28, 29) decrease voluntary ethanol drinking in this model is evidence of predictive value, as is the findings that stress hormones and subchronic nicotine instead increase ethanol intake (235).

Materials and Methods

Ethical considerations

All experiments in this thesis were approved by the local Ethics Committee for Animal Experiments in Gothenburg (diary numbers 5/04 and 337/06). All efforts were made in order to minimize the number of animals and their suffering in these studies.

Animals

The present studies used male Wistar rats purchased from BK Scanbur (Sollentuna, Sweden) or from Taconic (Denmark) and weighing 250-300 grams upon arrival. Animals were housed under controlled environmental conditions with 65% humidity and a constant room temperature of 22°C. Rats were housed with a 12-h light-dark cycle with light on at 8.00 am and light off at 8.00 pm in Paper II and III, whereas in Paper I and IV, rats were housed under reverse 12-h light-dark conditions, with light off at 8.00/9.00 am and light on and 8.00/9.00 pm. In the brain dialysis experiments (Paper II and III) rats were housed five per cage until probe implantation surgery and from then on separately for 48 hours until the dialysis experiment. Rats were housed in individual cages in the ethanol consumption studies (Paper

I and IV). Rats had continuous access to tap water and standard rat chow, from BK Feed, Sweden in Paper I and from Harlan Teklad Europe, UK in Papers II-IV. The home cages including the bedding material made of wood-cuttings were changed once a week.

Drugs and chemicals

Isoflurane (Baxter, Sweden) applied as 3.5-4.0% in air was used as anaesthetic. Orudis Gel, a 2.5% ketoprofel gel (Apoteket AB, Sweden) was applied as analgesic. Ringer solution: 140 mM NaCl, 1.2 mM $CaCl_2$, 3.0 mM KCl and 1.0 mM $MgCl_2$ was used as artificial CSF and perfused via the probe into the nAc during *in vivo* microdialysis. 95% ethanol (AB Svensk sprit, Paper I, Kamet AB, Haninge Sweden, Paper II-IV) was dissolved (2, 4 and 6% v/v) in regular tap water and presented in 250 ml plastic bottles for oral consumption. Ethanol was dissolved in Ringer solution at a concentration of 300 mM for local application in nAc by reversed microdialysis. For systemic administration, ethanol was dissolved in 0.9% NaCl at a concentration of 15% w/v and injected i.p. in the dose of 2.5 $g.kg^{-1}$. An ethanol perfusate concentration of 300 mM is shown to produce a dopamine elevation of ~30-40% in nAc, equivalent to that observed after 2.5 $g.kg^{-1}$ i.p. (138, 162, 236). In this laboratory, this dose is required for producing an adequate and detectable dopamine elevation and is not meant to mimic ethanol amounts in voluntary ethanol intake. The selective GlyT-1 inhibitors Org25935 and Org24598 were kindly provided by Organon Labs. Ltd , now Merck Sharp & Dohme, Newhouse, Lanarkshire, UK. Org25935 was dissolved in 0.9% NaCl and administered i.p. as 6 $mg.kg^{-1}$ in a volume of 2.0 $ml.kg^{-1}$ in Paper I and II and perfused via the dialysis probe at a concentration of 100µM in Ringer solution in Paper III. In Paper IV, Org24598 was dissolved in phosphate buffered saline and administered i.p. as 3, 6 and 12 $mg.kg^{-1}$ in a volume of 4 $ml.kg^{-1}$. Acamprosate, kindly provided by Merck (Lyon, France), was dissolved in 0.9% NaCl and administered as 50, 100 and 200 $mg.kg^{-1}$ in a volume of 4 $ml.kg^{-1}$. The GlyR antagonist strychnine was purchased from Sigma Aldrich (Stockholm, Sweden) and dissolved in Ringer solution for perfusion into nAc by reversed microdialysis in concentrations of 10 and 20 µM in Paper III. The NMDA receptor glycine site antagonist L-701.324 was purchased from Sigma Aldrich (Stockholm, Sweden) and dissolved in 10 % Polysorbate80 in 0.9% NaCl and administered as 5 $mg.kg^{-1}$ in a volume of 2 $ml.kg^{-1}$ in Paper II. The dose of L-701.324, 5 $mg.kg^{-1}$ i.p, was chosen based on studies showing antagonistic effects on relapse-like drinking in rats (260).

Voluntary ethanol consumption

Screening procedure

After one week of acclimatization to the new environment, rats were gradually familiarized to ethanol by giving them access to ethanol solution in an ascending concentration (2-4-6% v/v) over two weeks. They were then housed individually in clear plastic cages (40 x 24 x 15 cm) with two 250 ml plastic bottles with ball-valve nipples, one containing 6% ethanol solution and the other tap water. Two indexes for ethanol consumption were used, ethanol preference and ethanol intake. Ethanol preference was calculated as the amount of ethanol consumed (grams) in percent of total fluid intake (ml) consumed per drinking session whereas absolute ethanol intake was defined as ethanol (grams) consumed per kg rat body weight per drinking session. During screening, water and ethanol consumption was measured two times a week over a five-week period. Next, based on the rat's individual parameters for ethanol consumption during the baseline period, animals were characterized as ethanol high-preferring (preference quotient more than 60%), medium-preferring (preference quotient between 30-60%) and low-preferring (preference quotient less than 30%). Ethanol medium- and high-preferring rats were used in Paper I and IV. The subjects were randomly assigned to either control or treatment group, balanced for ethanol intake and preference quotients.

Limited access paradigm

Paper I

Since extracellular glycine levels peak approx. 50 minutes after i.p. administration of Org25935 (filed data, Organon Labs Ltd, now MSD), rats were presented with the bottles 40-60 min after drug injection. Based on information that Org25935 dosed as 6 $mg.kg^{-1}$ increased striatal glycine levels by 50-80% lasting about 2.5 hours (261), a 2.5 hour limited access (LA) drinking period was used to assure adequate blood drug concentrations during the drinking session. To achieve a stable fluid intake before the testing, rats were placed on the LA schedule where they were limited to drink ethanol (6% v/v) and water for 2.5 hours per day for one week prior to drug challenge; baseline LA drinking. All drinking sessions started when the light was turned off. High-and medium preferring Wistar rats selected from different batches were tested separately. First, high-preferring animals were treated with

Org25935 6 mg.kg^{-1} i.p. or vehicle for 12 days before being exposed to 14 days of AD when they had 24-h access to water but no ethanol. After the AD period rats were again placed on the LA drinking schedule with drug treatment for 16 days. After 7 days with full dose, the dose was gradually lowered (4 days with ½ dose, 2 days with ¼ dose and 3 days with vehicle injections). Next, a new set of medium-preferring rats was selected and in addition to fluid intake also rat weight and food intake were systematically monitored. These rats were challenged with daily vehicle injections also during the seven days of LA baseline drinking in order to evaluate tentative influence of stress caused by the injections. Medium-preferring rats were challenged with 12 days of 6 mg.kg^{-1} Org25935 or vehicle, followed by an AD period of 14 days. Since onset of drug effect was delayed among the high-preferring rats, rats were this time treated with Org25935/vehicle on the 4 last AD days while still allowed to drink only water. Following the AD period, the LA schedule with daily drug challenge was continued for 7 days.

Paper IV

Following the screening, ethanol high- and medium-preferring rats were placed on a LA drinking schedule also here lasting 2.5 hours. Following seven days of baseline LA drinking, the rats were treated with acamprosate (200 mg.kg^{-1}), Org 24598 (12 mg.kg^{-1}) or vehicle i.p. 40-60 min prior to the daily choice of ethanol and water for 2.5 h (9:30-12:00 am) for 12 days. Rats were then exposed to 14 days of AD during which they had free access to water but not ethanol. On the last three AD days, the rats were again injected daily with either acamprosate, Org24598 or vehicle but only allowed to drink water. After the AD period the rats continued to receive daily drug injections and were replaced on the LA schedule for 10 days. In order to examine whether the observed effect was dose-related, the acamprosate and Org24598 dosing were gradually lowered during the final 6 days (2 days with ½ dose, 2 days with ¼ dose and 2 days with vehicle. Rat weight was monitored weekly.

In vivo brain microdialysis

The technique

In vivo brain microdialysis is a popular and frequently used method in neuroscience (262, 263) which offers a unique way to investigate the extracellular environment in alert and freely moving animals while causing relatively little disturbance of the intracerebral environment. It can be used under different experimental conditions, as the sampling from a conscious animal can be correlated to behavioral and neurological states. In the present studies, effects of exogenous compounds on neurotransmitter levels, administered systemically or applied to restricted areas by reverse microdialysis, were assessed. Microdialysis measures free, unbound analyte concentration of the averaged overflow into the extrasynaptic space. A change in the extracellular level is believed to primarily reflect changes in synaptic levels provoked by synaptic release, but the method cannot detect the underlying event promoting such a change. Thus, it is assumed that an increased extracellular dopamine level in nAc primarily reflects activity of dopaminergic projections from VTA, comprising the major dopaminergic input to this brain region, however the influence of other events like changes in dopamine transporter efficacy, enzymatic degradation, autoreceptor activation and dopamine release from other neurons cannot be excluded.

A certain brain area is perfused with Ringer solution, a perfusate solution resembling the ionic composition of the extracellular fluid. In the present experiments, microdialysis was performed in awake, freely moving Wistar rats in order to measure extracellular concentrations of dopamine, glycine, taurine and β-alanine in nAc. Microdialysis has a relatively low temporal (determined by length of sampling interval) as well as spatial resolution (probe size). In the present studies, dialysates were sampled at 20 min intervals and rapid alternations in transmitter levels were not estimated. Also, the invasive nature of this technique poses some problems as probe insertion is known to cause scar tissue and microglial activation. Taking these acute effects of implantation traumas, as well as inflammatory responses and necrosis that may occur at a later time point into consideration, an optimal time window to perform dialysis is usually 24-48 hours post surgery (264, 265).

The method requires insertion of a dialysis probe, a catheter with a semipermeable hollow fiber membrane at its tip designed to mimic blood capillary. The sampling is based on passive diffusion and direction of analyte flow is determined by the concentration gradient. Equilibrium is not established as the probe is constantly perfused, which leads to dialysate concentrations lower than *in vivo* concentrations (recovery) (266). Recovery is mostly dependent on flow rate and molecular weight. The typical *in vitro* recovery of dopamine is found to be 5-10% in our laboratory (unpublished data), whereas recoveries of glycine, taurine and β-alanine have not been established. The data presented here are not corrected for recovery. A typical problem when performing reversed microdialysis is to estimate exact concentration of the perfused drug reaching the extracellular space outside the probe. As recovery of dopamine *in vitro* is 5-10% of the actual concentration outside the probe, we estimate the *in vivo* recovery/excovery to be ~1-5%, depending on molecular size, charge and area of the active space of the probe. We therefore estimate that perfusion of 100 µM Org25935 equals a concentration of 1-5 µM in the extracellular space. The concentration of Org25935 used was selected based on data obtained from Organon Labs. The perfusate concentrations of the GlyR antagonist strychnine (10 and 20 µM) were selected based on results obtained in previous studies applying strychnine by reverse dialysis (37, 194, 267).

The microdialysis probe

The microdialysis experiments were performed using probes custom made in the laboratory, a developed version of the former I-probe (268). The inlet and the outlet of the probes were made of 20 gauge PE plastic tubing (VWR, Sweden) and a glass rod attached by Super Epoxy glue between the inlet and outlet tubings was used as a holder. The dialysis membrane, prepared from a copolymer of polyacrylonitrile and sodium methallyl sulfonate with an o.d./i.d. of 310/220 µm (Hospal-Gambro, Sweden) was sealed with a glue plug and thread on the fused silica. The fused silica, extending 10 mm from the tip of the probe was covered with silicon glue (CAF 3; Rhodorsil Silicones, Saint-Fons Cedex, France) so that the length of the exposed tip, the active space, was 2.0 mm. The probes were rinsed before being implanted, by perfusion with 40 µl of ethanol (70%) followed by 120 µl Ringer solution (2.5 µl/min). The inlet and outlet tubes were sealed with heating and were stored at +4°C for a maximum of four days before being used.

Surgeries

Rats were anaesthetized by isoflurane (Baxter, 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 for placement of two anchoring screws and the dialysis probe. The dialysis probe with 2 mm active space was lowered into the nAc monolaterally; A/P: +1.85, M/L: -1.4, relative to bregma, and D/V: -7.8 mm relative to dura (94). These coordinates correspond to a core-shell borderline region, suggesting that sampling was done in both core and shell of the nAc. The dialysis probe and screws were fixed to the scull with Harward cement (DAB Dental AB; Gothenburg, Sweden) and a 2.5% ketoprofen gel was applied on the injured tissue to relieve inflammation and pain. Animals were injected with 2 ml saline post surgery to prevent dehydration and allowed to recover for 48 hours prior to the experiment.

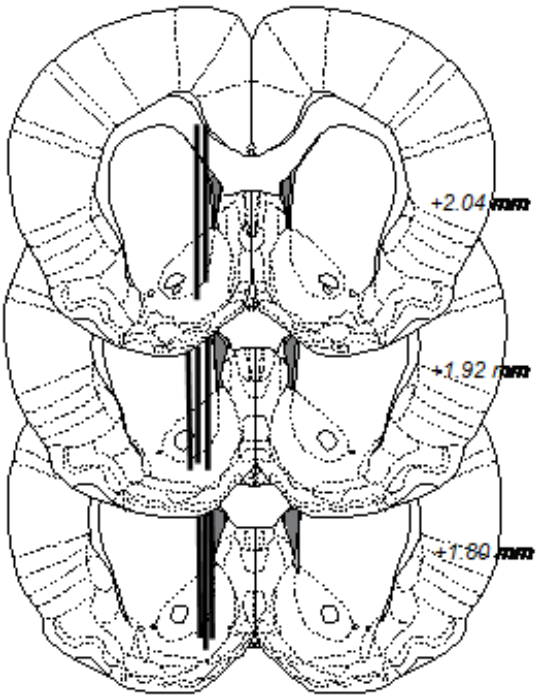

Figure 5. Coronal brain sections displaying representative accepted probe placements in nAc. The dialysis probe with 2 mm active space was lowered into nAc monolaterally; A/P: +1.85, M/L :-1.4, relative to bregma, and D/V: -7.8 relative to dura, corresponding to sampling from the nAc core/shell borderline region. The number in each section indicates millimeters anterior from bregma. The positions of the probes were verified by visual examination of brain slices cut by a vibroslicer. Brains displaying haemorrhage or incorrect probe placement were excluded from the study. Adapted from Paxinos and Watson, 2007 (94).

Neurochemical assay - dopamine

A high-pressure liquid chromatography system (HPLC) with electrochemical detection (ED) was used for the on-line separation and detection of dopamine content of the dialysate samples. This HPLC system consisted of a pump (Dionex P580, Kovalent AB, Sweden), a stainless steel column (2 x 150 mm) packed with Nucleosil, 5u SA 100A (Phenomenex Skandinaviska Genetec, Sweden) used at 32°C and an electrochemical detector (Decade, Antec Leyden, Kovalent AB, Sweden) operated at 0.40 V versus Hy-REF. A mobile phase (Citric acid; 58 mM, NaOH; 135 mM, Na_2-EDTA; 0.107 mM and 20% methanol) was used at a flow rate of 0.3 ml/min. The time of analysis was, injection to injection, 4.7-6 minutes. An external standard containing 3.25 fmol/µl of dopamine was used to identify the dopamine peak. For all experiments, microdialysis was performed during continuous monitoring of extracellular accumbal dopamine levels. When three stable values of dopamine were obtained (±10%), the mean of these was set to 100% for each individual animal and the time point was set to 0 min, all percentages throughout the experiments were related to this.

Neurochemical assay - glycine, taurine and β-alanine

To analyze glycine, which often co-elutes with threonine, a gradient HPLC method was developed where these two amino acids were separated in addition to taurine and β-alanine. The amino acids were allowed to react for one minute with o-phthaldialdehyde (OPA)/2-mercaptoethanol in a precolumn derivatisation, using a Waters 7179 plus autosampler, after which they were separated on two series connected Onyx C_{18} columns (4.6 x 50, 4.6 x100 mm) at 37°C, and were detected by a fluorescence detector (Perkin Elmer LC 240, Ex 330 nm, Em 420 nm). The HPLC pump (Perkin Elmer series 200) utilized a gradient both in the mobile phase (50 mM sodium phospate, pH 6.10 with phosphoric acid and 12.5-50% acetonitrile, both containing 1% tetrahydrofuran) and in the flow rate (2.2-4.0 ml/min). The time of analysis, from injection to injection, was 13 minutes. External standards in three concentrations (0.1-1.0 µM) were used to identify the different amino acid peaks. All samples received an addition of sodium azide (NaN_3) in order to maintain stability of the samples. The data are presented as percent of baseline values calculated in the same manner as for dopamine (see above).

Experimental procedures of *in vivo* microdialysis

On the experimental day, the sealed inlet and outlet of the probe were cut open and connected to a microperfusion pump (U-864 Syringe Pump, AgnTho's, Sweden) via a swivel allowing the animal to move around freely. The probe was perfused with Ringer solution at a rate of 2 µl/min and dialysate samples (40 µl) were collected every 20 minutes. Before sampling begun, the rats were perfused with Ringer solution for at least one hour to obtain a balanced fluid exchange. In the studies with amino acid detection (Paper II and IV), NaN_3 was added to the dialysate samples and stored at -80ºC for later analysis. Animals were sacrificed immediately after the experiment and the brains were removed. The positions of the probes were verified by visual examination of brain slices (after using a vibroslicer, Campden Instruments). The drug administration paradigms in the respective studies were as follows:

Paper II

Ringer solution was perfused in the nAc continuously throughout the experiments. Either 6 $mg.kg^{-1}$ Org 25935 or vehicle was administered i.p. Then, in order to examine a tentative Org25935-ethanol interaction, the rats received a systemic injection of either 6 $mg.kg^{-1}$ Org 25935 or vehicle at time point 0, followed by an injection of either 2.5 $g.kg^{-1}$ethanol i.p. or vehicle 100 min later. The time points were selected in order to optimize a possible interaction, based on information that the peak glycine level can be expected at approximately 50 min when administered at this dose and that the glycine levels remain increased for approximately 2.5 hours (261).

Paper III

Org25935 (100 µM) was perfused into nAc from time point 40 min while Ringer perfusion was maintained throughout the experiment in the control group. Next, in order to study the influence of pre-treatment with either strychnine (10 and 20 µM) or L-701.324 (5 $mg.kg^{-1}$ i.p.) on the effect of Org25935, perfusion of strychnine was initiated at time point 0 min as was injection of L-701.324, while perfusion of Org25935 started 40 min later, at time point 40 min. Lastly, in order to examine the influence of Org25935 on ethanol-induced dopamine increase, Org25935 (100 µM) was perfused from time point 0 min while ethanol (300 mM)

was perfused alone or co-perfused with Org25935 from time point 100 min and throughout the experiment.

Paper IV

In all animals, Ringer solution was perfused in the nAc continuously throughout the experiment. The same treatment groups as in the drinking study received either Org24598 (12 $mg.kg^{-1}$ i.p.), acamprosate (200 $mg.kg^{-1}$ i.p.) or vehicle at time point 0 min, followed by an injection of ethanol (2.5 $g.kg^{-1}$ i.p.) 40 min later.

Statistics

A probability value (p) less than 0.05 was considered statistically significant. All values are expressed as mean ± S.E.M. Due to the biological variability in the ability of Org25935 to elicit an accumbal dopamine response, separate time courses for the Org25935 responding and non-responding subgroups were analysed in addition to the group as a whole. The criteria for being an Org25935 responder was set to >10% increase in dopamine output while the remaining animals were classified as non-responders.

The following statistical methods were used as appropriate:

- One-way analysis of variance (ANOVA) (Paper I and IV)
- Analysis of variance (ANOVA) with repeated measures (Paper I, II, III, IV) over time periods
- Fisher's protected least significant difference (PLSD) *post hoc* test, (Paper I, II, III, IV)
- Paired and unpaired *t*-tests for adequate comparisons between specific time points (Paper I, II, III, IV)

Results and discussion

Paper I

The glycine reuptake inhibitor Org25935 decreases ethanol intake and preference in male Wistar rats.

Rationale

Exogenous glycine applied by bilateral perfusion into the nAc elevated dopamine levels and reduced ethanol intake in ethanol high-preferring rats, while accumbal perfusion of the GlyR antagonist strychnine instead decreased dopamine levels and increased ethanol intake. The finding that extracellular glycine levels modulate voluntary alcohol consumption encouraged us to investigate whether inhibition of glycine reuptake mechanisms may constitute a tentative new target for reducing alcohol consumption.

Experimental design

The effect of the selective and non-competitive GlyT-1 inhibitor Org25935 on voluntary ethanol intake in ethanol high-preferring male Wistar rats (>60% ethanol preference) was tested. In a confirmative study, Org25935 was tested in a similar manner in a set of ethanol medium-preferring (30-60%) rats, where food intake was also monitored. Rats placed on a LA drinking schedule with access to the bottles 2.5 hours per day starting with the circadian dark period were treated with daily Org25935 (6 $mg.kg^{-1}$) and vehicle (saline) injections for 12 days. This was followed by a 14-day AD period and a reintroduction of the drug regimen for 7 days. Among the ethanol high-preferring rats, the drug regimen was gradually phased out over a period of 9 days.

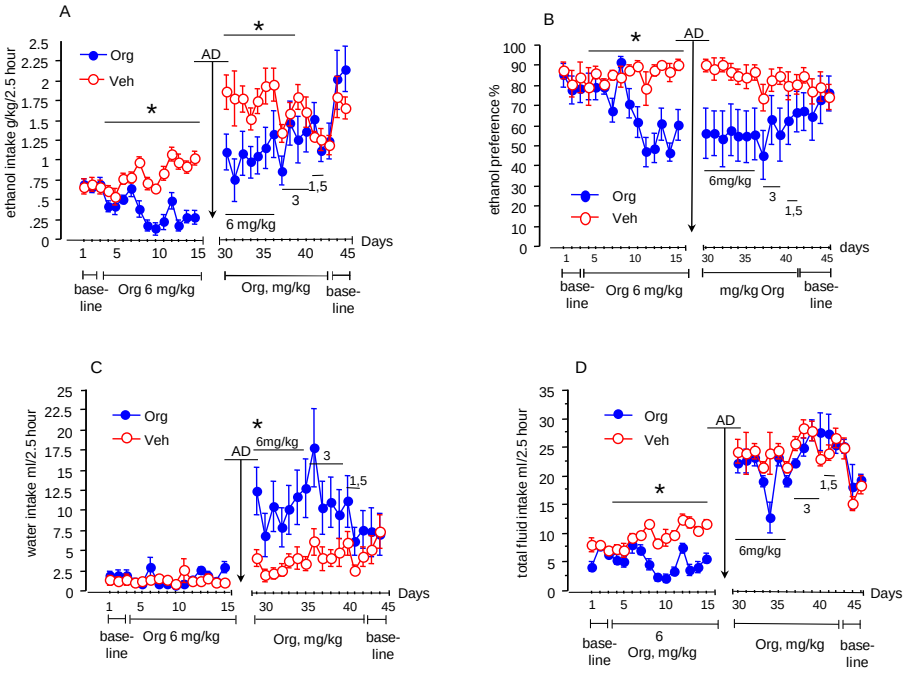

Figure 6. Org25935 treatment decreases voluntary ethanol intake and preference in ethanol high-preferring (>60%) Wistar rats, n=6-9. The graph displays the three last baseline days (d1-3), test period 1 with daily Org25935 6 mg.kg^{-1} i.p. injections for 12 days (d4-15), a 14-day alcohol-free period (d 16-29, not shown), test period 2 with reintroduction of daily treatment of Org25935 6 mg.kg^{-1} for seven days (d30-36), 3 mg.kg^{-1} for four days (d37-40), 1.5 mg.kg^{-1} for two days (d41-42) and baseline drinking for two days (d43-45). Stars indicate significant differences. Ethanol intake (A), ethanol preference (B), water intake (C) and total fluid intake (D).

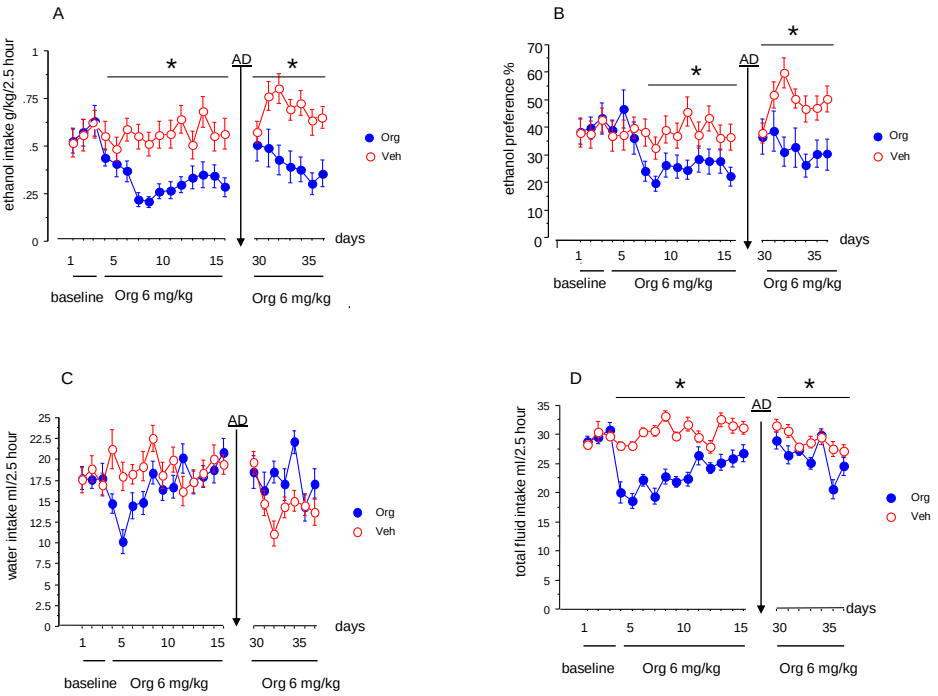

Figure 7. Org25935 treatment decreases voluntary ethanol intake and preference in ethanol medium-preferring (30-60% ethanol preference) Wistar rats, n=14-15. The graph displays the three last baseline days (d1-3), where rats received daily vehicle injection, test period 1 with daily Org25935 6 mg.kg^{-1} i.p. injections for 12 days (d4-15), a 14-day alcohol-free period (d 16-29, not shown) and test period 2 with reintroduction of daily Org25935 6 mg.kg^{-1} treatment for seven days (d30-36). Stars indicate significant differences. Ethanol intake (A), ethanol preference (B), water intake (C) and total fluid intake (D)

Findings and discussion

This was the first study to demonstrate that a GlyT-1 blocker influences alcohol drinking behavior. Daily injections of Org25935 produced a profound decrease of ethanol intake and ethanol preference, both in ethanol high- (Figure 6) and medium-preferring (Figure 7) rats compared to vehicle groups. The effect of Org25935 was fully reinstated after an AD period, which promoted increased ethanol intake in both groups. The effect on ethanol drinking

appeared dose-dependent, as the decreased ethanol intake was reversed by dose reduction and cessation. That the rats fully returned to their baseline drinking level after drug withdrawal (d 44-45, Figure 6) indicates that the effect is not due to chance fluctuations of ethanol intake. Onset of effect was delayed by four to five days among the high-preferring rats. Therefore, medium-preferring rats were treated with Org25935 also on the four last days of the AD-period prior to the second test period, which explains the earlier onset of effect in the second test-period among these rats. The long-lasting effect on ethanol intake contrasts to effects of other substances on voluntary ethanol intake in rats, such as the serotonergic drugs buspirone and citalopram (269, 270) and the functional NMDA receptor antagonist acamprosate (30), that display prompt onsets of action but not as durable effects on ethanol consumption. Ethanol intake temporarily increased after the AD period, in line with previous observations (249, 271, 272). This was true both for vehicle- and Org25935-treated animals, even though ethanol intake remained lower in the Org25935 group. However in control animals, the observed increase in total fluid intake was mainly accounted for by increased ethanol intake, possibly in order to experience its pharmacological effect. In contrast, ethanol and water intake contributed equally to the increased drinking drive in the Org25935 group, possibly reflecting a decreased or modulated experience of ethanol's reinforcing value.

In ethanol high-preferring rats, treatment with Org25935 did not significantly reduce the body weight, whereas body weight was lower among drug-treated medium-preferring rats compared to controls (see Figure 4., Paper I). Also food intake was transiently reduced among these rats compared to controls, observed during the first but not the second test period (see Figure 3., Paper I). This finding may imply that the drug influenced appetite or eating behavior, yet rat weight was not significantly reduced over time, but rather maintained at the same level in the Org25935 group. This may alternatively reflect a counteraction of over-eating in laboratory rats with unlimited food supply, but since rats subsequently gained weight during and after the AD period, this may be an initial effect only. Water intake remained stable or increased throughout the two test periods in both sets of rats, arguing against the presence of toxicological effects. From daily observations, rats appeared healthy and behaviorally unaffected by the dose of 6 $mg.kg^{-1}$ Org25935. This dose was selected based on previous preclinical testing at Organon Labs Ltd, now MSD. The initial

decreased water intake in medium-preferring rats may reflect a sedative response, but tolerance developed to this effect and water intake was comparable to control rats later on during treatment. A decreased water intake was not observed among high-preferring rats; instead an increased water intake was evident after the alcohol-free period. No obvious biological variance in the drinking response to Org25935 was observed and the treatment response to Org25935 was high (86%), as displayed in Figure 8.

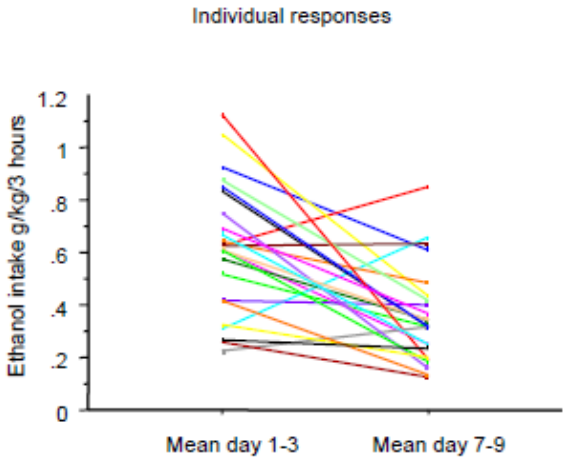

Figure 8. A consistent effect of Org25935 on ethanol intake was observed. Plot of individual responses to Org25935 on ethanol intake. Only 3 of 21 rats examined increased their ethanol intake when comparing the mean of day 1-3 (baseline drinking) versus mean of day 7-9, representing a time point in the middle of the first test period.

In conclusion the selective GlyT-1 inhibitor Org25935 produced a robust, long-lasting and reversible decrease in ethanol intake and preference in a two-bottle free choice model in the male Wistar rat. There was no tolerance development to the anti-alcohol intake effect of Org25935 in either test group and the drug effect was reinstated after alcohol withdrawal. This paper implies that GlyT-1 blockers may have a therapeutic role in reducing alcohol consumption.

Paper II

The glycine reuptake inhibitor Org25935 interacts with basal and ethanol-induced dopamine release in rat nAc

Rationale

Exogenous glycine as well as GlyT-1 inhibition has demonstrated alcohol intake reducing properties in rat, and glycine has been shown to modulate basal and ethanol-evoked release of dopamine in nAc. This evidence leads to the present study, which aimed to 1) test the effect of Org25935 on extracellular glycine in nAc and to 2) test whether Org25935 interacted with basal and ethanol-induced dopamine release in a similar manner as glycine.

Experimental design

The effects of Org25935 (6 mg.kg^{-1} i.p.) and/or ethanol (2.5 mg.kg^{-1} i.p) on dopamine efflux in nAc and the effect of Org25935 (6 mg.kg^{-1} i.p.) on glycine, taurine and β-alanine efflux in nAc were examined by means of *in vivo* microdialysis coupled to HPLC in freely moving male Wistar rats.

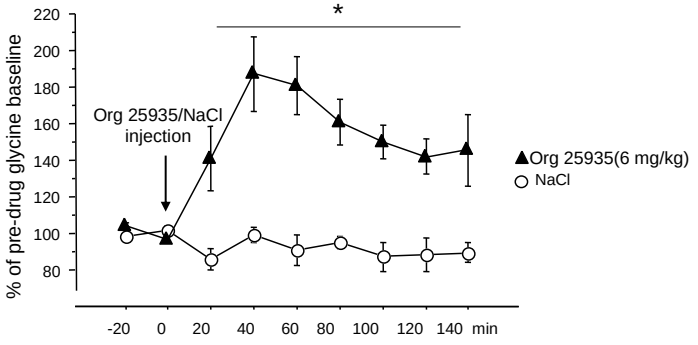

Figure 9. Systemic i.p. administration of Org25935 raises glycine levels in nAc.
The effect of acute 6 mg.kg^{-1} Org25935 i.p. administration on extracellular nAc glycine levels, as measured by *in vivo* microdialysis. The maximal increase in glycine levels was 87% observed 40 minutes after time of injection.

Findings and discussion

It was demonstrated that systemic administration of the GlyT-1 blocker Org25935 at the dose of 6 $mg.kg^{-1}$ i.p consistently increases glycine levels in nAc by maximally 87% (see Figure 9). This is similar to reported effects of Org25935 in the frontal cortex, hippocampus and striatum (261). That 3, 6 and 10 $mgkg^{-1}$ i.p Org25935 dose-dependently increases striatal glycine output by 25, 80 and 130% suggests that the dose used is within a relevant dose-response range (261). GlyT-1 is distributed throughout the CNS and is primarily expressed by astrocytes in the near vicinity of glycinergic and glutamatergic synapses, where it terminates post-synaptic responses (198). Organon Labs Ltd., now MSD, has demonstrated high radioligand binding of [^{3}H]Org25935 in several brain regions including the nAc. Org25935 is known to be a selective inhibitor of GlyT-1 over GlyT-2 and monoamine transporters. That Org25935 did not influence extracellular taurine or ß-alanine (both GlyR agonists) in this study (Figure 3b,c, Paper II) also underlines a selective action on the GlyT-1 protein. It is likely that the elevated glycine levels provoked by Org25935 induce the subsequent effects on dopamine output.

The study found that Org25935 increased dopamine efflux in nAc in a subpopulation of rats, 52% in one set of rats (Figure 1, Paper II) and 38% in another set, the latter displayed in Figure 10, when the criterion for being a Org25935 responder was set to a >10% increase of dopamine levels. Ethanol significantly increased dopamine levels (35%) among vehicle-receiving rats and slightly also in Org25935 non-responders (19%). Ethanol failed to further increase the dopamine levels in rats with a dopamine-response to Org25935. The consistent glycine-response to Org25935 indicates that the variability in dopamine response lies in the response to glycine and not in the function or expression of the GlyT-1 protein. This biological variation is similar to that previously observed, when glycine applied in nAc increased dopamine levels in some but not all rats and counteracted ethanol-induced dopamine release after local ethanol in all rats, as well as in some rats after systemic ethanol (233). In this study, strychnine conversely suppressed accumbal dopamine levels by itself and prevented ethanol-evoked dopamine release (233).

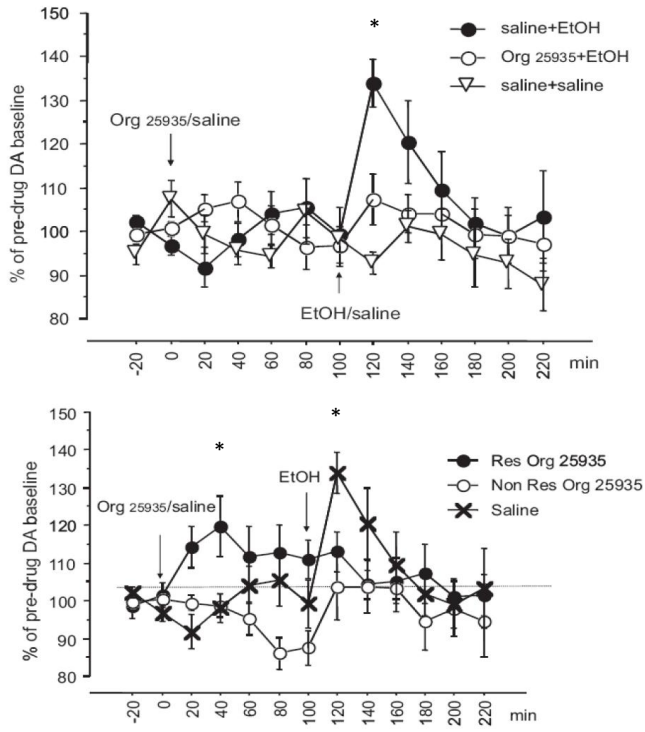

Figure 10. Upper graph displays the effect of acute ethanol, 2.5g/kg, i.p, on extracellular nAc dopamine levels in rats injected with Org25935 (6 mg/kg, i.p.) or vehicle, n=8-13. Drug and ethanol were administered at time point 0 and 100, respectively. Basal dopamine concentrations for saline-EtOH, Org 25935-EtOH groups and saline-saline group were 2.04nM±0.22, 2.09nM±0.17 and 1.80nM±0.19, respectively. **Ethanol induced dopamine in the group pretreated with vehicle, but not in Org25935-treated rats.** Lower graph displays the effect of ethanol on extracellular dopamine in the responding (n=5) and non-responding (n=8) subgroup and in vehicles (n=8). Basal dopamine concentrations for Res Org25935, Non-Res Org25935 and saline were 2.48nM±0.20, 1.90nM±0.21 and 2.04nM±0.22, respectively. **Org25935 induced a dopamine increase in responders. Ethanol induced a dopamine increase in non-responders**.

That the previous findings with glycine pointed to an overrepresentation of glycine responders among ethanol high- compared to low-preferring rats (245) may imply that Org25935-evoked dopamine increase is linked to the effect of Org25935 on ethanol drinking behavior. Unfortunately at this time we were not set up to monitor glycine levels in parallel to dopamine (glycine levels were performed in another set of rats) which would have allowed investigation of whether the variability in dopamine response reflects a variability in endogenous glycine levels. For instance, a rat with high basal glycine levels and thus high basal saturation of the GlyR could be less prone to evoke a subsequent dopamine effect of GlyT-1 blockade and is perhaps less prone to experience the reinforcing effects of ethanol, a 'low-dopamine responsive' phenotype. Alternatively, since blockade of the uptake carrier for glycine potentiates the time course of synaptically released glycine, Org25935 is dependent on the preceding glycine release and the variability may as well lie here.

Responding rats may thus represent a phenotype with a highly responsive dopamine system. Yet it appeared that regardless of dopamine response to glycine, the subsequent ethanol-induced dopamine activation was either blocked or at least reduced (non-responders). Thus the mechanism by which ethanol elevates dopamine has been hampered by pre-exposure to glycine, and apparently more efficiently so when the glycine exposure has produced dopamine activation by itself.

Taken together, this study shows that Org25935 in a dose that reduces ethanol consumption in the rat, 1) increases glycine but not ß-alanine or taurine levels in the nAc, 2) may increase dopamine levels in the nAc and 3) counteracts ethanol-induced accumbal dopamine elevations. The results support the proposed involvement of the neurotransmitter glycine in modulation of basal dopamine levels and in ethanol-induced dopamine release in nAc. We propose an extension of the neurochemical effects to the behavioral outcomes on ethanol consumption by suggesting that the effects of the GlyT-1 blocker on alcohol consumption may be partly mediated via modulation of mesolimbic dopamine activity. The working hypothesis is that the Org 25935-induced increase of extracellular glycine levels activates inhibitory GlyRs in the nAc and/or in the VTA, which may disinhibit GABAergic feed-back inhibition onto VTA dopamine neurons and/or onto acetylcholinergic neuronal terminals in the VTA, and thereby allow an increased firing of dopamine neurons.

The results further indicate that GlyT-1 inhibition represents a combined substitution (in the nAc; stimulating GlyRs and elevating dopamine levels) and antagonism treatment (preventing further GlyR activation and dopamine elevation by ethanol) for alcohol dependence. The substituting effect appears to be of most importance for reducing ethanol consumption, since neither glycine non-responders nor strychnine-treated animals reduced their ethanol drinking in a previous study, despite that ethanol-evoked dopamine release was largely prevented by both treatments. In the human situation a low-grade substitution treatment paired with antagonism of further activation may be beneficial, as illustrated by the excellent effects of the partial agonists buprenorphine (273) and varenicline (274) in the treatment of opiate and nicotine dependence, respectively.

Paper III

A role for accumbal glycine receptors in modulation of dopamine release by the glycine transporter-1 inhibitor Org25935

Rationale

Systemic administration of the GlyT-1 inhibitor Org25935 elevated dopamine levels in nAc, an effect that totally prevented a further ethanol-induced dopamine elevation. There is significant evidence that glycine uptake blockers potentiate activity of the NMDA receptor complex through an indirect action at the NMDA glycine-binding site. As previous data point to an involvement of accumbal GlyRs in glycine's dopamine-modulating effect, it was important to test also a tentative interaction with the NMDA receptor in the effect of Org25935 on dopamine levels. The study tested 1) whether the Org25935-ethanol interaction with respect to dopamine levels seen after systemic drug challenge (Paper II) is evident also after drug and ethanol applications on site in nAc, and 2) whether indirect activation of GlyRs and/or NMDA receptors contribute to the Org25935-evoked dopamine elevation.

Experimental design

In vivo microdialysis coupled to HPLC-ED in freely moving rats was used to monitor dopamine efflux in nAc after local perfusion by reverse dialysis of 1) Org25935 (100 μM) alone, 2) Org25935 combined with ethanol (300 mM) and 3) Org25935 perfusion after pre-treatment with either the GlyR antagonist strychnine (10 and 20 μM) perfused in nAc, or administration of the NMDA glycine site antagonist L-701.324 (5 $mg.kg^{-1}$ i.p.).

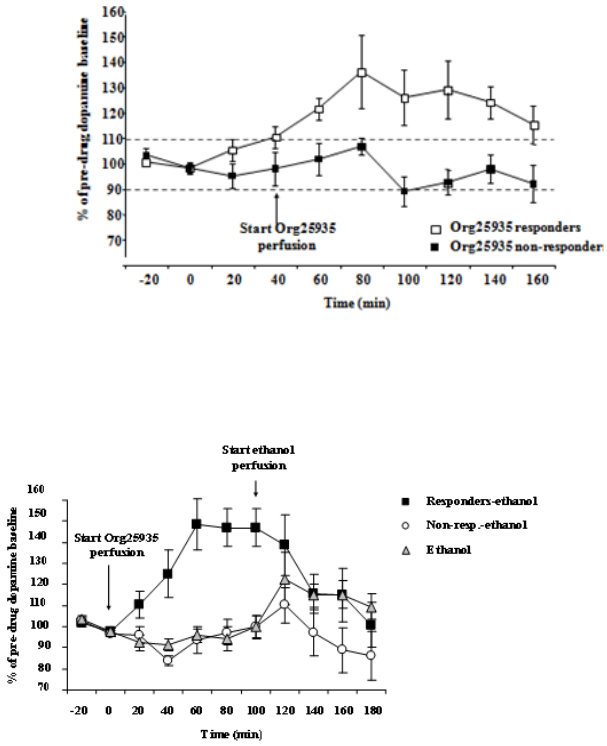

Figure 11. Upper graph: Org25935 on site in nAc increased accumbal dopamine levels, as measured by in vivo microdialysis after perfusion of 100 µM Org25935 into nAc in the responding (n=7) and non-responding (n=6) subgroup, mean ± SEM. Arrow indicates start of drug perfusion. Basal dopamine concentrations for responders and non-responders were 3.02±1.24 and 2.37±0.55 nM, respectively. **Lower graph: Org25935-evoked increase in dopamine levels in nAc prevents ethanol-induced dopamine activation**, as measured by in vivo microdialysis after perfusion of 300 mM ethanol (n=7) and after Org25935 perfusion followed by ethanol co-perfusion in the responding (n=8) and non-responding (n=9) subgroup, mean±SEM. Org25935 perfusion started at time point 0 min and ethanol perfusion at 100 min, indicated by arrows. Basal dopamine concentrations for ethanol, responders and non-responders were 2.91±0.64, 2.13±1.4 and 2.14±0.22 nM, respectively.

Findings and discussion

This study demonstrated a role for accumbal GlyRs in the dopamine-modulating effects of the GlyT-1 blocker Org25935. Local application of the GlyT-1 blocker Org25935 in nAc increased dopamine output in the rat nAc in a subpopulation of dopamine-responsive rats, when the criterion for being a dopamine responder was a >10% Org25935-evoked dopamine increase (upper graph, Figure 11). In these rats, Org25935 on site in nAc provoking an elevated dopamine tone prevented a subsequent ethanol-induced dopamine elevation (lower graph, Figure 11). That the drugs were perfused locally in nAc and yet produced results almost identical to those observed after systemic drug challenge, underlines the

ethanol-interaction (Paper II) and further localizes the Org25935-ethanol interaction to the nAc. This corroborates previous results pointing to the GlyR in nAc not only as a modulator of basal dopamine levels in nAc, but as an access point for ethanol to the mesolimbic dopamine system (233, 245). In addition to the robust prevention of ethanol-induced dopamine output in Org25935-responding animals, Org25935 mitigated ethanol's effect also among the Org25935 non-responding animals, and such a trend was also seen after systemic administration (Paper II). Thus the ethanol-interaction seems to take place at least to some extent regardless of the variability in the strength of the Org25935-induced dopamine response as such. Excessive glycine availability and thus saturation of the glycine binding site provokes a conformational change in the GlyR that in turn may hinder ethanol from binding and allosterically modulating the GlyR (243, 275, 276).

Org25935 produced an inconsistent dopamine response, as observed also after systemically administered Org25935 (Paper II) and after nAc glycine perfusion (194, 233). Org25935 increased accumbal dopamine output by 26% in all rats, and by 36% in responders, as shown in Figure 11. Biological responses may well lie in receptor desensitization or internalization processes, however recent studies have indicated that the GlyR not may be prone to compensatory alterations induced neither by alcohol (206), nor by repeated Org25935 injections (more on biological variability, see General discussion).

The dopamine response to Org25935 was antagonized by strychnine but was not affected by antagonism of the glycine site of the NMDA recpetor, as displayed in Figure 12. This finding implies that the dopamine increase provoked by the GlyT-1 blocker is rather mediated via accumbal GlyR activation than via NMDA receptor signaling. Perfusion of glycine as well as taurine and ß-alanine (also endogenous GlyR agonists) into nAc also increased accumbal dopamine levels (194, 267, 278), and co-perfusion of glycine with strychnine concentration-dependently reversed the suppressing effect of strychnine on dopamine output in nAc (194), all events that support GlyR involvement in the Org25935-evoked dopamine elevation.

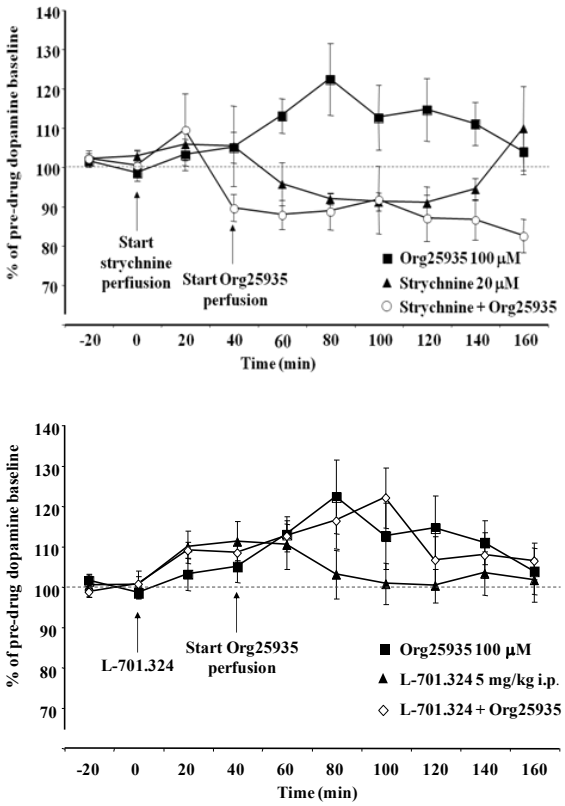

Figure 12. The Org25935-induced dopamine response is prevented by the GlyR antagonist strychnine. Upper graph: Strychnine in nAc prevents Org25935-induced increase in accumbal dopamine levels, as measured by *in vivo* microdialysis after perfusion of 20 µM strychnine (n=8), 100 µM Org25935 (n=13) and after strychnine perfusion followed by Org25935-perfusion (n=11), shown as mean ±SEM. Basal dopamine conc for Org25935, strychnine and strychnine-Org 25935 were 2.66±0.66, 1.34±0.57 and 2.26±0.44 nM, respectively.

Lower graph**: L-701.324 did not influence the Org25935-induced increase in accumbal dopamine levels**, as measured by in vivo microdialysis after L-701.324 5 mg/kg i.p (n=7), 100 µM Org25935 (n=13) and after systemic L-701.324 followed by Org25935 perfusion (n=10), shown as mean ± SEM. Basal dopamine conc for Org25935, L-701.324 and L-701.324-Org 25935 were 2.66±0.66, 3.70±0.56 and 2.92±0.70 nM, respectively

Besides serving as a GlyR agonist, glycine is an essential co-agonist of glutamate at the NMDA receptor. There is evidence that glycine reuptake inhibitors potentiate activity of the NMDA receptor complex through action at the NMDA receptor glycine binding site (279), a mechanism which also could be involved in the dopamine-modulating effect of Org25935. The present study reported that the NMDA receptor glycine site antagonist L-701.324 did not affect dopamine-responses to Org25935. Indeed, glutamate signaling is implicated in alcohol and other drug addictions and L-701.324 given in the same dose prevents relapse-like drinking behavior (260) as well as ethanol-induced conditioned place preference (280), effects believed to derive from NMDA receptor blockade. However, NMDA receptor-evoked dopamine responses do not appear clear-cut; while withdrawal from chronic ethanol is consistently associated with elevated glutamate levels in nAc (281) there is controversy regarding acute effects of alcohol on glutamate (282). NMDA receptor antagonists and thus a negative NMDA receptor modulation has been suggested to be useful for alcohol addiction pharmacotherapy (58, 283), a view that is not in concordance with a tentative NMDA

receptor mechanism by Org25935 since GlyT-1 blockade rather potentiates than antagonizes the receptor. Further, that alcohol itself inhibits the NMDA receptor contrasts to the NMDA-potentiating effect of Org25935 and argues against a tentative ethanol-substituting mechanism for Org25935 on this receptor. In summary, these effects favor a GlyR mechanism rather than an NMDA receptor mechanism underlying Org25935's as well as alcohol's interference with mesolimbic dopamine.

Alcohol's activation of mesolimbic dopamine activity, and especially dopamine release in nAc, is linked to ethanol reinforcement and reward, which in turn may contribute to development and maintenance of alcohol dependence (71, 73, 91). Chronic exposure to alcohol is known to result in allostatic and functional changes in the VTA-nAc pathway that in part underlie the addictive actions of alcohol (18, 70). That Org25935 attenuates ethanol-induced increase in nAc dopamine levels implies Org25935-interference with ethanol's rewarding and reinforcing effects, a tentative beneficial effect for an alcohol intake reducing therapy. However, also other neurochemical events are implicated in regulation of alcohol intake. We thus suggest that the Org25935-dopamine interaction primarily involves the GlyR, whereas also Org25935-evoked effects mediated by the NMDA receptor could be involved in the alcohol intake reducing effect of the GlyT-1 blocker Org25935. This view is supported by recent findings that Org25935 treatment persistently reduces relapse-like drinking behavior and that this durable effect may result from restoration of both glycinergic and glutamatergic signaling (180).

The results lend further support to the previously suggested mechanism (se Paper II) that Org25935 displays indirect, "partial" agonistic properties on the GlyR, i.e. Org25935 acts as an antagonist when ethanol is present, competing with ethanol and producing a net decrease in GlyR activation compared to ethanol alone. The stabilization of extracellular glycine levels, which in turn elevates dopamine in Org25935 responders or preserve/stabilize dopamine levels in non-responding subjects, may reduce GlyR stimulation by excess amounts of alcohol. Such a partial agonistic pharmacologic profile is suggested to be beneficial in clinical therapy (284). The study adds to the growing evidence for the GlyR as an important player in the dopamine reward circuitry and in ethanol's effects within this system.

Paper IV

The glycine reuptake inhibitor Org24598 and acamprosate reduce ethanol intake in the rat; tolerance development to acamprosate but not to Org24598

Rationale

Glycine availability modulates alcohol consumption and ethanol-induced dopamine responses within the reward circuitry, and the glycine reuptake inhibitor Org25935 displays excellent anti-alcohol drinking effects in experimental animals. Increasing evidence thus supports the notion that GlyT-1 inhibition may constitute a new concept for pharmacotherapy of alcohol addiction. It was now judged of importance to explore whether the effect on drinking behavior is substance-bound (to Org25935) or class-bound (to GlyT-1 inhibitors), and also to compare the effect to a drug currently in clinical use for alcohol addiction. The present study examined the effect of a different selective GlyT-1 inhibitor, Org24598, on ethanol consumption in rats and compared the effect to that of acamprosate.

Experimental design

The effects of daily injections of Org24598 and acamprosate on ethanol consumption were studied in rats in a LA two-bottle preference model for 12 days, followed by alcohol deprivation for 14 days before a second test-period of 10 days, as shown in Table 2. The study used selected male Wistar rats with an ethanol preference >30%, corresponding to medium- and high-preferring rats in Paper I. After monitoring of the ethanol consumption, the effects of the respective drugs and ethanol on extracellular dopamine, glycine, taurine and β-alanine levels in nAc were examined by means of *in vivo* microdialysis.

Table 2.

Experimental design of the study

Time	Procedure	Access to
Week 1-6	Screening Free choice drinking	Food, tap water, 6% ethanol Free access
Day -7-0	LA baseline drinking	Food, tap water, 6% ethanol LA 9.30-12.00 a.m.
Day 1-12	Drug treatment + LA baseline drinking	Food, tap water, 6% ethanol LA 9.30-12.00 a.m.
Day 13-26	2 weeks deprivation	Food and tap water
Day 27-36	Drug treatment + LA baseline drinking	Food, tap water, 6% ethanol LA 9.30-12.00 a.m.
Day 37-40	Drug washout	Food, tap water, 6% ethanol
Day41-45	3 treatment days with microdialysis day 3	Free access

Findings and discussion

Daily injections of Org24598 profoundly reduced ethanol intake from 1.2 to 0.3 $g.kg^{-1}$ per drinking session, the full effect was developed after three days and remained throughout the first treatment period of 12 days (Figure 13). The effect was reinstated after 14 days of AD and gradually declined with dose reduction and treatment termination, suggesting a dose-response relationship between Org24598 and reduction of alcohol consumption. That the effect was maintained for the entire treatment period indicates that the effect is not due to initial sedation affecting drinking behavior in general. Acamprosate promptly reduced ethanol intake from 1.2 to 0.5 $g.kg^{-1}$ per drinking session, but after three days complete tolerance developed to this effect and acamprosate failed to influence alcohol consumption during the second test period.

Neither Org24598 nor acamprosate reduced water intake but Org24598-treated rats displayed higher water intake compared to the acamprosate and vehicle groups during dose reduction and termination (day31-36 Figure 13). The Org24598-group displayed reduced total fluid intake during the first treatment days, probably reflecting the sudden reduction in ethanol consumption. The rat weight gain, displayed in Figure 14, did not differ between the groups over time, and no behavioral and physical signs of toxicity were found in doses up to 16 $mg.kg^{-1}$ of Org24598 (unpublished tests in the present laboratory) nor in doses up to 30 $mg.kg^{-1}$ (286). From this we propose that the GlyT-1 blocker Org24598 exhibits alcohol intake

reducing properties, supporting the concept that GlyT-1 inhibition decreases ethanol consumption. However the anti-alcohol properties of GlyT-1 inhibitors need to be further investigated. For instance, the two structural classes of GlyT-1 inhibitors, sarcosine derivatives versus non-sarcosine based compounds, have different binding sites that affect the pharmacological profiles of the drugs (287). Non-sarcosine based compounds have less respiratory and motor effects but have only been available for a shorter period of time. The compounds so far tested on ethanol consumption in rats (Org25935 and Org24598) are both sarcosine derivatives and are apparently non-competitive inhibitors, as they exert inhibition independent of glycine concentration (287).

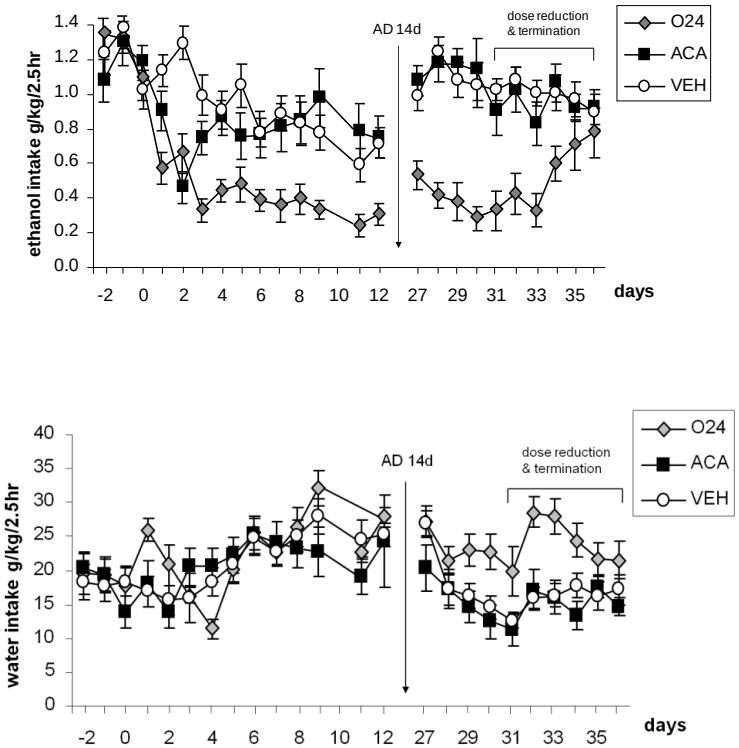

Figure 13 Org24598 and acamprosate reduce ethanol consumption. Effects of Org24598 and acamprosate on (upper graph) ethanol intake and (lower graph) water intake in rats with ethanol preference >30% (medium- and high-alcohol consuming rats). Org24598 reduced ethanol intake and the effect lasted throughout the treatment period and was also reinstated after alcohol withdrawal. Acamprosate reduced ethanol intake but after three days complete tolerance developed to this effect and the effect was not reinstated after AD. Water intake did not differ between the groups during the first period (d1-12) but the Org24598-group displayed higher water intake during dose reduction and termination (d31-36). Treatment day 10 was excluded due to incorrect performance of the LA procedure. LA schedule: 7 BL days where last 3 days are shown. Day1-12: Org24596 (12 mg.kg^{-1}) and acamprosate (200 mg.kg^{-1}) inj. 40 min prior to LA. Day 13-26: AD. Day 27-30: Reintroduction of LA and daily drug treatment, full dose. Day 31-32: LA and ½ dose. Day 33-34: LA and ¼ dose. Day 35-35: LA and vehicle injections.

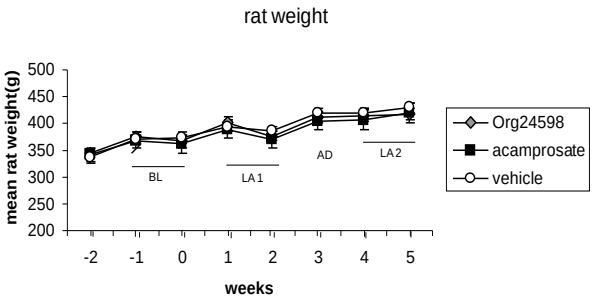

Figure 14. Rat weight. Mean rat body weight ±SEM in each treatment group monitored once a week throughout the experiment. No differences between the groups were observed.

Next, the findings suggest that GlyT-1 inhibition may represent a pharmacological treatment principle for alcohol dependence that is superior to acamprosate. Acamprosate promptly decreased ethanol on treatment day two but on the fourth day complete tolerance had developed and the effect was not reinstated after the alcohol withdrawal period. This finding is in consensus with previous reports that repeated injections cause rapid tolerance development to acamprosate's effect on voluntary ethanol drinking and operant ethanol self-administration (288, 289). It has been shown that acamprosate reduces the AD effect (288), yet in this study, ethanol intake was increased equally in the control group and in the acamprosate group after 14 days of ethanol withdrawal, as visualized in Figure 15. This is most likely due to tolerance development to the acamprosate effect already during the preceding subchronic administration period.

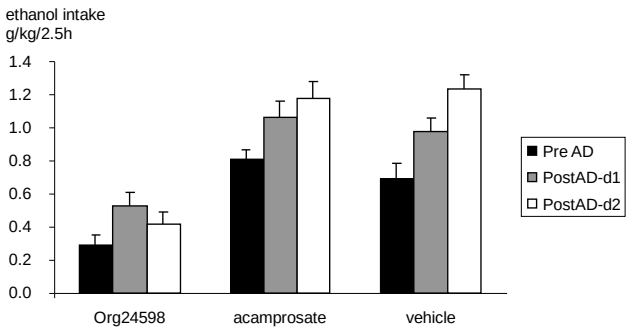

Figure 15. Ethanol intake on the last drinking day (PreAD) before the 14-day alcohol deprivation (AD) and on the first and second day after AD (Post AD-d1 and d2**). Ethanol intake was significantly increased on d1 in all groups, and on d2 in the vehicle- and acamprosate group but not in the Org24598 group, as compared to PreAD.**

Acamprosate is believed to interact with ethanol through a glutamatergic mechanism (41, 290). A recently proposed alternative mechanism is that the homotaurine derivative acamprosate interacts with ethanol by exerting taurine-like effects (267), secondarily interacting with GlyRs (37, 38). It has previously been shown that acamprosate elevates

extracellular taurine levels (291), but no such effect was observed in the present study. The subchronic acamprosate exposure prior to the microdialysis experiment may explain why acamprosate was not able to elevate taurine levels, as tolerance may have developed to the taurine response. If acamprosate indeed produces action by mimicking taurine, or by elevating taurine levels, this may in turn be linked to the short-lived reduction of ethanol intake imposed by acamprosate. Also ethanol has been shown to increase taurine in nAc (292), yet here, ethanol injection did not influence taurine levels in nAc, neither in vehicle- nor drug- treated animals (Figure 2c, Paper IV). The rats´ preceding access to alcohol may have caused tolerance also to ethanol's taurine-releasing effects. That taurine release produced by alcohol, and possibly also by acamprosate, rapidly declines could be related to taurine's primary role as an osmoregulator rather than as a neurotransmitter. Yet, whether taurine release by glial cells really is a short-lived event remains unknown.

Systemic administration of Org24598 produced a 54% increase in extracellular glycine levels (Figure 2b, Paper IV), peaking approximately 1 hour after injection, whereas neither Org24598 nor acamprosate treatment altered accumbal taurine or β-alanine levels. This result is in line with previous findings, where Org24598 in a dose of 10 $mg.kg^{-1}$ increased glycine levels in a similar manner in rat hippocampus and striatum, whereas the levels of glutamate, GABA, alanine and taurine were unaltered (293). Basal glycine levels were not increased 24 hrs after the last Org24598-injection. Regional differences in glycine response have been demonstrated by two different GlyT-1 blockers, that produced a transient increase in glycine levels in prefrontal cortex (peaked after 3 h and then returned towards baseline) in contrast to a sustained elevation in cerebellum (178), and also similar differences in the glycine response to Org24598 have been reported (293). Assuming that the short-lived increase in glycine levels in nAc in the present study reflects that GlyT-1 inhibition is more forceful in hindbrain versus forebrain regions, this corresponds to the distribution of GlyT-1 in the CNS, where higher levels of GlyT-1 were found in the spinal cord, brain stem and cerebellum and moderate levels in forebrain regions (224).

A second aim of the study was to determine if ethanol consumption over 9-10 weeks altered the ethanol-induced dopamine response, and whether Org24598 and/or acamprosate influenced the ethanol-induced dopamine signal. The control group responded with a typical 45% increase in dopamine levels after 2.5 $g.kg^{-1}$ i.p. ethanol, but acamprosate/Org24598 pre-

treated rats did not respond to ethanol to the same extent. Thus Org24598 tended to interact with dopamine output in the same manner as acute administration of the GlyT-1 inhibitor Org25935 (Figure 2a, Paper IV), which produced a dopamine elevation *per se* while antagonizing the ethanol-induced dopamine increase. Following repeated drug injections, Org24598 and acamprosate were still able to mitigate ethanol-induced dopamine release in nAc. This suggests that the ability of GlyT-1 inhibitors to counteract ethanol-induced dopamine output, is present, although perhaps not to the same extent, also in subjects exposed to repeated injections, in this case Org24598. This argues against rapid tolerance development to this effect, which could be related to the durable effect on ethanol intake, and which could indicate rigidity of the GlyR and the GlyT-1 in terms of adaptational changes. However, also acamprosate attenuated ethanol's dopamine-elevating effect, and yet tolerance developed to the anti-alcohol drinking effect. This observation may argue against accumbal dopamine involvement in the anti-alcohol drinking effect of Org24598, and may indicate that Org24598 and acamprosate produce their anti-alcohol effects through different mechanisms.

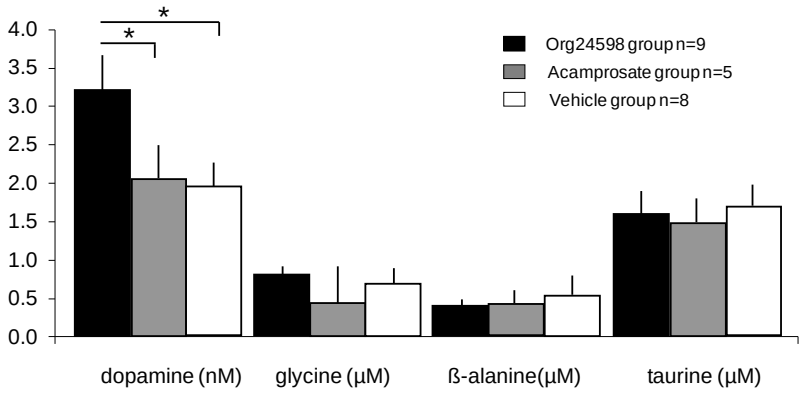

Figure 16. Org24598-treated rats displayed significantly higher dopamine basal dopamine levels in nAc compared to the acamprosate and the vehicle group. Endogenous basal levels of dopamine, glycine, taurine and β-alanine in nAc, shown as groups mean ± SEM of the two last stable baseline values from each individual animal. The sampling was done 24 hours after injections of Org24598 and acamprosate.

Interestingly, Org24598-treated animals displayed higher endogenous baseline levels of dopamine compared to the acamprosate and the vehicle group, even though these basal levels were not determined by the preferred no-net flux technique (Figure 16). It is established that withdrawal from chronic administration of ethanol reduces extracellular dopamine levels (77) and that this is probably due to a homeostatic adaptation to repeated stimulation of the system (74). The lower basal dopamine levels in the acamprosate and the control group may thus be explained by their higher ethanol exposure relative to the Org24598-treated animals. The low ethanol intake in the Org24598-group may have prevented adaptive reduction of basal dopamine levels and also the chronic Org24598 treatment has apparently failed to reduce basal dopamine levels. An alternative understanding is that Org24598 elevates basal dopamine levels by itself, and that this elevation sustains 24 hrs after drug administration (rats were last injected the day prior to probe sampling). This explanation may however appear less plausible as Org24598 failed to increase baseline levels of glycine, which is the primary target of Org24598 and the elevation of which probably precedes that of dopamine (Paper III). Since reduced dopamine levels during alcohol withdrawal is believed to promote further alcohol intake, the increased dopamine levels observed in the Org24598 treated group may in any case be related to the sustained ethanol intake-reducing effect of Org24598.

In conclusion, the present study demonstrated robust and long-lasting alcohol intake reducing properties of the GlyT-1 blocker Org24598 and together with previous findings with the GlyT-1 blocker Org25935 (Paper I) the results propose GlyT-1 inhibition as a new concept for reducing ethanol consumption. It is suggested that an interaction between elevated glycine levels provoked by Org24598 and mesolimbic dopamine may be a central event underlying the anti-alcohol effect of Org24598. Moreover, GlyT-1 inhibition may represent a new pharmacological treatment principle for alcohol dependence that is superior to acamprosate.

Summary of results

- The selective GlyT-1 inhibitors Org25935 and Org24598 demonstrated excellent ability to decrease ethanol intake and preference in selected alcohol high-and medium-preferring rats.
- The effects of both Org24598 and Org25945 on alcohol drinking behavior were rapidly reinstated after an alcohol free period, demonstrating a long-lasting effect that could prevent relapse occurrence.
- The alcohol intake-reducing effect of Org24598 appears superior to that of acamprosate, a drug in current use for treating alcohol dependence.
- The GlyT-1 blocker Org25935 produced a consistent increase in extracellular glycine levels by 87% in nAc.
- In a subpopulation of rats, the GlyT-1 blocker Org25935 raised accumbal dopamine levels and totally prevented ethanol-evoked dopamine increase in this brain region. Org25935 mitigated the effect of ethanol on dopamine levels in nAc also in the subpopulation not responding with a dopamine increase after Org25935 per se.
- The ability of Org24598 to elevate accumbal dopamine levels was maintained following nine weeks of alcohol exposure and a subchronic drug regimen with Org24598.
- Recruitment of GlyR mediated activity in nAc, rather than NMDA signaling, is involved in mediating the effect of glycine reuptake blockade by Org25935 on dopamine levels.
- Rats treated with Org24598 displayed higher endogenous dopamine levels in nAc compared to acamprosate- and vehicle-treated rats. Thus subchronic Org24598 treatment either raised basal dopamine levels in nAc or, possibly by reducing alcohol consumption, protected against a tentative down-regulation of accumbal dopamine levels following the 9 weeks of ethanol exposure.

General discussion

The overall finding in the present thesis is that two different GlyT-1 inhibitors possess robust alcohol intake-reducing effects and in addition attenuate alcohol's dopamine stimulating effect. The present thesis further suggests that GlyT-1 inhibitors interact with accumbal dopamine levels through a GlyR mechanism in a similar manner as glycine, and indicates that the dopamine-interaction is at least partly related to the anti-alcohol drinking properties of GlyT-1 inhibitors. The latter statement also receives some support from results obtained in the below described studies.

The strychnine reversal study

In addition to the alcohol intake-sparing effect in rats (Paper I), Org25935 demonstrated ability to modulate ethanol-induced dopamine output in nAc (Paper II). It was further shown that accumbal GlyRs most likely mediated the dopamine-modulating effect (Paper III), but the influence of accumbal GlyRs on the modified drinking behaviour was not probed. In order to validate tentative accumbal GlyR involvement in the anti-alcohol intake effect of Org25935, we examined if strychnine on site in nAc was able to antagonize the effect on alcohol drinking. By using the LA ethanol consumption model described in Papers I and IV, the selected ethanol high- and medium preferring rats were implanted with guide cannulae into nAc, as previously described (38). Rats were exposed to Org25935 (6 mg/kg i.p.) for 5 days, followed by 3 days of micro-injection of either strychnine or Ringer solution into the nAc 30 min prior to Org25935 injection and access to ethanol and water. Strychnine application in nAc did not produce a clear effect on the reduced alcohol intake in the Org25935-group, displayed in Figure 17. It appeared that some rats responded to strychnine with a partial reversal and others did not, i.e. strychnine did not consistently reverse the reduced ethanol intake produced by Org25935. The interpretation was complicated by the fact that vehicle-treated animals also increased their ethanol intake following strychnine application, though only on the last day of strychnine application. The results imply that accumbal GlyRs may play a role for the alcohol intake-reducing effect, but that GlyRs elsewhere, or alternatively NMDA mechanisms provoked by GlyT-1 blockade, also may contribute. Alternatively, the study design did not allow the hypothesized response to take place, i.e. the reduced ethanol intake could not be reversed by glycine antagonism, since the

GlyR, the access point for alcohol, is already pre-blocked by strychnine and therefore may obstruct reversal of the reduced alcohol intake.

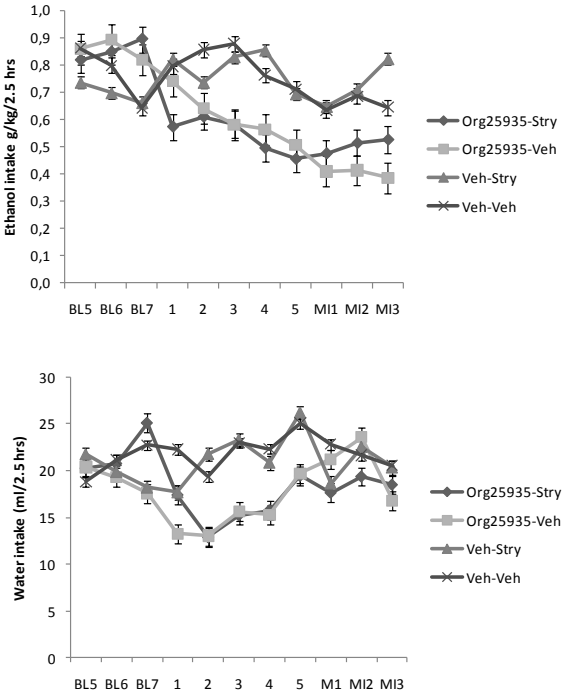

Figure 17. Blockade of accumbal GlyRs partly reverses the anti-alcohol intake in rats treated with the GlyT-1 blocker Org25935. Upper graph displays ethanol intake and lower graph displays water intake on 3 last days of LA baseline drinking (BL), 5 days of Org25935 (6 mg/kg i.p.) or vehicle (saline) injections followed by 3 days of strychnine or vehicle (Ringer solution) microinjection locally into nAc. Blockade of accumbal GlyRs produced a partial reversal of the reduced ethanol intake in the Org25935-group (n=15-20).

Strychnine in the VTA and dopamine levels in nAc - a pilot study

In a parallel study, a similar strychnine application regimen in nAc as described above completely reversed the ethanol intake-reducing effect of acamprosate (38). Local strychnine in nAc may have influenced the effect also of Org25945 on ethanol consumption, but did not convincingly reverse the anti-alcohol drinking response. It is possible that GlyRs also in other brain regions may contribute to the dopamine-modulating effects of Org25935 as well as the effect on ethanol drinking. Indeed, glycine has previously been shown to produce disinhibition of VTA dopaminergic neurons and of nigrostriatal dopamine neurons, via reduction of GABAergic inhibitory activity due to interference with GABAergic terminals in the VTA (294). In order to investigate whether GlyRs in VTA may interact with accumbal

dopamine levels, a pilot study using *in vivo* microdialysis was performed, where dopamine levels in nAc were analyzed after application of the GlyR antagonist strychnine in VTA by reverse microdialysis. Strychnine concentration-dependently decreased accumbal dopamine levels (Figure 18), suggesting that GlyRs located in VTA also tonically interact with the dopamine VTA-nAc pathway. The involvement of GlyRs also in VTA may be related to why blockade of GlyRs in nAc did not clearly prevent the anti-alcohol effect of Org25935. These preliminary results need to be followed up by studying the effects of glycine applied in the VTA, and a tentative interaction with ethanol application in nAc. Also, in order to localize the GlyRs involved in Org25935´s effects on ethanol drinking behavior, additional strychnine reversal studies with local microinjections of strychnine in both nAc and VTA will be required, an experimental design that represents a major challenge.

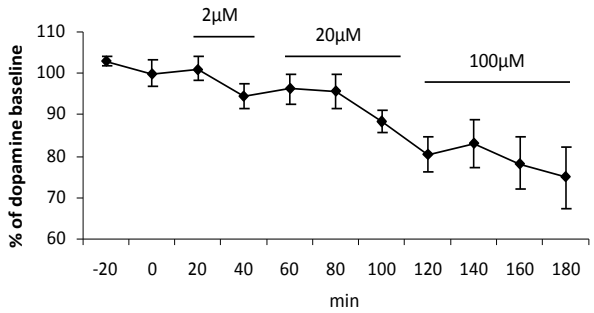

Figure 18. The effect of strychnine perfusion in VTA on accumbal dopamine levels. n=7. Probe placement VTA: A/P-5.4, L/M -0.7, V/D -8.4 , nAC: A/P +1.85, L/M -1.4 V/D -7.8. nAc probe was perfused with Ringer solution throughout the experiment. Strychnine administered locally in VTA by reverse microdialysis concentration-dependently decreased accumbal dopamine levels, maximally by 23.5% at time point 180 min.

NMDA receptor contribution to the GlyT-1 inhibitor-ethanol interaction

That NMDA receptor antagonists can substitute for ethanol in drug-ethanol discrimination tests suggests that NMDA receptors are involved in mediating the subjective effects of ethanol (168, 295). Activity of the mesolimbic reinforcement system is under heavy control of glutamatergic neurotransmission and the NMDA receptor constitutes a primary binding site for ethanol (159, 260, 296). However the relevance of alcohol's effect on glutamatergic transmission for the interaction with the dopamine VTA-nAc pathway is not fully established (160). It cannot be excluded that alcohol also accesses the VTA-nAc pathway through the

NMDA receptor. Glycine reuptake blockers potentiate the activity of the NMDA receptor complex and evidence support that this receptor and other glutamatergic receptors are involved in alcohol drinking behavior (20, 58). Since effects of glycine on the GlyR and the NMDA receptors appear to occur at similar doses in rodents (297) it cannot be excluded that NMDA receptor mechanisms are involved in the anti-alcohol properties of GlyT-1 inhibitors. As discussed earlier, it has been reported that L-701.324 prevents relapse drinking behavior (260) and ethanol-induced conditioned place preference (280). In spite of the presented evidence that glycine regulates alcohol consumption via a GlyR mechanism, the effect of GlyT-1 blockers on alcohol consumption thus could be attained by a dual mechanism involving both GlyRs and NMDARs. This view is supported by a recent study showing that Org25935-treatment reduces relapse-like drinking, an effect that persisted into treatment-free periods and suggested to result from restoration of ethanol-induced changes in glycinergic and glutamatergic signaling-related genes (180). Potentiation of the NMDA receptor by GlyT-1 inhibitors and glycine agonists has shown potential in the treatment particularly of the negative and cognitive symptoms of schizophrenia (230, 279), and glycine therapy in healthy humans is reported to improve working memory (298). In the transition to drug addiction, ethanol's dopaminergic reinforcing effects connects to learning and memory processes where glutamate signaling has a central role (85). A speculation is therefore that GlyT-1 inhibitors may have a beneficial impact on dysfunction in frontal glutamate projections to the VTA-nAc through an NMDA receptor mechanism, implicated both in compulsivity and in the cognitive impairment observed in the addicted subject. Possibly, the effects of GlyT-1 blockers can be distinguished into (1) anti-psychotic/ and pro-cognitive properties through facilitation of NMDA receptor signaling and (2) the anti-alcohol drinking properties, initially through GlyR-modulation of alcohol's effects on mesolimbic dopamine and later, as alcohol drinking becomes compulsive (not mimicked in this model), through pro-executive glutamatergic mechanisms restoring prefrontal function. This dual mechanism of action that arises upon enhancement of extracellular glycine levels may be the strength of therapy with GlyT-1 inhibition, as it may extend to recent evidence for a glycine-glutamate cross-talk as important fine-tuning between excitatory and inhibitory actions in the brain.

The loop theory for GlyRs in the mesolimbic dopamine system

The findings in the present thesis and in the thesis of Anna Molander (2005) together provide evidence for the role of glycine mechanisms in modulation of alcohol consumption and in ethanol's effects on dopamine in nAc. There is now substantial evidence for the involvement of GlyRs in these events, particularly in tonic modulation of dopamine levels in nAc. The expression of GlyRs in the forebrain including nAc and in the midbrain including VTA is well demonstrated (188, 194, 206, 299), but the cellular localization of GlyRs within the VTA-nAc pathway remains to be determined. Electrophysiological studies have suggested that accumbal GlyRs are expressed both on GABAergic neurons projecting to VTA and other regions (193, 299) and on the large aspiny cholinergic interneurons (191,192). Medium spiny GABAergic neurons constitute 95% of the nAc cell population and the evidence for GABA and glycine receptor co-localization as well as co-release indicates that GABA and glycine use the same presynaptic terminal (175). Presumably, synaptic GlyR heteromers may be co-localized with GABA receptors on GABAergic terminals, whereas extrasynaptically α-homomers may well be expressed elsewhere along the GABA-neuron, including on cell-bodies in nAc (see Figure 4). Our research group has suggested that dopamine release results from activation of inhibitory GlyRs located on the soma of inhibitory GABAergic feed-back neurons projecting from the nAc to the VTA, leading to inhibition of the negative feed-back tone on the dopamine neurons in VTA, ultimately producing, presumably via acetylcholine release in the VTA (165, 300), enhanced firing of the dopamine projections to nAc (138, 139, 162, 236, 245, 267). The VTA dopamine pathway is also under tonic control from ventral pallidum-GABAergic projections and from local interneurons within the VTA (102, 107) and accumbal GABAergic neurons also project to other areas such as ventral pallidum, which in turn is connected via the thalamus to prefrontal cortex as well as the striatum (see Introduction). An alternative route of action for the GlyR-dopamine interaction in nAc could therefore be interference with other non-VTA outward projecting neurons, with in turn may modulate glutamatergic afferents to these areas. Therefore, the proposed VTA-nAc feed-back loop theory for ethanol-induced dopamine elevation in nAc provoked by GlyRs, remains a theory that as to date has not been firmly confirmed (proposed neurocircuitry is reviewed in detail in Söderpalm *et al*, 2009 (246)).

GlyR expression directly on dopaminergic cells has not been reported. If GlyRs were located on dopamine terminals they would have to be excitatory in order to promote dopamine release, requiring a reverse chloride transmembrane gradient to allow chloride efflux (GlyR ion channels are selective for chloride ions). This would most likely not be the case under normal physiological conditions, but could theoretically be true when homeostasis is interrupted in the presence of alcohol. This scenario challenges the traditional view of the GlyR as exclusively an inhibitory receptor causing cell hyperpolarization by chloride influx. On a higher level, this implies that GlyR action (and GABA receptor activity) may obtain a different meaning, or perhaps become of higher importance in an altered electrochemical milieu during e.g. alcohol intoxication. Glycine transporter proteins may operate in reverse direction causing release of glycine instead of reuptake, which imply that also receptors (at least ion channel receptors) may act in reverse operation under altered physiological conditions (218). This may have implications for understanding the role of the GlyR as a homeostatic regulatory mechanism tuning functional balance between synaptic dopaminergic signaling and GABAergic inhibition in the reward pathway. Given that neuronal excitability is controlled by a balance between excitatory and inhibitory neurotransmitters, this may also apply to CNS activity more generally, in a comparable manner to the inhibitory feed-back function of GlyRs in the brain stem and spinal cord. This tempting view blurs the distinction between excitatory and inhibitory receptors but cannot be further elaborated upon in the present thesis.

Acamprosate mechanisms

The taurine derivate acamprosate (calcium acethylhomotaurinate) increases extracellular dopamine levels when applied locally in the nAc (37, 301) in a similar manner as taurine itself (267). Taurine released from glial cells is implicated in maintaining physiological conditions through osmoregulation, neuroptotection and neuromodualtion. Endogenous taurine is suggested to be a modulator of the effects of ethanol (302,303) and ethanol increases taurine levels in the nAc (282, 303). Studies in the present laboratory have shown that the GlyR is involved both in the dopamine elevating and alcohol intake-reducing effects of acamprosate (37, 38). That blockade of GlyRs in nAc alone totally antagonized the

ethanol-reducing effect of acamprosate could reflect a fragile anti-alcohol mechanism which in turn is prone to rapid tolerance development, and which possibly may be connected to alcohol's interference with the function of taurine. However, acamprosate is suggested to interfere also with other receptors, such as the NMDA and the GABA receptor and other ion channels, which further complicates matters. In a clinical perspective, the rapid tolerance development to acamprosate's alcohol drinking-reducing effects implies that using acamprosate as an intermittent, on demand preventive therapy could be of benefit. However such a strategy may be hindered by the low bioavailability of the drug requiring several days of treatment for accumulation and pharmacological effect.

Inconsistent dopamine responses

The variability in dopamine response observed in the present studies probably lies in the response to the elevation of glycine levels as such and not in glycine removal capacity, since both Org25935 and Org24598 produced consistent increases in glycine levels, Paper II and IV (261, 293). The phenomenon of a large biological variation in dopamine response to Org25935 is similar to the observed effects after exogenous glycine (194, 233, 245). Several factors may account for the variance in dopamine response, such as magnitude of GlyR expression and GlyR subunit composition as such, and their responsiveness due to propensity to desensitize or internalize. Also various factors down-stream to direct GlyR actions may differ between the animals, since we know that e.g. nACh receptors in the VTA are also involved in the dopamine effect. Interestingly, a treatment regimen of daily injections of Org25935 for 19 days produced no significant changes in the expression of any GlyR subunit or GlyT-1 mRNA in several brain regions, including nAc (Figure 19). To date the dominating GlyR subunit composition in forebrain regions is poorly explored. Since glycine binds to the α-subunit it is conceivable that extrasynaptic α1-homomers are more sensitive to an increase in extracellular glycine levels compared to heteromeric GlyRs and the α1-homomer is also the subtype most sensitive to alcohol. It should be investigated whether GlyR expression remains unaltered after Org24598-treatment and whether this is true also for acamprosate treatment.

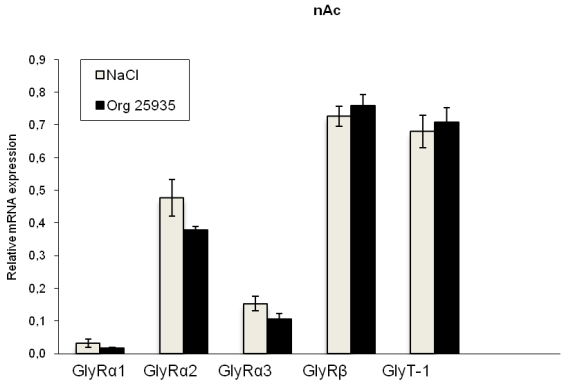

Figure 19. Subchronic Org25935 treatment does not alter GlyR subunit expression or GlyT-1 expression in nAc. The figure displays relative mRNA expression ± S.E.M, n=6 (normalized quantities) of four examined glycine receptor subunits and GlyT-1 in the rat nAc after subchronic treatment with Org25935 (6 mg/kg, i.p.) daily for 19 days. No significant differences were observed in nAc or in other relevant reward-related brain regions.

A commonly held view is that chronic alcohol exposure causes changes in gene expression and receptor function in neuronal systems targeted by alcohol. For example, the tolerance development to GABAergic effects of alcohol are thought to involve changes in $GABA_A$ receptor subunit expression and function (304). That GlyRs and extracellular glycine are involved in regulation of voluntary ethanol intake would therefore imply that long-term alcohol exposure induces changes in the glycinergic system. A recent study found that genetically high versus low alcohol-preferring AA (Alko, Alcohol) rats display very few differences in mRNA GlyR expression despite the diversity in drinking behavior (206). Clearly, GlyR changes may be evident after long-term alcohol exposure (exceeding the alcohol drinking period in the present studies), as underlined in a study reporting that Org25935 attenuated molecular changes in glycinergic signaling provoked by long-term ethanol exposure (180). Modelling chronic conditions is a challenge in preclinical research and the alcohol-exposure in the present studies is not likely to produce neurochemical alternations similar to those underlying alcoholism in man, as transition to alcohol addiction often requires several years of high alcohol consumption. Nevertheless, the exposure to GlyT-1 inhibitors in the present studies did not produce tolerance, and the durable effect of GlyT-1 blockade on alcohol drinking (up to 40 days) argues against adaptational changes in the GlyR system within this time course. The apparent resistance of the GlyR to rapid changes may offer a great advantage for GlyR pharmacotherapy in terms of tentative problems with tolerance development, a profile contrasting to the rapid tolerance development observed

to the alcohol intake reducing effects of a variety of substances (acamprosate, serotonin reuptake inhibitors, 5-HT1A agonists and opioid antagonists).

Consistent anti-alcohol drinking responses

Opposite to the inconsistent dopamine-stimulating effect of Org25935, both GlyT-1 blockers consistently decreased ethanol consumption in the vast majority of ethanol medium-and high-preferring rats. This contrasts to the observed variability in behavioral response to exogenous glycine perfusion, which decreased ethanol drinking in animals responding to glycine with a dopamine elevation, but not in the non-responders (245). GlyT-1 blockers affect the protein via discrete binding sites, and sarcosine-based inhibitors such as Org25935 and Org24598 exhibit a non-competitive mode of action, meaning that their effect is independent of basal glycine concentrations (287). This profile may stabilize extracellular glycine levels both in high- and low-glycinergic subjects and an independency of the rat's endogenous glycine level may partly underlie the consistent anti-alcohol intake effect of GlyT-1 blockade. Moreover, an overrepresentation of glycine-dopamine responders in ethanol high-preferring rats compared to naïve rats has been reported (245), suggesting that a pronounced glycine-dopamine interaction, which in turn produces significant dopamine increases (dopamine responders), may be linked to high ethanol preference and ethanol reinforcement.

The addictive and neurotoxic potential of GlyT-1 inhibitors

Drugs that increase dopamine levels in nAc after systemic administration may be associated with risk for abuse. There is no published literature available on the effects of Org25935/Org24598 on place preference or self-administration, but as we stand today several points argue against an addictive potential of GlyT-1 blockade. The GlyT-1 inhibitor Org25935 has previously been tested in clinical trials initiated by Organon Labs. (now Merck, Sharp and Dohme) and it is reported that chronic elevation of extracellular glycine in humans appears safe in the clinic (178, 305, 306). It was found that Org25936, like ethanol, elevates dopamine levels in nAc, yet Org25935 provokes a modest and slow increase in dopamine levels. It has been shown in humans that the time course for the dopamine increment is crucial, *i.e.* dopamine levels need to be increased rapidly in order to be associated with a reported 'high', which is considered an important feature of a potentially addictive drug

working via the dopamine system (146). Org25936 has ethanol-like effects on accumbal dopamine, but the brain perceives ethanol through several known primary targets and not only through the GlyR. Therefore, although Org25935 has similar properties as alcohol on accumbal dopamine levels, the interoceptive cues of Org25935 and ethanol in the brain are most likely not the same. Also the indirect mode of increasing dopamine levels by blocking reuptake of glycine rather potentiates the effect of naturally released glycine, which in turn tonically affects dopamine levels. This mechanism may also argue against an abuse-potential of GlyT-1 inhibitors. Nevertheless, in order to minimize the risk for abuse of GlyT-1 inhibitors, e.g. in the treatment of alcohol dependence, they may preferably be administered in slow-release formulae.

GlyT-1 blockers are being developed as potential treatments for schizophrenia and there is considerable literature covering this field of research. Overall, the reported behavioral effects of concern are respiratory depression and motor deficits observed in rodents, rather than the abuse risk potential (287). Several studies have for example evaluated antipsychotic effects of oral administration of glycine, glycine agonists and GlyT-1 inhibitors in man, and in a recent review the question of tentative addictive properties of GlyT-1 blockers was never raised (279). Yet, given that NMDA receptors are implied in the neurotoxicity of excitatory amino acids, also possible neurotoxic effects of GlyT-1 blockers should be considered. A reduction in specific types of Ca^{2+} channels has been reported as a general adaptation to long-term, high dose glycine treatment (306) but overall no major neurotoxic effects (such as excitotoxic damage or degeneration) have been reported for glycine enhancing drugs, including GlyT-1 blockers.

Glycine and dopamine responses placed in a context

Based on the present findings, it is relevant to examine the tentative association between basal glycine levels and basal dopamine levels. Rats with a reactive dopamine system (dopamine responding rats in the present thesis), may respond stronger to ethanol-induced dopamine activation, and thus possibly also to alcohol´s rewarding and positive reinforcing effects. The higher ethanol-liking and reinforcement may in turn result in higher ethanol intake and preference. This trait may reflect a hedonic dopamine-responsive phenotype with vulnerability to develop addictive behavior. Opposite, subjects with lower mesolimbic

dopamine system reactivity may experience less positive pharmacological effects of alcohol (non-responding rats). This could possibly be explained by higher basal glycine levels, which partly substitute and protect the GlyR against ethanol's actions. Consequently alcohol has lower access to the mesolimbic dopamine system, which protects against alcohol liking (see Table 3.)

Table 3. A dopamine high- versus low-responsive phenotype hypothesis

The dopamine high-responsive phenotype Higher hedonic effects of alcohol	Low basal dopamine levels and a higher dopamine responsiveness	Low basal glycine levels and higher dopamine response from GlyT-1 inhibition	Stronger ethanol reinforcement and ethanol liking and higher ethanol preference	Vulnerability to develop addictive behavior
The dopamine low-responsive phenotype Lower hedonic effects of alcohol	High basal dopamine levels and lower dopamine responsiveness	High basal glycine levels and higher saturation of GlyR binding site which partly substitutes and partly antagonizes ethanol-effect on the GlyR	Less prone to ethanol reinforcement and lower ethanol preference	Protection against development of addictive behavior

This interpretation rests on the association between home-cage drinking and ethanol-induced reinforcement (248, 259). The dopaminergic pathway to nAc is implicated in motivation and goal-directed behavior (72, 73, 307, 308) and several lines of evidence link ethanol activation of this system to alcohol consumption (71, 126, 139, 259, 300) . Moreover, GlyT-1 inhibitor- and glycine-interference with the mesolimbic dopamine system represent a central, but probably not the only, event underlying ethanol reinforcement and ethanol drinking behavior. Indeed, alcohol has other neurochemical access points to the mesolimbic dopamine system, and $GABA_A$–, glutamate-, serotonin- and ACh- receptor engagement as well as endocannabinoid and endogenous opioid engagement may also be important for acquisition of alcohol reinforcement.

The heterogeneity of alcohol addiction disorders is reflected in the diversity of symptoms and the great variability in treatment response - only 20-30% respond to acamprosate and

naltrexone - and it is suggested that different profiles of alcohol addiction behavior require tailored pharmacological treatment. This is exemplified by naltrexone, which works better in a subgroup of patients (50). Since GlyT-1 inhibition appears especially effective in reducing alcohol drinking, and since GlyT-1 inhibition has the profound ability to prevent ethanol-induced dopamine activation, a biomarker reflecting 'the dopamine high-responsive phenotype' could be of potential benefit in the process of predicting GlyT-1 treatment response.

The potential of GlyT-1 inhibitors as treatment of alcohol dependence

Since alcoholism is a progressive condition with a full-blown addiction as the end-stage, treatment response may depend on progression state. An implication of the profound GlyT-1 inhibition-dopamine interaction is that GlyT-1 blockers should be applied during the early stage of the addiction cycle, when dopaminergic reward mechanisms still are critical for alcohol drinking behavior (see Introduction, Pathophysiology). Moreover, that the effects of both Org24598 and Org25935 on alcohol drinking were rapidly reinstated after an alcohol-free period implies that GlyT-1 inhibition possibly also could prevent relapse occurrence. Interestingly, a recent study reported a large and consistent increase in glycine levels in nAc during operant responding for ethanol-containing gelatin, suggesting that increases in accumbal glycine levels are related to anticipation of alcohol reinforcement (309). This may in turn imply that a stabilization of glycine levels with GlyT-1 inhibition could interact with alcohol cue-induced dopamine activation and thereby affect craving. Nevertheless, the excellent effect on alcohol consumption proposes that the drug toolkit for alcohol dependence may be expanded from anti-craving and anti-relapse agents, to also include alcohol intake-reducing agents, which would preferably aid to reduce excessive alcohol consumption before the subject has developed a full-blown addiction. On a broader scale one might speculate as to whether a future reward-modulating drug will also be effective in treating other addictive and compulsive behaviors, since a dysfunctional dopaminergic system is seen as a core deficit linked to a number of addictive behavioral profiles.

Hopefully, the development of 'anti-alcohol drinking' pharmacological treatments will provide both support for the patients and aid insight into the underlying pathophysiological mechanisms. As alcohol addiction represents a major health problem and a global concern, a

continued research effort to develop improved medication is warranted. We have found that GlyT-1 blockers are effective in reducing voluntary alcohol consumption in experimental animals, that the availability of extracellular glycine is crucial for regulating alcohol consumption and that the GlyR is involved in alcohol's rewarding and reinforcing effects. This is supported by the findings in the present thesis and in the thesis of Anna Molander (2005). This suggests that the glycinergic system could be an interesting target for 'anti-alcohol drinking' drugs, and thus a good complement to the existing 'anti-relapse' and 'anti-craving' compounds. Of course, a more detailed understanding of the role of glycine as a neurotransmitter in the forebrain, and how it impacts on not only the mesolimbic dopamine system, but on all neuronal activity, is needed.

In summary, the present thesis proposes that elevated glycine levels produced by Org24598 and Org25935 are responsible for the dopamine-interactions and for the effects on alcohol consumption produced by these GlyT-1 inhibitors. In line with the findings pointing to the GlyR as a major modulator of basal mesolimbic dopamine levels and as an access point for ethanol to the mesolimbic dopamine system (reviewed in Söderpalm *et al*., 2009 (246)), the thesis proposes that these effects are mediated primarily by accumbal GlyRs but that also GlyRs in the VTA could be involved. Glycine reuptake blockade maintains stable levels of extracellular glycine, which in turn preserves stability of the dopamine levels. In dopamine high-responsive subjects (responders) this may also result in a significant increase in accumbal dopamine levels. In relation to alcohol, this results in a blocking/saturation mechanism that prevents positive allosteric GlyR modulation by ethanol and a further ethanol-mediated dopamine elevation. The present thesis points to the great potential of GlyT-1 inhibition as a new treatment principle for alcohol dependence or for preventing development of addiction by reducing excessive alcohol consumption. This concept has recently been confirmed by other strands of experimental evidence indicating the potential use of GlyT-1 inhibition in the modification of ethanol drinking (180).

Populärvetenskaplig sammanfattning

Prekliniska studier av GlyT-1 inhibition som ett nytt behandlingskoncept för alkoholberoende

- hur glycinåterupptagshämmare påverkar alkoholintag och alkohols effekter i hjärnans belöningssystem

Alkoholism

Alkoholberoende är en av våra stora folksjukdomar och har vittgående medicinska och sociala konsekvenser, för individen själv, för de närstående och för samhället. Enorma resurser används för vård av alkoholrelaterade sjukdomar och för hantering av alla sociala och ekonomiska konsekvenser. Totalt uppskattas konsekvenser av alkoholkonsumtion kosta det svenska samhället ca 100 miljarder kronor per år. Forskning på alkoholens effekter på hjärnan har resulterat i två läkemedel, naltrexon och akamprosat. Dessa preparat minskar risken för återfall till alkoholmissbruk genom att dämpa begäret efter alkohol, men tyvärr fungerar de endast för 20-30% av alla patienter som behöver hjälp och behovet av nya och mer effektiva läkemedel är stort. Vidare forskning kring alkohols effekter på hjärnan och hur alkohol leder till ett beroende kan ligga till grund för utveckling av nya behandlingsprinciper för denna kroniska återfallssjukdom.

Hjärnans belöningssystem

Hjärnan består av miljarder nervceller som är organiserade i ett invecklat nätverk. Cellerna kommunicerar genom att en ändring i cellens spänningspotential frisätter signalämnen från nervändarna som aktiverar närliggande nervcellers receptorer, små proteiner utanpå cellen, som fångar upp och vidarebefordrar signalen. Därmed aktiveras den nya nervcellen via en spänningspotential, som i sin tur leder till att en signalsubstans utsöndras. Nervceller kan skicka och ta emot olika signalämnen som dopamin, glutamat, glycin, gamma-aminosmörsyra (GABA) och olika små peptider för att kommunicera med varandra. Signalsubstansen dopamin identifierades av Arvid Carlsson, professor emeritus vid Göteborgs Universitet, som år 2000 mottog Nobelpriset i medicin för sitt arbete med

dopaminet och dess funktioner i hjärnan. Utöver sin roll i hjärnans belöningsmekanismer spelar dopamin en avgörande roll i kontroll av motorik och mentala funktioner, något som avspeglas i hur man effektivt kan behandla Parkinson's sjukdom och schizofreni med läkemedel som påverkar dopaminets aktivitet.

Belöningssystemet tillhör den gamla delen av hjärnan och är evolutionärt välbevarat. Genom att förmedla en känsla av belöning när en individ ägnar sig åt beteenden som gynnar dess överlevnad, till exempel att fortplanta sig eller äta, bidrar belöningssystemet till att säkra artens överlevnad. Än idag är mekanismerna hos människa och djur förvånansvärt lika och det gör råtthjärnan till en utmärkt modell för att studera belöningssystemet och dess funktion. Det urgamla systemet var alltså egentligen till för att främja livsviktiga aktiviteter men idag använder människan även olika substitut för att framkalla lustfylldhet och välbehag, fysiologiska såväl som artificiella. Hjärnans belöningssystem är ingen anatomiskt avgränsad del utan snarare olika nervbanor mellan olika hjärnregioner. Nervceller i ventrala tegmentala arean (VTA; strax ovanför hjärnstammen) som sträcker sig till en del som kallas nucleus accumbens (nAc) i framhjärnan, där signalämnet dopamin frisätts, anses utgöra den centrala delen av belöningssystemet. När dopamin utsöndras i nAc ger det en känsla av belöning och lustfylldhet och det leder i sin tur till att man gärna vill upprepa aktiviteten. Jämfört med en ”naturlig” belöning ger alkohol och andra beroendeframkallande droger ett snabbare och starkare dopaminpåslag, som på sikt kan få allvarliga konsekvenser.

Alkohol aktiverar hjärnans belöningssystem

Att vi dricker alkohol är inget nytt påhitt, så långt tillbaka som vi kan följa människan så har hon konsumerat och överkonsumerat alkohol. Kemiskt sett så är den alkohol vi dricker, etanol, en liten och ospecifik substans som påverkar de flesta av hjärnans nervkretsar och som därmed gör alkoholen svår att studera. Däremot är det visat att alkohol aktiverar hjärnans belöningssystem genom att frisätta signalsubstansen dopamin i hjärnregionen nAc och att detta resulterar i en känsla av välbefinnande och kan göra oss glada, upprymda och stimulerade. En dopaminökning i nAc är kopplad till alkoholens positivt förstärkande effekt och utgör med detta en drivkraft till att vilja dricka alkohol. Vid ett kroniskt och långvarigt alkoholintag anpassar sig nervcellerna till alkoholen och belöningssystemet byggs om så att alkoholen inte längre ger en dopaminökning utöver det normala utan snarare krävs för att

bibehålla en normal aktivitet i systemet. Övergången från att alkohol ger en känsla av belöning till att den krävs bara för att man ska må som vanligt tros vara en grundläggande förändring vid utveckling av ett alkoholberoende. Teorin stöds av att man har påvisat lägre aktivitet i belöningssystemet under normala förhållanden hos beroendesjuka och reducerade dopaminökningar som svar på naturliga belöningar så som mat, social samvaro och sex. Aktivering av hjärnans belöningssystem är också viktig för minne och inlärning, där begäret efter alkohol kan ses som ett sjukligt överinlärande. Hjärnans belöningssystem borde alltså vara en lämplig måltavla för utveckling av nya läkemedel för behandling av alkoholism.

Avhandlingsarbetet

Exakt hur alkohol frisätter dopamin i nAc är inte fastställt. Det är känt att alkohol direkt aktiverar jonkanalreceptorer i hjärnan och att det efter denna första aktivering följer en kaskad av sekundära effekter som påverkar hjärnan och tillsammans förmedlar alkoholens olika effekter. Forskning i gruppen för Beroendemedicin, under ledning av professor Bo Söderpalm, har visat att en receptor för signalämnet glycin, glycinreceptorn (GlyR), är involverad i alkoholens aktivering av belöningssystemet och att GlyR även håller mängden dopamin på en viss nivå genom att indirekt kontrollera aktiviteten i dopaminnervcellerna som sträcker sig från VTA till nAc. Anna Molanders avhandlingsarbete från 2005 visade att signalsubstansen glycin påverkar såväl basal som alkoholinducerad frisättning av dopamin och vidare att extracellulära halter av glycin reglerar alkoholkonsumtion. Med extracellulära halter menas den mängd av glycin som finns tillgänglig i utrymmet mellan den cell som skickar en signal med hjälp av glycin och den cell som tar emot signalen med hjälp av GlyR. Dessa resultat tyder på att alkoholens belönande effekter kan moduleras genom läkemedel som höjer eller stabiliserar glycinnivån i hjärnan. Under normala omständigheter regleras extracellulära glycinhalter huvudsakligen av glycintransportör-1 proteinet (GlyT-1) som sitter på närliggande celler och som rensar upp glycin och därmed reducerar glycinhalterna till normal nivå efter frisättning i utrymmet mellan cellerna, synapsen. Org25935 och Org24598 är två GlyT-1 hämmare som höjer glycinhalterna genom att stänga av GlyT-1.

Med avsikt att utreda om man genom att hämma GlyT-1 kan utarbeta en ny behandlingsprincip för alkoholberoende studerar avhandlingsarbetet effekten av att stänga av GlyT-1 på alkoholkonsumtion och på alkoholinducerad utsöndring av dopamin i nAc. Härmed kopplas effekter på alkoholkonsumtion samman med effekter på alkoholens aktivering av hjärnans belöningssystem, som tros vara en viktig neurobiologisk faktor för utveckling av ett beroende. Effekten av två olika GlyT-1 hämmare på alkoholkonsumtion studerades i en drickmodell där råttor fritt fick välja mellan att dricka vatten eller en 6 % alkohollösning. För att undersöka effekter av alkohol och olika behandlingar med GlyT-1 hämmare i nAc användes mikrodialys på vakna råttor. Prover av den extracellulära vätskan togs lokalt i hjärnan och analyserades för att studera förändringar i dopamin-och glycinnivåer.

Det här avhandlingsarbetet stödjer tidigare resultat om att GlyR i nAc utgör en viktig angreppspunkt för alkohol vad gäller dess dopaminaktiverande och belönande effekter. Resultaten pekar på att signalsubstansen glycin, som hittills varit mest känd för sina funktioner i ryggmärgen, också har en betydelsefull roll i framhjärnan. Två olika GlyT-1 hämmare ger en robust och långvarig sänkning av alkoholintag, stabiliserar dopaminhalterna i nAc och motverkar alkoholens dopaminstimulerande effekt i belöningssystemet, troligtvis genom att höja och stabilisera glycinnivån i hjärnan. Tillsammans föreslår resultaten GlyT-1 blockad som ett nytt behandlingskoncept för ett etablerat alkoholberoende och/eller för att reducera riskfylld alkoholkonsumtion i syfte att förhindra utvecklingen av alkoholberoende.

Acknowledgements

I would like to especially thank the following people who have helped me create this thesis:

My supervisor professor **Bo Söderpalm**, for all your intelligent thoughts and for continuous enthusiasm and support during the ups and downs of the present project. For believing in me and introducing me to the field of alcohol research.

My co-supervisor **Mia Ericson**, for plenty of help, for your sharp mind and the ability to put ideas into practice.

All my great colleagues at **Addiction Biology Unit** at Section for Psychiatry and Neurochemistry. I am especially grateful for good collaboration, technical assistance, scientific discussions and all other help from: **PeiPei Chau, Susanne Jonsson, Rosita Stomberg, Chris Pickering, Louise Adermark, Elin Löf** and from the former members of Addiction Biology Unit: **Anne Fagerberg, Rhona Clarke, Nora Kerekés and Anna Molander**.

Hugh Marston and **Glenn Walker**, for great collaboration and assistance and for valuable intellectual input (Merck Sharp & Dohme, former Organon Labs. Ltd., Newhouse, Lanarkshire, UK).

My husband **Henrik**, for love, support and encouragement and for coping with all my peculiarities. My daughter **Liv**, for the infinite love I have to you and for making life joyful and meaningful. The child I am bearing, for being such good company when writing the thesis. Pappa **Jan**, my sister **Kaja** and my parents in law **Maud** and **Tommy**, for love and great support.

All family and friends outside the lab, no one mentioned and no one forgotten.

The work was financial supported by Merck Sharp & Dohme, former Organon Labs. Ltd., Newhouse, Lanarkshire, UK, the Swedish Medical Research Council and government support under the LUA/ALF agreement, the Swedish Labor Market Insurance (AFA) support for biomedical alcohol research, the Alcohol Research Council of the Swedish Alcohol Retailing Monopoly, Bror Gadelius Foundation, Sigurd and Elsa Goljes Foundation, Wilhelm and Martina Lundgrens Scientific Foundation, Lars Hiertha foundation, and Gunnar and Märta Bergendahl Foundation.

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