The role of taurine-related compounds on the effects of ethanol in the rat mesolimbic dopamine system

Karin Ademar

Department of Psychiatry and Neurochemistry

Institute of Neuroscience and Physiology

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2025

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karin.ademar@gu.se

ISBN 978-91-8069-947-1 (PRINT)
ISBN 978-91-8069-948-8 (PDF)
http://hdl.handle.net/2077/84036

Printed in Borås, Sweden 2025
Printed by Stema Specialtryck AB

Till Pappa

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Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology
Sahlgrenska Academy, University of Gothenburg
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ABSTRACT

Alcohol use disorder (AUD) is a major contributor to the global burden of disease. The reinforcing properties of alcohol involve enhanced dopamine activity in the nucleus accumbens (nAc), an important part of the mesolimbic dopamine system. For ethanol to induce accumbal dopamine elevation, a concomitant increase in endogenous taurine within the nAc appears to be required. However, the cellular origin of this ethanol-mediated increase in extracellular taurine levels remains unknown. Interestingly, one of the available treatments for AUD is a homotaurine derivative, acamprosate (Campral®; calcium-bis(*N*-acetylhomotaurinate)). Although it has been used for decades, the exact mechanisms underlying acamprosate's anti-relapse effects are still controversial and unknown. The overall aim of this thesis was not only to define neurobiological mechanisms contributing to the rewarding properties of ethanol, but also to identify novel targets for pharmacological treatments. To this end, *in vivo* microdialysis, combined with pharmacological, chemogenetic and metabolic approaches, and behavioral paradigms were performed in male Wistar rats. We demonstrate that the ethanol-induced elevation of extracellular taurine levels in the nAc was blocked by local administration of an L-type calcium channel antagonist and not prevented by inhibition of action potential firing, while specific manipulation of astrocytes did not affect the evoked release. Acamprosate elevated nAc dopamine levels in a glycine receptor-sensitive manner, possibly through a simultaneous taurine release. Moreover, co-administration of calcium and *N*-acetylhomotaurine produced an enhanced dopamine and taurine output compared to the drugs

administrated alone, suggesting that the two components act in concert on a neurochemical level and that both parts of acamprosate in fact are biologically active. The acute effect of a combination of calcium and *N*-acetylhomotaurine administration abolished the alcohol deprivation effect (ADE), while long-term treatment did not. Following acute and repeated calcium treatment, the same effects on ADE outcome were observed. Additionally, the dopamine-elevating properties of calcium were lost after sub-chronic calcium administration. Thus, tolerance development was evident in long-term calcium-treated rats. In conclusion, the results indicate that ethanol-induced taurine elevation may originate from neurons in an action-potential-independent manner. Furthermore, this thesis shows that both calcium and the *N*-acetylhomotaurine in acamprosate have important roles in the mechanism of action of the drug, while calcium appears prominent for the tolerance development previously observed for acamprosate. Even if tolerance development is observed, the results implicate an important role of calcium on the acute effects of ethanol, and that it may serve as a treatment supplement for some patients with AUD.

Keywords: Acamprosate, dopamine, ethanol, nucleus accumbens, taurine

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ISBN 978-91-8069-948-8 (PDF)

SAMMANFATTNING PÅ SVENSKA

Betydelsen av taurin-relaterade substanser för alkohols effekter i det mesolimbiska dopaminsystemet hos råtta

Alkoholbrukssyndrom är en stor bidragande orsak till den sjukdomsbörda som ses världen över. De belönande och förstärkande egenskaperna som alkohol har, involverar ökad dopaminaktivitet i hjärnregionen nucleus accumbens (accumbenskärnan), vilket är en viktig del av hjärnans belöningssystem och det mesolimbiska dopaminsystemet. För att alkohol ska kunna skapa en ökning av dopamin i nucleus accumbens, verkar en samtidig ökning av endogent taurin inom samma hjärnregion vara nödvändig. Vilka celler som bidrar med den extracellulära ökningen i taurin efter exponering av alkohol är däremot inte kända. Intressant nog innehåller ett av de läkemedel som används för alkoholbrukssyndrom taurinmolekylen, nämligen akamprosat (Campral®; kalcium-bis(*N*-acetylhomotaurinat)). Trots att akamprosat har använts i flera årtionden, är de exakta mekanismerna bakom dess förmåga att förhindra återfall till alkoholintag fortfarande kontroversiella och inte helt kända. Det övergripande syftet med denna avhandling var inte bara att definiera neurobiologiska mekanismer som bidrar till alkohols belönande egenskaper, utan också att identifiera nya mål för farmakologiska behandlingar. I detta syfte användes *in vivo* mikrodialys i kombination med farmakologiska, kemogenetiska och metaboliska strategier för att provta och analysera dopamin samt taurin i hjärnan, och även beteendemodeller för att studera alkoholkonsumtion hos Wistar råttor. Vi fann att den alkoholinducerade ökningen av extracellulära taurinnivåer blockerades av en kalciumkanalantagonist av L-typ, vilken administrerades lokalt i nucleus accumbens, och att taurinökningen inte förhindrades vid hämning av nervcellers aktionspotentialavfyrning. Specifik manipulering av astrocyter påverkade inte den alkoholframkallade taurinökningen, som var fortsatt intakt. Akamprosat förhöjde dopaminnivåerna i belöningssystemet genom förstärkt aktivering av glycinreceptorer, möjligtvis genom en samtidig frisättning av taurin. Dessutom resulterade den kombinerade administreringen av kalcium och *N*-acetylhomotaurin i ett förstärkt dopamin- och taurinsvar jämfört med när dessa substanser administrerades var för sig, vilket tyder på att de två komponenterna som utgör akamprosat samverkar på neurokemisk nivå och att de båda enheterna i själva verket är biologiskt aktiva. Den förväntade ökning i alkoholintag som normalt ses hos råttor efter två veckors avhållsamhet uteblev helt vid akut behandling med kombinationen av kalcium och *N*-

acetylhomotaurin, medan en sådan ökning sågs hos de djur som fått upprepad behandling. Vid akut och upprepad behandling med enbart kalcium, sågs samma effekter på det förväntade ökade alkoholintaget efter påtvingad avhållsamhet som för kombinationsbehandlingen. Närmare bestämt förhindrade akut behandling ett ökat alkoholintag, medan upprepad behandling inte gjorde det. Dessutom förlorade kalcium sina dopaminhöjande egenskaper efter subkronisk kalciumadministrering. Således visade råttor som blivit behandlade med kalcium över längre tid en påtaglig toleransutveckling. Sammantaget indikerar resultaten att alkoholframkallad ökning av taurin i nucleus accumbens kan ha sitt ursprung från nervceller via en mekanism som är oberoende av aktionspotential. Vidare visar data att både kalcium och *N*-acetylhomotaurin i akamprosat har viktiga roller för läkemedlets verkningsmekanism, medan kalcium verkar spela en framträdande roll för den toleransutveckling som tidigare noterats för akamprosat. Trots att toleransutveckling observeras, visar resultaten på en viktig roll för kalcium på alkoholens akuta effekter och att kalcium förslagsvis kan fungera som ett kompletterande behandlingstillägg för en viss grupp av patienter med alkoholbrukssyndrom.

LIST OF PUBLICATIONS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Ademar K**, Ulenius L, Loftén A, Söderpalm B, Adermark L, Ericson M.
Separate mechanisms regulating accumbal taurine levels during baseline conditions and following ethanol exposure in the rat. *Scientific Reports* 2024 Sep 26:14(1):24166

II. **Ademar K**, Adermark L, Söderpalm B, Ericson, M.
Sodium acamprosate and calcium exert additive effects on nucleus accumbens dopamine in the rat. *Addiction Biology* 2022 Sep;27(5):e13224

III. **Ademar K**, Loftén A, Nilsson M, Domi A, Adermark L, Söderpalm B, Ericson M.
Acamprosate reduces ethanol intake in the rat by a combined action of different drug components. *Scientific Reports* 2023 Oct 19;13(1):17863

IV. **Ademar K**, Danielsson K, Söderpalm B, Adermark L, Ericson M.
The effects of sub-chronic calcium treatment on ethanol-induced dopamine elevation and the alcohol deprivation effect in the rat. *Submitted 2024.*

The original publications and manuscripts are appended at the end of the thesis.

TABLE OF CONTENTS

ABBREVIATIONS

5-HT_3R	5-hydroxytryptamine (serotonin) subtype 3 receptor
ADE	Alcohol deprivation effect
ANOVA	Analysis of variance
ARRIVE	Animal Research: Reporting of *In vivo* Experiments
AUC	Area under the curve
AUD	Alcohol Use Disorder
Ca^{2+}	Calcium
CaAcamp	Acamprosate
$CaCl_2$	Calcium chloride
CIN	Cholinergic interneuron
CNO	Clozapine *N*-oxide dihydrochloride
CNS	Central nervous system
D1	Dopamine receptor type 1
D2	Dopamine receptor type 2
DCPIB	Oxobutyric acid
DMSO	Dimethyl sulfoxide
DREADD	Designer receptor exclusively activated by designer drug
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th edition
EMA	European Medicines Agency
EtOH	Ethanol
FC	Fluorocitrate
FDA	Food and Drug Administration
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid receptor subtype A
$GABA_BR$	γ-aminobutyric acid receptor subtype B
GES	Guanidinoethyl sulfonate
GFAP	Glial fibrillary acidic protein
GLP-1	Glucagon-like peptide-1
GlyR	Glycine receptor
GPCR	G protein-coupled receptor
HPLC	High performance liquid chromatography
ICD-11	International Statistical Classification of Diseases and Related Health Problems, 11th revision
i.p.	Intraperitoneal

LDTg	Laterodorsal tegmental nucleus
LRRC8	Leucine-rich repeat-containing protein 8A
LTCCs	L-type calcium channels
Mem	Memantine
mGluR	Metabotropic G protein-coupled glutamate receptor
MSN	Medium spiny neuron
NaAcamp	Sodium acamprosate
nAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NCD	Nicardipine
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
OPRM1	Opioid Receptor Mu 1
PAT1	Proton-coupled β-amino acid transporter
PFC	Prefrontal cortex
PPN	Pedunculopontine nucleus
PREPARE	Planning, Research and Experimental Procedures on Animals: Recommendations for Excellence
TauT	Taurine transporter
TLV	The Dental and Pharmaceutical Benefits Agency
TTX	Tetrodotoxin
VRAC	Volume regulated anion channel
VTA	Ventral tegmental area
WHO	World Health Organization

1 INTRODUCTION

By activating the brain reward system, alcohol increases extracellular dopamine levels, which in turn is associated with reward and addictive responses. This section will provide an overview of alcohol and the brain reward system as well as the interaction between the two.

1.1 ALCOHOL AND ALCOHOL USE DISORDER

Recreational consumption of alcohol has taken place worldwide for thousands of years. Today, the level of drinking is geographically connected to high-income countries (WHO 2024a), where the experience of pleasure, relaxation and disinhibition in combination with reduced anxiety and stress often is the driving force for moderate intake. However, even when alcohol is consumed in moderation, it is related to health risks and harm. Globally, alcohol use is considered as one of the leading risk factors for disease and mortality, and approximately 3 million people die annually due to alcohol-related complications (WHO 2024a). In addition to causing significant health problems to the afflicted individual, alcohol consumption also causes social issues, including suffering for their significant others, and poses an economic burden to the society (Manthey et al 2021, Rehm & Shield 2019). In 2017, the societal costs due to alcohol consumption in Sweden were estimated to 103 billion SEK, including direct costs as healthcare expenses and indirect costs as loss of productivity (Folkhälsomyndigheten 2024). Most individuals are capable to consume alcohol in moderation without developing an addiction, however, when the intake for some people progresses, becomes compulsive and uncontrolled despite negative consequences, alcohol use disorder (AUD) has developed. This disease is a chronic and relapsing brain disorder, thus the individual can go into remission but not be cured. The diagnosis of AUD is based on meeting a certain number of criteria gathered into diagnostic manuals, such as the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5) by the American Psychiatry Association (APA 2013), or the International Statistical Classification of Diseases and Related Health Problems 11th revision (ICD-11) by the World Health Organization (WHO) (WHO 2024b). The diagnostic manuals differ in their included criteria and while the DSM (Table 1) is restricted to cover mental disorders and emphasize the actual diagnostic criteria, ICD has a broader scope covering overall health,

including mental disorders, and emphasizes clinical judgement in making diagnoses (Tyrer 2014). Worldwide, AUD is one of the most prevalent psychiatric disorders, with approximately four percent of the Swedish (CAN 2022) and eleven percent of the American (SAMHSA 2022) adult population meeting the diagnostic criteria.

Table 1. *Diagnostic and Statistical Manual of Mental Disorders, 5th edition guidelines for diagnosis of Alcohol Use Disorder. The diagnosis is met when two of the symptoms are fulfilled over a 12-month period. The severity of the disorder can be defined as mild (2-3 criteria), moderate (4-5 criteria) or severe (>6 criteria) depending on the number of criteria fulfilled.*

1	Alcohol is consumed in larger quantities or for longer time periods than desired
2	Inability to cut down or control the drinking despite a desire to do so
3	A substantial amount of time is spent on obtaining, using or recovering from alcohol use
4	Craving or an intense desire to use alcohol
5	Frequent alcohol use leading to an inability to manage work, school, or home
6	Continued alcohol use despite knowledge that it results in recurring social or interpersonal problems
7	Reducing or giving up important social, professional or enjoyable activities because of alcohol use
8	Persistent alcohol use in physically hazardous situations
9	Continued alcohol use despite knowledge that a persistent physical or mental problem has been caused or worsened by alcohol use
10	Tolerance – the need to use increased amounts of alcohol to achieve the desired effects, or undesired effects when consuming the same amount as before
11	Withdrawal – the occurrence of a characteristic group of physical effects or symptoms that emerges when the amount of alcohol in the body decreases

Long-term alcohol use and misuse is associated with more than 200 medical conditions, including various forms of cancers, intentional and unintentional injuries, cardiovascular and circulatory diseases, gastrointestinal diseases such

as liver cirrhosis and pancreatitis, infectious diseases and other psychiatric disorders (Rehm et al 2017), where the outcome is related to drinking pattern and amount of alcohol consumed (Shield et al 2013). Thus, preterm death and reduced lifetime expectancy is a common consequence following harmful alcohol use and the related medical conditions.

1.1.1 ETIOLOGY AND RISK FACTORS

The development of AUD is an ongoing process, taking place over several years characterized by excessive alcohol consumption, ultimately leading to maladaptive behaviors. It can manifest differently between individuals, making AUD a heterogeneous disorder, as patients can meet different diagnostic criteria and not share any symptoms and still end up with the same diagnosis. The exact etiology of AUD is unknown, it is, however, known to be a multifactorial disorder where the susceptibility for development is controlled by both genetic and environmental factors and the complex interplay between the two. In addition, epigenetic adaptations are likely to play a role in the progress of the disorder (Qiang et al 2014, Shukla et al 2008). Altogether, there is a great individual variability, with influence of various risk factors, in the propensity to develop AUD.

The hereditary component of the disorder is estimated to approximately 50 percent for both men and women as shown by several family-, twin- and adoption studies (Cloninger et al 1981, McGue et al 1992, Prescott et al 1999, Verhulst et al 2015), with the remaining 50 percent attributed to environmental factors. A childhood characterized by heavy parental drinking will influence the functionality of the family, the parent-child relationship as well as parental supervision and discipline skills, leading to an adverse development of the afflicted children and increased risk for AUD progression during adolescence or later in life (Latendresse et al 2008). Childhood abuse or trauma and social stressors such as low socio-economic status and racial discrimination are also risk factors for AUD development (Amaro et al 2021, Shin et al 2009). Genetic variation, polymorphism, in numerous genes has been linked to predisposition of AUD. Several polymorphisms linked to alcohol intake have been associated with genes involved in brain signaling systems, such as genes encoding dopamine type 2 receptors (Blum et al 1990), γ-aminobutyric acid receptors type A ($GABA_AR$) (Edenberg et al 2004), muscarinic acetylcholine receptors (Luo et al 2005), opioid receptors (Ray & Hutchison 2004), and in the metabolism of alcohol, such as genes encoding alcohol dehydrogenase and aldehyde dehydrogenase (Shen et al 1997). However, polymorphisms in the

latter genes have been shown to protect against AUD, in contrast to the former ones, as an accumulation of the toxic alcohol metabolite acetaldehyde occurs leading to aversive effects following alcohol intake. These genetic variations are more common in east Asian populations (Wolff 1972), but are also present in populations worldwide (Dick & Bierut 2006), and the consequence of the altered enzymatic activity is generally lower or no alcohol consumption in these individuals. Attempts to subtype AUD were made early, where the most frequently used is the heredity-based type I-type II typology (Cloninger et al 1981, Cloninger et al 1988). Type II AUD is distinguished by showing a prominent genetic component without environmental interference, an early onset, and the association with impulsive traits and antisocial behavior. In the type I subpopulation, the onset is later in life and the genetic predisposition is relatively low, whereas environmental factors are dominating and proposed to be the limiting factor for development of the disorder (Cloninger et al 1988).

1.1.2 PHARMACOTHERAPY

Today, the pharmacotherapeutic range of medications approved for treatment of AUD is small, with currently four compounds approved for treatment by the European Medicines Agency (EMA), and their effect sizes are limited (Jonas et al 2014). Beyond the approved substances, there are other drugs prescribed off-label, such as topiramate, aripiprazole, varenicline, ondansetron, and baclofen, of which the latter is approved for AUD in France (de Beaurepaire & Rolland 2022). Despite the high prevalence of AUD, it is an undertreated disorder with a large treatment gap (Kohn et al 2004, Mekonen et al 2021). Even when people with AUD seek help from the healthcare, pharmacological medication is only offered to a minority of the afflicted individuals (Mekonen et al 2021). Finding novel pharmacological treatment targets for AUD is crucial to improve and broaden the current treatment arsenal and to reduce the treatment gap.

Disulfiram

Disulfiram (Antabuse®) was the first pharmacological compound available for treatment of AUD, launched during the 1950s. The mechanism of action is based on the substance blocking the normal degradation of alcohol in the liver by inhibiting acetaldehyde dehydrogenase, causing an accumulation of the toxic metabolite acetaldehyde that produces aversive effects following alcohol consumption (Hald & Jacobsen 1948). The avoidance of the discomforting disulfiram-alcohol reaction is the primary motive for staying abstinent from alcohol.

Naltrexone

Naltrexone (Revia®, Vivitrol®) acts as an opioid receptor antagonist, with an affinity for both the μ-, κ-, and δ-opioid receptor, leading to blockade of mesolimbic dopamine release following alcohol consumption. This mechanism decreases alcohol's rewarding effects and reduces heavy drinking, but also the motivation for alcohol use, and thereby craving, and the risk for relapse (Sinclair 2001). Naltrexone has been suggested to be more effective in and benefit individuals who carry a specific gene variant encoding the μ-opioid receptor (OPRM1) (Garbutt et al 2014), but recent human studies do not confirm a positive correlation between OPRM1 and better treatment response to the drug (Witkiewitz et al 2019). Another hypothesis is that naltrexone benefits individuals whose drinking primarily is driven by positive reinforcement (reward drinkers) compared to those whose drinking is driven by negative reinforcement (relief drinkers) (Mann et al 2018).

Nalmefene

Nalmefene (Selincro®) is approved for treatment by EMA in Europe, but not by Food and Drug Administration (FDA) in the US, and, like naltrexone, it is an opioid receptor antagonist. In contrast to naltrexone, nalmefene acts as a partial agonist at the κ-opioid receptor (Swift 2013), and can be taken "as-needed" when alcohol cravings are the hardest (Gual et al 2013). This makes nalmefene a suitable option for individuals who do not require daily treatment. However, nalmefene is not funded by The Dental and Pharmaceutical Benefits Agency (TLV) in Sweden due to lack of long-term studies comparing the effects of naltrexone and nalmefene as well as nalmefene being more expensive (TLV 2015).

Acamprosate

Acamprosate (Campral®) is a derivative of homotaurine and even though being available as a treatment for AUD since 1989 (Mason 2001), the precise mechanism of action is still not entirely understood. Initially, acamprosate was suggested to attenuate the hyperglutamatergic state associated with alcohol withdrawal, by modulating glutamatergic receptors (Rammes et al 2001), and/or to act by normalizing altered GABAergic- and glutamatergic neurotransmission evoked by chronic alcohol consumption (Mann et al 2008, Pierrefiche et al 2004). Other studies have suggested that acamprosate mimics alcohol's effects in the nucleus accumbens (nAc) via interaction with glycine receptors (GlyR) (Chau et al 2010a, Chau et al 2018), and therefore partially acts as a substitution therapy. The mechanism of action has further been ascribed to the calcium part of acamprosate, whereas the homotaurine moiety

has been proposed to be biologically inert (Spanagel et al 2014). Clinically, acamprosate reduces the risk of abstinent individuals to return to any drinking by decreasing craving, and thus works as relapse prevention (Maisel et al 2013, Rösner et al 2010).

Pipeline research

Research advancements have found a selection of promising pharmacological targets for treatment of AUD including the neuropeptides glucagon-like peptide-1 (GLP-1), orexin, somatostatin, nociceptin, and neuropeptide S (Brockway & Crowley 2024). Targeting either the specific peptide receptor or receptors located on the specific peptide neurons both show positive results for treatment of various alcohol-related conditions (Brockway & Crowley 2024). Beyond finding novel targets and developing new pharmacological compounds, the concept of repurposing drugs can be applied. This means that already available substances can include new indications if promising results are found in preclinical and clinical studies. One interesting candidate is the GLP-1 receptor agonist semaglutide (Ozempic®, Wegovy®) approved for diabetes mellitus type II and obesity. Preclinical studies demonstrate that semaglutide reduces alcohol intake in both female and male rodents (Aranäs et al 2023, Chuong et al 2023), and multiple clinical trials examining the effect of semaglutide on alcohol intake are ongoing (ClinicalTrials.gov 2024). In addition, recently the combined administration of the partial nicotinic acetylcholine receptor (nAChR) agonist and smoking cessation compound varenicline and the catecholamine reuptake inhibitor and antidepressant agent bupropion has shown promising clinical results, and further clinical development is pending (Söderpalm et al 2023).

1.2 NEUROBIOLOGY OF ADDICTION

Addiction is a complex human disorder with neurobiological alterations at multiple levels in various brain structures and circuits involved in behavior and motivation, which ultimately result in long-term manifest neuronal abnormalities in connecting pathways. These neuroadaptations are interacting in a complicated manner and typically characterize the addictive phenotype through loss of control in limiting drug intake, compulsion to seek and take drug despite negative consequences, and development of a negative emotional state when taking the drug is hindered (Koob & Le Moal 1997, Koob & Volkow 2010, Koob & Volkow 2016). The neurobiology of addiction is multidimensional, consequently different neurobiological theories of addiction have been proposed in line with continuous ongoing research. Common for all theories is the definition of addiction as a chronic disorder, the theories are thus not mutually exclusive but instead extend and improve earlier ones (Alvarez-Monjaras et al 2019, Ferrer-Pérez et al 2024). This thesis will cover the idea of dopamine as a central neurotransmitter in addiction, thereby focusing on the mesolimbic dopamine system, known as a part of the brain's reward system with dopaminergic neurons originating in the ventral tegmental area (VTA) in the midbrain projecting to the nAc in the striatum (Fig. 1). The attention will focus on the brain region nAc as well as the neurotransmitter dopamine and the neuromodulator taurine.

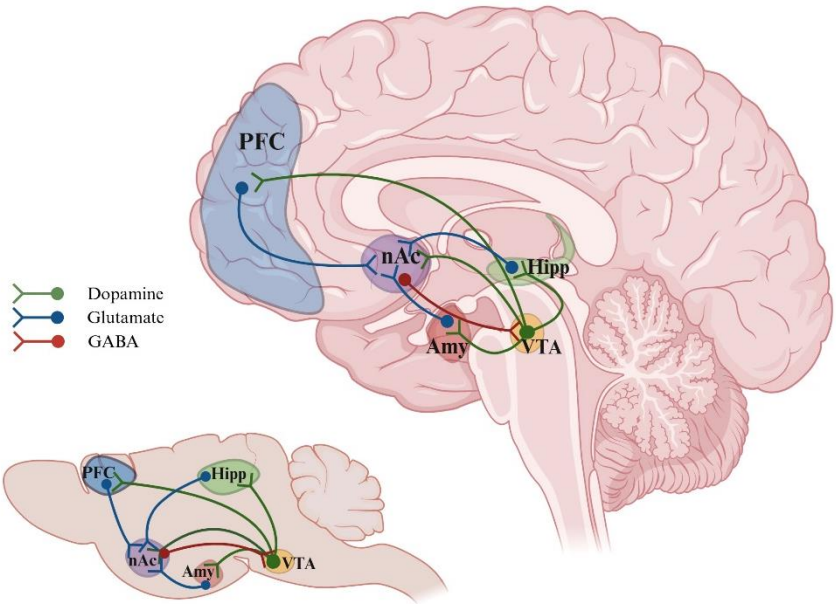

Figure 1. The major dopaminergic pathways in the brain. *Schematic representation of dopaminergic projections in the human and rat brain. Dopaminergic cell bodies in the ventral tegmental area (VTA) project axons to the nucleus accumbens (nAc), prefrontal cortex (PFC), amygdala (Amy), and hippocampus (Hipp). Visualized are also glutamatergic afferents to the nAc, and GABAergic efferents from the nAc. Adapted from (Loftén 2024). Image created using BioRender.com.*

1.2.1 NEUROCIRCUITRY OF DRUG ADDICTION

Addiction has been described as a progression from impulsive to compulsive drug intake in a recurring cycle of three different stages comprising binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation, where each stage is associated with some central brain regions and their integrated networks (Koob & Le Moal 1997, Koob & Volkow 2010, Koob & Volkow 2016). Consistent with repeated drug exposure, the cycle intensifies, resulting in greater physical and psychological harm (Fig. 2).

The binge/intoxication stage involves sub-regions of the striatum, primarily the nAc and dorsal striatum. At the time of initial drug use, the mesolimbic dopamine pathway is activated, leading to increases in extracellular dopamine

levels in the nAc (Di Chiara & Imperato 1988, Wise & Rompre 1989). This is a critical event for mediating the acute rewarding and reinforcing effects of the drug, but also for natural rewards such as eating and reproduction (Koob & Volkow 2010, Volkow et al 1999). Along with increased dopamine signaling, the brain's opioid system is also activated by drugs of abuse. The subjective perception of the acute effect is highly dependent on the pharmacokinetic properties of the drug, where the rate, amount, and duration of the dopamine increase is associated with greater intensity of euphoria or "high" (Koob & Volkow 2010, Volkow et al 1999). The reason as to why most drugs of abuse are smoked or injected can also be related to pharmacokinetics, as these routes of administration generate a more rapid drug delivery. If the initial drug exposure is associated with pleasure, this positively reinforces the drug use and increases the likelihood for the behavior to be repeated. During the binge/intoxication stage, a previous neutral stimulus can become associated with the rewarding effect of a drug, referred to as conditioned reinforcement (Schultz 1997). Over time, by means of associative learning, this paired stimulus will display reinforcing properties in its own, and acts as a cue for drug-seeking behavior, which in turn increases the risk for drug use. The motivation and desire for rewards elicited by drug-associated cues consequently triggering drug-seeking behavior is defined as incentive salience (Robinson & Berridge 1993). Both conditioned reinforcement (stimulus acquiring reinforcing properties) and incentive salience (motivation for or 'wanting' a rewarding stimulus) have been suggested to be involved in cue-induced drug-seeking, self-administration behavior, and probably also in the shift to habit-like compulsive drug-seeking behavior (Koob & Volkow 2016). During the emergence of compulsive and habitual drug use, from impulsive and reward-driven, the dorsal striatum appears to be engaged and have a central role (Everitt et al 2008).

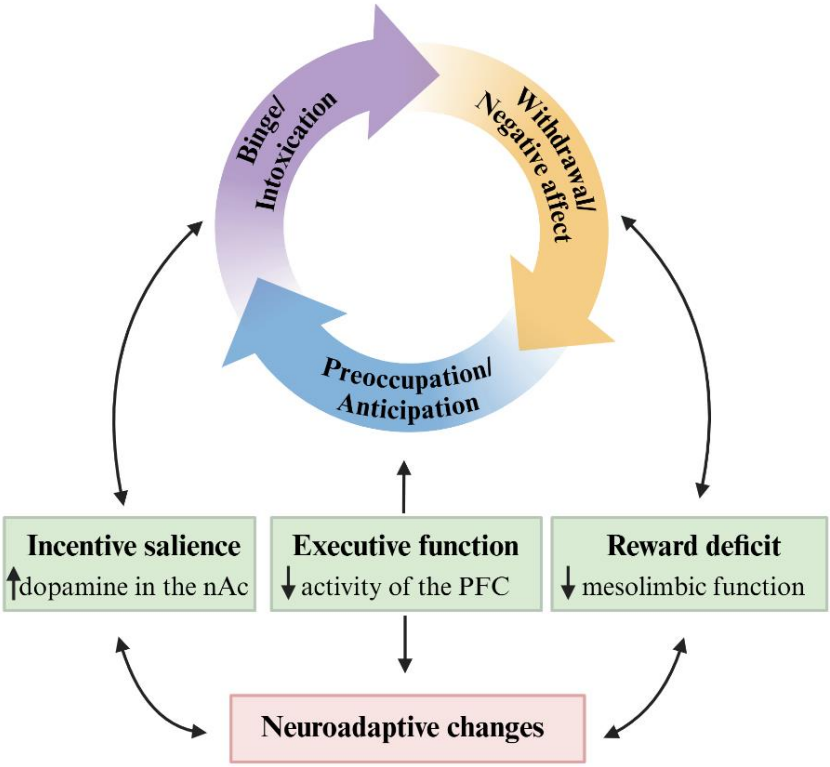

Figure 2. Simplified model of the neurocircuitry of addiction. *Drug intake generates intoxication, and the increased nucleus accumbens (nAc) dopamine levels mediate reward. At this stage, the drug is paired with cues, which trigger drug-seeking behavior and the likelihood of continuous drug administration through incentive salience. With time, a negative affect state emerges when the drug is not on board, as the activity in the mesolimbic dopamine system now is reduced. In addition, the drug-induced dopamine response is reduced, thus a decreased reward and reward deficit. The activity in the prefrontal cortex (PFC) is compromised, resulting in executive control disturbances, further encouraging the incentive salience of continued drug intake. All stages in the reoccurring cycle are associated with neuroadaptive changes, contributing to disorder progression. Modified from (Wise & Koob 2014). Image created using BioRender.com.*

In the withdrawal/negative affect state, there is a shift from reduced activity of the reward circuitry in the mesolimbic dopamine system to increased activity of stress systems in the extended amygdala via stress neurotransmitters such

as dynorphin and corticotropin-releasing factor (Koob & Le Moal 2008, Koob & Volkow 2010). When drug use is discontinued, discomforting withdrawal symptoms emerge as a consequence of drug-induced neuroadaptations, which can be both physical and psychological in nature. The negative emotional state that now fills the individual is a driving force or motivation to continue taking the drug, in order to avoid discomfort. Thus, drug use is now driven by relief and negative reinforcement rather than reward and positive reinforcement (Fig. 2; Fig. 3) (Koob & Le Moal 1997, Koob & Volkow 2010). The dysfunction induced in the mesolimbic dopamine system following long-term drug intake has been shown in human studies to be associated with reduced availability of dopamine D2 receptors, which persists for months after drug withdrawal (Volkow & Fowler 2000, Volkow et al 1997). Together with decreased dopamine response to drug administration (Rossetti et al 1992, Weiss et al 1992), the overall reduced sensitivity of the mesolimbic dopamine system also applies to natural rewards as these depend on the same system and brain circuits for rewarding effects (Garavan et al 2000).

Relapse to drug use is a major problem for individuals with addiction, where reinstatement of drug-seeking behavior and return to compulsive drug intake occur following a period of abstinence. The preoccupation/anticipation stage of the addiction cycle covers the relapse phase, where immense craving is a hallmark driven by both environmental cues in combination with internal states associated with negative emotional state and stress (Koob et al 2014, Koob & Volkow 2010). This stage involves the prefrontal cortex (PFC), the region controlling executive functions, i.e. decision making, working memory, self-regulation, and behavioral inhibition, thus, important for making appropriate choices whether to use drugs or not, and its disruption increases the risk of relapse. When neuronal circuitries in the PFC are triggered by drug-paired stimulus at this stage, it involves neuronal activity in both glutamatergic and dopaminergic pathways projecting from and to the PFC, respectively, leading to an urge to use the drug (Koob & Volkow 2016, Volkow et al 2011). Habitual responses in the dorsal striatum for taking the drug are also promoted, as deficits in inhibitory pathways from the PFC have evolved, and stress neurotransmitters are increased due to intensified activity in the extended amygdala.

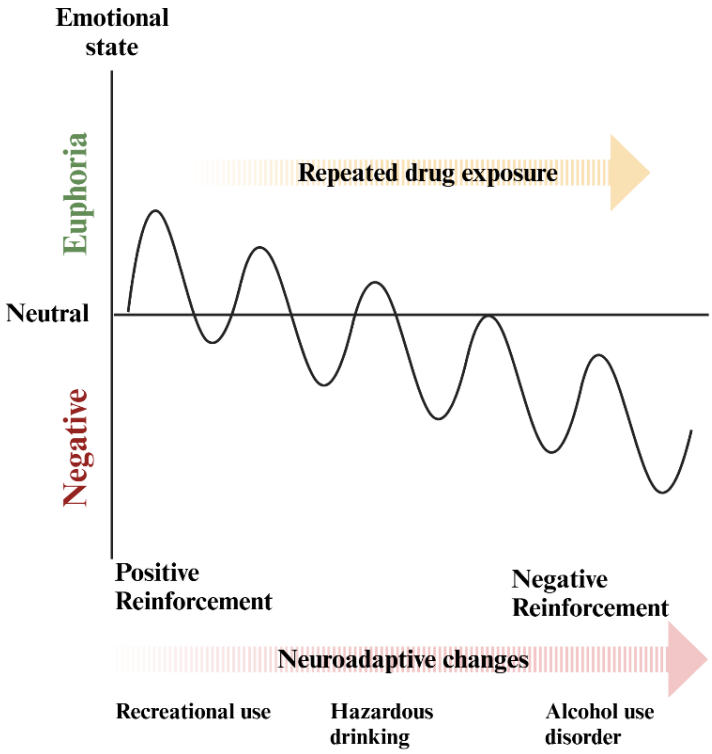

Figure 3. Conceptual overview of long-term drug use and related consequences. *The initial drug experience is associated with and driven by positive reinforcement and reward. With time, the drug intake is driven by negative reinforcement to avoid the negative emotional state that occurs when the drug intake ceases. This is a consequence of the neuroadaptive changes induced by repeated drug exposure. Adapted from (Koob & Le Moal 2001, Loftén 2024). Image created using BioRender.com.*

The progression of addiction includes major neuroadaptive changes in brain regions, as well as multiple neurotransmitter systems, involved in motivated behaviors, stress regulation, and emotions, leading to a pathological state. Reward and positive reinforcing effects of drugs are replaced with compulsion and negatively reinforced drug use. For alcohol and AUD, the mesolimbic dopamine system is assumed to be involved in both the development and maintenance of the disorder. Consequently, the mesolimbic dopamine system is a tentative target for new pharmacological tools for AUD, targeting either alcohol reward or mechanisms underlying relapse after periods of abstinence.

1.2.2 BRAIN REWARD SYSTEM

As depicted above, the brain reward system is involved in regulating reward and motivation, and it is highly essential for the survival of the species as it motivates eating and reproduction. It was in the 1950s that Olds and Milner discovered what later was going to be the modern understanding of the brain reward system (Olds & Milner 1954). Their experiments revealed that rats self-administer electrical currents into certain brain areas by pressing a lever, which implicated the presence of neural structures that provided reinforcement. Shortly after Olds and Milner's discoveries, similar findings were observed in humans (Bishop et al 1963). Since then, several brain structures and neuronal pathways have been defined to be part of mediating reward and reinforcement of both natural rewards and drugs of abuse (Milner 1991). The mesocorticolimbic dopamine system is recognized as the primary pathway within the brain reward system, originating with dopaminergic cell bodies in the VTA with related axons projecting predominantly to the nAc as well as to the PFC, amygdala and hippocampus (Fig. 1). The reinforcing properties of natural rewards and most drugs of abuse are associated with the elevated levels of extracellular dopamine within the nAc obtained after activation of the reward pathway (Di Chiara & Imperato 1988, Wise & Rompre 1989). However, the discrepancy between natural rewards and more or less all drugs of abuse, is that the increase of dopamine in the nAc by natural rewards is essential for survival of the species, as mentioned in the beginning of this section, whereas the dopamine response to drugs of abuse is considerably enhanced and is the reason these substances have the property to "hijack" the reward pathway (Boileau et al 2003, Tan et al 2024, Wise & Rompre 1989).

1.2.2.1 REGULATION OF THE NUCLEUS ACCUMBENS

The nAc, a part of the ventral striatum in the basal ganglia, is considered to consist of a homogeneous composition of cells compared to other brain regions. Out of the neuronal cell types present in the nAc, approximately 95% are GABAergic medium spiny neurons (MSN), whereas the remaining portion constitutes GABAergic interneurons and cholinergic interneurons (CIN) (Robison & Nestler 2011). The MSNs are the accumbal output neurons and important for regulating reward-related behaviors, including alcohol intake (Strong et al 2020). Upon exposure to almost all drugs of abuse, the MSNs change in number, shape and size of dendritic spines (Robinson & Kolb 2004) leading to altered connectivity of the neurons. The GABAergic interneurons and the CINs are localized within the brain region and control local circuit activity as well as provide a modulatory function to both the MSNs, afferent

neurons, and non-neural cells such as astrocytes, microglia, oligodendrocytes and endothelial cells (Chen et al 2021, Robison & Nestler 2011). Astrocytes are actively involved in brain physiology, providing metabolic- and structural support, neuroprotection, and extracellular homeostasis. They are not generating action potentials although they have a hyperpolarized membrane, but still release gliotransmittors such as taurine, glutamine, and aspartate (Benarroch 2005). Astrocytes regulate synaptic transmission and neuroplasticity through the clearance of amino acids by the release of gliotransmittors, and there are indications that astrocytes in the nAc are important for dopaminergic signaling in the brain reward circuit (Corkrum et al 2020). Input to the nAc originates from several brain structures, such as glutamatergic neurons from the PFC, amygdala, and hippocampus mediating excitatory control as well as dopaminergic projections from the VTA modulating the activity and output signal of the accumbal MSNs (Fig. 1).

1.3 DOPAMINE IN THE MESOLIMBIC DOPAMINE SYSTEM

Dopamine synthesis in the central nervous system (CNS) takes place both in neuronal terminals and in cell bodies of dopaminergic neurons in various brain regions, but mainly in VTA and the substantia nigra (Dahlström & Fuxe 1964). The neuronal release is primarily dependent on an action potential to be transmitted through the neuron, resulting in dopamine being released from vesicles into the synaptic cleft by exocytosis, leading to dopamine exerting its effects by binding to dopamine receptors. That the catecholamine dopamine was a neurotransmitter was discovered by Arvid Carlsson and colleagues in the 1950s (Carlsson et al 1958).

1.3.1 DOPAMINE PATHWAYS

The dopaminergic system consists of four main pathways: the mesolimbic-, mesocortical-, nigrostriatal-, and tuberoinfundibular dopamine system. As mentioned above, the midbrain including the VTA and substantia nigra is rich in dopaminergic neurons, making it the origin for three out of four pathways (Dahlström & Fuxe 1964, Moore & Bloom 1978). The mesolimbic dopamine system is involved in reward and reinforcement and its cell bodies originates in the VTA projecting to, via the medial forebrain bundle, limbic structures such as ventral striatum (i.e. the nAc), amygdala, and hippocampus (Fig. 1). In contrast, the dopamine neurons projecting from the VTA to the PFC is called the mesocortical dopamine system and regulates motivation and executive functions. These two pathways, with origin in the VTA, are sometimes referred to as the mesocorticolimbic dopamine system (Russo & Nestler 2013). The nigrostriatal dopamine system comprises projections from the substantia nigra to mainly the dorsal striatum and is involved in motor control (Obeso et al 2008). The tuberoinfundibular pathway has its origin in the arcuate nucleus of the hypothalamus with projections to the pituitary gland, controlling prolactin synthesis and secretion (Fitzgerald & Dinan 2008). Common for the midbrain-originating pathways are their involvement in the pathophysiology of AUD.

The firing pattern of midbrain dopamine neurons is characterized by two distinct modes: tonic firing and phasic burst firing (Grace 2000). Tonic firing is associated with single spike firing patterns, which maintain the baseline level of extracellular dopamine and varies over minutes to hours at lower frequencies (1-5 Hz) (Grace & Bunney 1984b, Hyland et al 2002, Morikawa & Morrisett 2010). When dopamine neurons fire at higher frequencies (10-30

Hz), which fluctuates over faster timescales, burst firing occurs and dopamine levels are rapidly increased (Grace & Bunney 1984a, Morikawa & Morrisett 2010). The maintenance of baseline dopamine levels is dependent on action potential-mediated exocytosis, as exposure to tetrodotoxin (TTX), a sodium channel blocker inhibiting action potential firing, induces a massive drop in extracellular dopamine levels (Jonsson 2012, Ulenius 2019). VTA dopaminergic cells are under excitatory control of mainly glutamatergic projections from the PFC, the pedunculopontine nucleus (PPN), the laterodorsal tegmental nucleus (LDTg), and the lateral hypothalamus (Omelchenko & Sesack 2007), while inhibitory control is maintained by local GABAergic interneurons and GABAergic neurons projecting from the nAc and the ventral pallidum (Conrad & Pfaff 1976). In addition, dopamine neurons are regulated by cholinergic input to the VTA from PPN and LDTg via activation of nAChR (Blaha et al 1996).

1.3.2 DOPAMINE RECEPTORS

In humans, five different dopamine receptor subtypes have been characterized, further divided into two subgroups depending on the pharmacological, structural, and signaling properties. They are termed D1-D5, being sub-grouped as D1-like (D1 and D5) and D2-like receptors (D2, D3, and D4). All of the receptors are G protein-coupled cell-surface receptors, with the difference that the D1-like receptors are Gq coupled, exerting excitatory effects upon activation, while the D2-like receptors are Gi coupled, exerting inhibitory effects upon activation. The receptors are localized postsynaptically (heteroreceptors), however the expression of the D2-like receptors are also found presynaptically and can act as autoreceptors on dopamine neurons (Beaulieu & Gainetdinov 2011). The autoreceptors are predominantly found at extrasynaptic sites (Sesack et al 1994) and provide feedback mechanisms (inhibition) regulating synthesis, release, metabolism, and uptake of dopamine (Ford 2014). Mice genetically lacking D2-receptors are reported to have altered extracellular dopamine levels (Schmitz et al 2002) and show reduced preference for alcohol (Phillips et al 1998). The D2-like autoreceptors are, in comparison to the D1-like postsynaptically positioned receptors, proposed to be more sensitive to dopamine as a consequence of lower exposure to dopamine concentrations (Ford 2014).

1.4 ALCOHOL IN THE MESOLIMBIC DOPAMINE SYSTEM

The alcohol used as a recreational drug by humans is the small two-carbon containing molecule ethanol (C_2H_5OH). It easily crosses biological membranes, including the blood-brain barrier, due to its hydrophilic and lipophilic nature, thereby exerting a number of pharmacological effects throughout the body. Ethanol exposure at low to medium concentrations elicits disinhibiting, simulative, and relaxing effects, while sedative and depressant effects are associated with medium to high concentrations. Very high doses can eventually lead to death (Hendler et al 2013).

In the CNS, ethanol is reported to interact, directly or indirectly, with various receptor types, primarily ligand-gated ion channels but also with metabotropic receptors. Decisive factors for the outcome of the interaction are which receptor subtype that is involved, leading to either inhibition or potentiation of their function (Abrahao et al 2017), the subunit composition of the receptor, the concentration of ethanol, and the time duration of the exposure (Vengeliene et al 2008). Generally, the acute psychotropic effects of ethanol involve inhibition of the N-methyl-D-aspartate receptor (NMDAR) function (Lovinger et al 1989), whilst the function of the $GABA_AR$, the GlyR, the 5-hydroxytryptamine (serotonin) subtype 3 receptor ($5\text{-}HT_3R$), and the nAChR are enhanced (Lovinger 1999, Mihic 1999, Narahashi et al 1999). In addition to receptor interactions, ethanol opens G protein-coupled inward rectifying potassium channels (Kobayashi et al 1999, Lewohl et al 1999) and interacts with the L-type voltage-gated calcium channel (LTCC) (Morton & Valenzuela 2016, Wang et al 1991). The interaction of ethanol with some of these receptor systems, relevant for this thesis, is briefly outlined below.

The response of several neurotransmitter systems is exposure-dependent, i.e. short- or long-term exposure, where glutamate and GABA are the most studied in this context. Short-term ethanol exposure decreases the glutamatergic activity by NMDAR antagonism, while chronic ethanol intake increases the receptor expression and function, probably to counterbalance the inhibitory effects induced by ethanol (Hoffman et al 1990). These adaptive changes may be the cause of the hyperexcitable state of the CNS evoked during ethanol withdrawal (Gass & Olive 2008). Studies investigating antagonists targeting not only NMDARs, but also metabotropic G protein-coupled glutamate receptors (mGluR), suggest that a decreased glutamatergic activity is central for suppressing relapse-like behavior (Bäckström et al 2004, Vengeliene et al

2005). In contrast to glutamate, the $GABA_AR$ function is increased at the acute state of ethanol intake, while prolonged exposure has opposing effects caused by counteradaptive processes (Mihic 1999). The $GABA_AR$ density is reduced, and subunit composition is altered (Golovko et al 2002). By targeting $GABA_AR$ in the VTA, alcohol-preferring P rats demonstrate decreased ethanol consumption, indicating the impact of ethanol on GABAergic transmission in the mesolimbic dopamine system (Nowak et al 1998). Ethanol is further proposed to act as an allosteric modulator on the GlyR at clinically relevant doses, thereby enhancing its function and potentiating the action of the endogenous ligand glycine (Perkins et al 2010). The GlyR shows a high expression pattern in infratentorial structures, but is also expressed within the nAc conceivably on MSNs (Jonsson et al 2012, Molander & Soderpalm 2005b, Muñoz et al 2020). The ability of ethanol to potentiate the receptor depends on the subunit composition (Mascia et al 1996), where the pentameric GlyR can be either α-homomer or α-β-heteromer. α1-containing receptors, rather than α2 and α3 containing receptors, have been suggested to display higher sensitivity for ethanol (Förstera et al 2017, Yevenes et al 2010). Contrariwise, more recent studies demonstrate that α1, -2, and -3 containing GlyRs in the nAc and amygdala are all implicated in ethanol consumption and ethanol-related behaviors (Blednov et al 2015, Gallegos et al 2021, Muñoz et al 2020). By modulating the glycinergic system using glycine reuptake inhibitors, voluntary ethanol intake is reduced in rats by increasing extracellular glycine levels (Lido et al 2012, Molander et al 2005). Another GlyR ligand is the amino acid taurine, which levels are increased in the nAc following ethanol exposure (Adermark et al 2011, De Witte et al 1994, Ericson et al 2011, Ericson et al 2020), and will be discussed more in the subchapter “Taurine and Ethanol”. Central nAChRs are activated, directly or indirectly, by ethanol and are suggested to be important for the reinforcing effects of ethanol, indicated by the effects of the non-specific nAChRs antagonist mecamylamine on reducing ethanol consumption (Blomqvist et al 1996, Farook et al 2009). Further, nAChRs located in the VTA are indirectly involved in increased nAc dopamine levels upon ethanol administration (Blomqvist et al 1997, Ericson et al 2008, Ericson et al 2003).

1.4.1 DOPAMINE AND ETHANOL

Administration of ethanol induces increased levels of extracellular dopamine in the nAc regardless of systemic (Di Chiara & Imperato 1988, Ericson et al 2020), oral (Weiss et al 1993) or local (Ericson et al 2003) drug delivery, as shown by means of *in vivo* microdialysis. Likewise, the anticipation of ethanol

has the potential to elicit an accumbal dopamine elevation (Löf et al 2007, Söderpalm et al 2009, Weiss et al 1993), indicative of the role of the mesolimbic dopamine system in cue-induced drug-seeking and relapse to ethanol drinking. However, by which exact mechanisms ethanol alters accumbal dopamine levels, mediating the rewarding and reinforcing effects of ethanol, are not entirely clear.

1.4.1.1 DOPAMINE ENHANCEMENT BY ETHANOL VIA THE NAC-VTA-NAC CIRCUITRY

Studies performed by our research group for over two decades have endeavored to target the mechanistic principles of ethanol-induced dopamine elevation in the reward system, which has resulted in the hypothesis of a neuronal circuitry involving nAChRs in the VTA and GlyRs in the nAc (Soderpalm et al 2009). The importance of nAChR in the VTA was observed when administration of the nAChR antagonist mecamylamine, systemically and locally into the VTA, prevented ethanol-mediated accumbal dopamine increase (Blomqvist et al 1993, Blomqvist et al 1997, Ericson et al 1998). Perfusion of mecamylamine directly into the nAc did however not prevent the ethanol-induced dopamine release (Blomqvist et al 1997). Further, following voluntary ethanol intake in the rat, increased levels of extracellular levels of acetylcholine within the VTA and dopamine within the nAc were observed (Larsson et al 2005). This suggests that activation of nAChRs located in the VTA, by an overflow of acetylcholine, induces dopamine release in the nAc. In parallel, when GlyRs in the nAc are blocked by local perfusion with the GlyR antagonist strychnine in the same region, basal dopamine levels are decreased, and the ethanol-induced dopamine elevation is completely abolished (Jonsson et al 2014, Molander & Soderpalm 2005a, Molander & Soderpalm 2005b). Furthermore, local nAc perfusion with glycine, agonizing GlyRs, increases dopamine levels, but prevents further ethanol-induced increase of accumbal dopamine following co-perfusion with ethanol (Molander & Soderpalm 2005a). In summary, ethanol is suggested to activate accumbal GlyRs, putatively located on GABAergic projecting neurons, leading to a reduced GABAergic tone, and thereby disinhibition of cholinergic axons synapsing on dopaminergic cell-bodies in the VTA. The release of acetylcholine, probably in concert with ethanol (Löf et al 2007), leads to nAChR activation in the VTA and increased firing of dopaminergic neurons, resulting in increased extracellular dopamine levels within the nAc (Fig. 4).

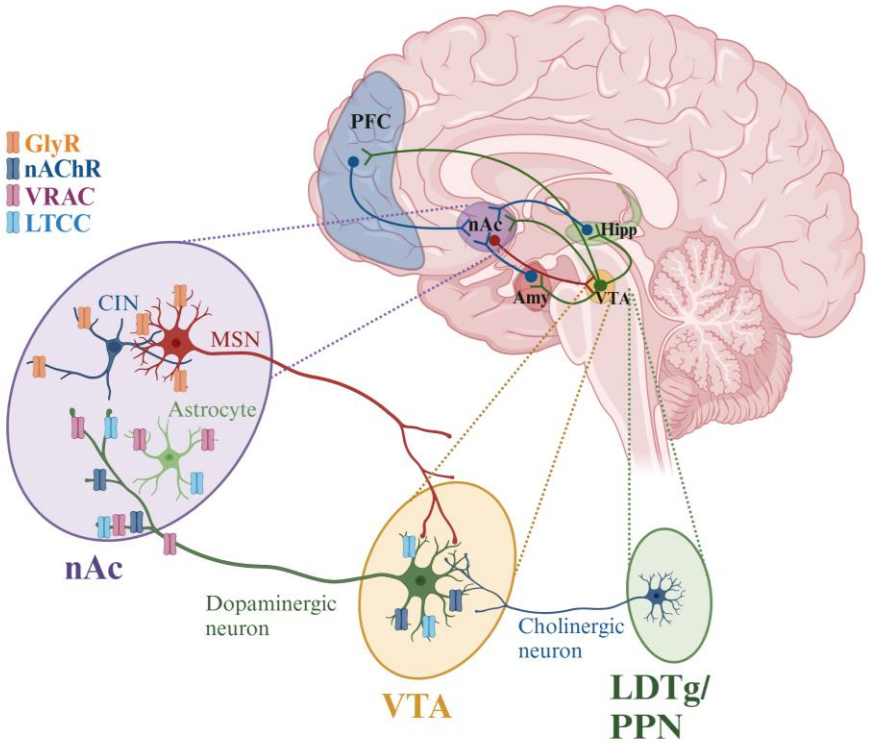

Figure 4. Schematic overview of the hypothesized neuronal circuitry involved in ethanol-induced release of dopamine in the nAc. *Ethanol activates glycine receptors (GlyRs) found on GABAergic projecting neurons in the nucleus accumbens (nAc), mediating disinhibition of cholinergic projections from the LDTg/PPN to the VTA. The subsequent activation of nicotinic acetylcholine receptors (nAChRs) located on dopaminergic neurons within the ventral tegmental area (VTA), induces nAc dopamine release. Modified from (Chau 2011, Loftén 2024). Image created using BioRender.com. CIN=cholinergic interneurons, LDTg=laterodorsal tegmental nucleus, LTCC=L-type calcium channels, PPT=pedunculopontine nucleus, VRAC=volume regulated anion channels.*

1.4.2 TAURINE AND ETHANOL

Taurine is a semi-essential amino acid, where the characteristic carboxyl group is interchanged to a sulfo group. The synthesis primarily occurs in the liver and, to some extent within the CNS, where astrocytes are the main cells responsible for production (Vitvitsky et al 2011). Taurine acts as a zwitterion,

and has high hydrophilicity and low lipophilicity. The taurine molecule is thus membrane impermeable and requires cellular transport mechanisms (Huxtable 1992). The transporters mainly responsible for taurine uptake into cells are the sodium- and chloride-dependent taurine transporter (TauT) and the proton-coupled β-amino acid transporter (PAT1). In addition, cellular release of taurine occurs via volume-sensitive and volume-insensitive pathways, involving the volume regulated anion channel (VRAC) being located on both neurons, primarily on axons and dendrites (Fields & Ni 2010, Wang et al 2017), and astrocytes (Fig. 4) (Lambert et al 2015, Mongin 2016).

1.4.2.1 PHYSIOLOGICAL FUNCTIONS OF TAURINE

Taurine is one of the most abundant amino acids in the brain and is known to have several essential physiological functions, such as being involved in osmoregulation, modulation of calcium, antioxidative effects, and serving as a transcription factor (Lambert et al 2015). The intracellular concentration of taurine is higher than extracellular levels, and upon reductions in external osmolarity taurine is released into the extracellular space from both neurons and astrocytes making taurine an organic osmolyte (Huxtable 1992, Oja & Saransaari 2017). Efflux of taurine occurs under hypotonic conditions when cells undergo regulatory volume decrease due to hypotonic-induced cell swelling (Oja & Saransaari 1992, Oja & Saransaari 1996, Pasantes-Morales et al 1993, Vitarella et al 1994). VRACs are activated by cell swelling and are important for hypoosmotic taurine, glutamate and aspartate release (Choe et al 2012, Jentsch 2016, Mongin 2016). These channels are formed as a heterohexamer complex, discovered as late as 2014, belonging to the leucine-rich repeat-containing 8 (LRRC8) protein family (Qiu et al 2014, Voss et al 2014), and VRACs containing the LLRC8A subunit are associated the with swelling-activated release of taurine in rat astrocytes (Hyzinski-García et al 2014). Other factors can also activate VRACs, such as reactive oxygen species (Shimizu et al 2004). Another important physiological property of taurine is to ensure calcium homeostasis. Taurine regulates intracellular calcium concentrations by inhibiting calcium release from internal stores and modifying the influx via outer cell membrane mechanisms (Junyent et al 2010, Schaffer et al 2002, Wu et al 2009). Excitotoxicity, involving a massive influx of calcium ions into neurons that ultimately leads to neuronal damage, is mediated by excitatory amino acids binding to their receptors, primarily glutamate, and is reduced and prevented by taurine (Foos & Wu 2002, Wu & Prentice 2010). This is proposed to involve different mechanisms (Ramírez-Guerrero et al 2022), and supports the neuroprotective effects suggested for taurine.

The presence of a specific taurine receptor in the human brain has long been debated (Frosini et al 2003, Jakaria et al 2019, Ripps & Shen 2012, Wu et al 1992), as well as whether or not taurine's actions are functionally the same as that of a neurotransmitter. Regardless of its functional classification, taurine has agonistic properties at both the GlyR and the $GABA_AR$ (Haas & Hösli 1973, Okamoto & Sakai 1980, Taber et al 1986), while exhibiting antagonistic properties at the NMDAR (Kurachi et al 1983, Lehmann et al 1984). Agonistic actions of taurine on the $GABA_B$ receptor ($GABA_BR$) have also been suggested, but the functional role of this interaction is unknown (Albrecht & Schousboe 2005).

1.4.2.1 TAURINE ENHANCEMENT BY ETHANOL IN THE NUCLEUS ACCUMBENS

Taurine influences several ethanol-evoked behaviors, including ethanol-induced locomotion and sedation as well as ethanol consumption (Aragon et al 1992, Ferko & Bobyock 1988, McBroom et al 1986, Olive 2002). The effects of taurine have been closely related both to the taurine dose used for treatment and the dose as well as the exposure time of ethanol. In general, taurine reduces ethanol-induced behaviors produced by a low dose of ethanol, while being ineffective at higher doses of ethanol.

Ethanol exposure is associated with increasing extracellular levels of taurine in the nAc, which was first discovered by the use of *in vivo* microdialysis (Dahchour et al 1994, De Witte et al 1994). Later, elevated accumbal taurine levels have been demonstrated following both systemic (Dahchour et al 1996, De Witte et al 1994, Ericson et al 2011, Quertemont et al 2003) and local nAc administration of ethanol (Adermark et al 2011, Ericson et al 2011, Loftén et al 2023) in a dose-dependent manner. Moreover, rats exposed to operant ethanol self-administration have been found to have increased taurine levels in the nAc, with the taurine increase positively correlated with the amount of ethanol consumed (Li et al 2008). Beyond the dopamine- and taurine-elevating properties of ethanol, taurine has the ability to influence accumbal dopamine levels on its own. Local perfusion with taurine in the nAc increases accumbal dopamine levels, an elevation blocked by local administration of mecamylamine in the VTA and strychnine in the nAc, as previously shown for ethanol (Ericson et al 2006). Conversely, when ethanol is diluted in a hypertonic (3.6% NaCl) saline solution and given systemically, taurine elevation is prevented, and the ethanol-induced dopamine increase is also lost. However, when a low dose of taurine was locally perfused into the nAc, the dopamine-elevating effect of ethanol could be restored (Ericson et al 2011).

The increase in extracellular levels of taurine in the nAc in response to ethanol has been suggested to be necessary for ethanol-induced dopamine elevation (Ericson et al 2011), possibly indicating an important role for taurine in regulating ethanol-mediated effects in microcircuits within the nAc. However, the mechanism underlying the increase of endogenous taurine levels in response to ethanol administration is not known.

1.4.3 ACAMPROSATE AND ETHANOL

The pharmaceutical compound Campral® (calcium-bis[*N*-acetylhomotaurinate]; acamprosate; Fig. 5) is a homotaurine derivative sharing structural similarities with both GABA and taurine (Lambert et al 2015, Littleton 1995), and one of the agents available to prescribe for patients with AUD. Acamprosate acts primarily by reducing the frequency and severity of relapse to ethanol drinking (Littleton 1995), but the mechanism of action has been a matter of debate for many years and still remains elusive. The first study on acamprosate was published during the 1980s and found acamprosate to suppress ethanol intake in the rat by a hypothesized interaction with the GABAergic system (Boismare et al 1984). Since then, extensive research has sought to unravel the underlying mechanism of action. The hyperglutamatergic state, a condition associated with ethanol withdrawal caused by neuroadaptations to long-term ethanol exposure, is thought to be attenuated by acamprosate via the interaction with NMDARs and/or mGluRs (al Qatari et al 1998, Harris et al 2002, Mann et al 2008, Naassila et al 1998, Rammes et al 2001). Acamprosate may also act by restoring the imbalance between glutamatergic and GABAergic neurotransmission resulting from chronic ethanol consumption (Chabenat et al 1988, Dahchour & De Witte 2000, Mann et al 2008, Pierrefiche et al 2004).

Previous research from our group has implied the GlyR in the nAc to be an important target for the actions of acamprosate (Chau et al 2010b). In response to both systemic and local nAc administration of acamprosate, extracellular accumbal dopamine levels are increased. This effect is antagonized by local perfusion with mecamylamine in the VTA and strychnine in the nAc (Chau et al 2010b), which are results mimicking what previously has been shown for ethanol and taurine. In an accompanying study, acamprosate reduced voluntary ethanol consumption in the rat, and microinjections of strychnine into the nAc completely reversed the ethanol-intake reducing effect of the drug (Chau et al 2010a). Further, acamprosate has been demonstrated to prevent ethanol from further elevating accumbal dopamine levels when acamprosate already is on-

board, which is a phenomenon linked to the ethanol-intake decreasing effect of acamprosate (Chau et al 2018). However, following long-term treatment with acamprosate, tolerance is developed to acamprosate's ethanol-intake reducing effect and acamprosate's ability to increase accumbal dopamine is also abolished (Chau et al 2018). This suggests that acamprosate partly could substitute for ethanol and its dopamine-elevating properties in the nAc-VTA-nAc circuit in the acute state. Conversely, following long-term acamprosate treatment, the drug no longer increases accumbal dopamine and tolerance develops to the ethanol-intake reducing effect.

The mechanistic principles of acamprosate, responsible for the clinical outcome in humans, have also been demonstrated to be attributed to the calcium part of acamprosate (Melugin et al 2022, Pradhan et al 2018, Spanagel et al 2014). Furthermore, the acetylhomotaurine molecule of the compound is proposed to be biologically inert, based on lack of effect of the sodium salt of acamprosate observed in different models examining ethanol drinking, seeking, and relapse-like behavior in rats and on ethanol-induced cognitive deficits in mice (Pradhan et al 2018, Spanagel et al 2014). Clinical investigations using calcium supplementation have found decreased craving and relapse in individuals with AUD, a result associated with reduced plasma calcium concentrations induced by long-term ethanol consumption (Schuster et al 2017, Schuster et al 2021). Consequently, more mechanistic and behavioral studies investigating the role of *N*-acetylhomotaurine and calcium of acamprosate are needed.

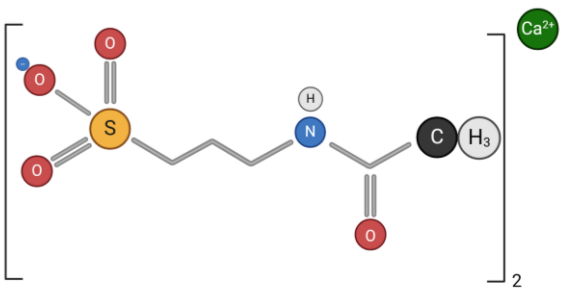

Figure 5. Structural formula of acamprosate. *Acamprosate comprises two N-acetylhomotaurine molecules connected to one calcium ion. The acetyl-group facilitates oral absorption. Image created using BioRender.com.*

2 AIM

The overall objective of this thesis project was to investigate the mechanistic principles of taurine-related compounds in the nucleus accumbens and the neurochemical effects induced by ethanol. For the individual papers, the specific aims are as follows:

1. To determine the origin of ethanol-induced increase of extracellular taurine levels in the nAc

2. To investigate if the dopamine elevating properties of acamprosate in the nAc are mediated by the *N*-acetylhomotaurine molecule or the calcium moiety

3. To evaluate the effects of *N*-acetylhomotaurine in combination with calcium on ethanol intake and relapse-like drinking

4. To explore the acute and chronic effects of calcium on ethanol-induced dopamine output in the nAc and relapse-like drinking

3 METHODS AND MATERIALS

The following section covers a brief description of the methodology used in the thesis, including reflective discussions regarding each individual method. Detailed descriptions of the material and methods are to be found in the individual papers.

3.1 ANIMALS AND ANIMAL MODELS

In medical research, animal models, especially the use of rodents such as rats and mice, have been instrumental. The rate of reproduction including a short gestational time and the small size of rodents are factors making them well suited as model organisms. Within the field of addiction, rodents are an appropriate animal model as the brain reward system, and its associated structures, are well conserved between species through evolution and therefore rather similar to that of humans (Mullins & Mullins 2004). As mentioned in the introduction, the brain reward system has a significant impact on the survival of the species as essential behaviors are constantly motivated. Even though no animal model of AUD fully emulates the human pathological process, animal models allow the study of specific parts of the progress of AUD that can be defined by modeling different stages of the addiction cycle. However, the validity of the model must be considered. There are different perspectives of validity, where the three most commonly discussed are face-, construct-, and predictive validity. Face validity is defined as how well the characteristics (symptoms or behavior) that are observed in the animal model resemble those in the human condition. Construct validity refers to how similar the underlying pathophysiology is in the animal model compared to the human situation. Lastly, predictive validity is defined as how well treatment effects in the animal model can be predicted or translated to the human subjects. The usage of several different models is necessary to simulate the diverse aspects of the AUD in order to make valid interpretations of the human state, not the least because AUD generally is a complex and heterogeneous disorder. Consequently, it is also important to carefully select models that are appropriate for the research question at hand.

The animals used in all studies presented here are outbred male Wistar Han rats, purchased from Envigo, the Netherlands, which changed name to Inotiv during 2024, or Taconic, Denmark. The use of outbred rat strains, in contrast to inbred rats, means that genetic variability is expected to be observed among

the animals, in order to better reflect the heterogeneity present in the human population with regards to ethanol consumption and the vulnerability to develop AUD (Stewart & Kalueff 2015). In Paper I, Wistar rats from different vendors were used, owing to the fact that Taconic, Denmark discontinued their Wistar strain. Consequently, a change of vendor became inevitable and Envigo, the Netherlands, was selected as the new supplier based on pilot studies screening a number of Wistar rats from different vendors in the microdialysis set-up. The use of animals from the same vendor is, however, preferable to be able to compare results between experiments and studies, as vendor-dependent differences have been reported with respect to both behavioral and neurochemical attributes for the outbred Wistar rat (Momeni et al 2015, Palm et al 2011, Palm et al 2012). Nonetheless, different outcomes can potentially also be observed when using animals from the same vendor due to genetic variation between batches over time. The data presented in this thesis should not have been significantly affected by the use of different vendors in Paper I, as the animals originating from Taconic only comprised a small number given the large total number of animals used.

In the human population, there are differences in terms of ethanol consumption pattern between men and women (Erol & Karpyak 2015), sex differences which are also observed in rodents. The majority of clinical and preclinical biomedical research, including neuroscience and alcohol research, have historically been influenced by sex bias favoring the use of male animals over female animals. The impact of the estrous cycle in female animals to take into account has been a limiting factor for their inclusion (Beery & Zucker 2011, Will et al 2017), where both ethanol-related behaviors and neurobiological factors have been observed to fluctuate depending on state of the estrus cycle (Dazzi et al 2007, Ford et al 2002, Forger & Morin 1982, Lorrai et al 2019, Vandegrift et al 2017). However, more recently, the significance of sex differences has received more attention, and the impact of gonadal hormones on ethanol-related behaviors and the underlying mechanisms has been studied in greater detail (Radke et al 2021). In the studies presented in this thesis, only male subjects were used, which is a drawback, and the inclusion of female animals would have been valuable.

All studies performed within this thesis are performed according to protocols approved by the Ethics Committee for Animal Experiments, Gothenburg, Sweden and in agreement with Swedish Legislation on Animal Experimentation. As part of using animals in research, the wellbeing of the animals is constantly considered. Animal technicians, together with the

responsible researcher, observe health status and ensures food and water on a daily basis to guarantee animal wellbeing and reduced suffering. Further, our research group follows the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) and PREPARE (Planning, Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines when planning, design and presenting our experiments (Percie du Sert et al 2020, Smith et al 2018).

3.2 DRUGS AND CHEMICALS

Ethanol

Drinking solutions for the intermittent drinking paradigms (Paper III, IV) were prepared from 95-96% ethanol diluted in tap water to a 12% (v/v) ethanol solution. For the microdialysis studies (Paper I, IV), ethanol was diluted in Ringer's solution (consisting of (in mM): 140 NaCl, 1.2 $CaCl_2$, 3.0 KCl, 1.0 $MgCl_2$) to a concentration of 300 mM and administered by local perfusion via the microdialysis probe or dissolved and diluted in 0.9% NaCl to a concentration of 2.5 g/kg and administered systemically via an intraperitoneal (i.p.) injection. The systemically administered dose of 2.5 g/kg and locally perfused dose of 300 mM have been demonstrated to elevate extracellular dopamine levels in the nAc to the same magnitude, to about 30-40% above baseline (Blomqvist et al 1997, Ericson et al 2003).

Tetrodotoxin

Tetrodotoxin (TTX), a sodium channel blocker inhibiting action potential firing, was dissolved in Ringer's solution, diluted to a concentration of 1 μM, and administered by local perfusion via the microdialysis probe. The dose of 1 μM TTX has frequently been used for the investigation of different extracellular amino acids in the brain (PFC, VTA, and hypothalamus) (Singewald et al 1993, Timmerman et al 1999).

Memantine

Memantine (Mem), a non-competitive NMDA receptor antagonist, was dissolved in Ringer's solution, diluted to a concentration of 100 μM, and administered by local perfusion via the microdialysis probe. A dose-range of 50-100 μM has previously been applied for *in vivo* microdialysis both in the spinal cord and in the striatum of the rat (Hesselink et al 1999, McAdoo et al 2005), and a dose-response study performed in our lab found 100 μM to be a relevant dose.

GES

Guanidinoethyl sulfonate (GES), a competitive taurine transporter (TauT) inhibitor, was dissolved in Ringer's solution, diluted to a concentration of 5 μM, and administered by local perfusion via the microdialysis probe. A previously performed dose-response study found a concentration-dependent increase in taurine levels in the nAc following GES treatment (Olive et al 2000), which also was performed in our lab, and 5 mM was considered an appropriate concentration for our experiments.

DCPIB

Oxobutyric acid (4-[2-Butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl]; DCPIB), a VRAC inhibitor, was dissolved in dimethyl sulfoxide (DMSO), diluted in Ringer's solution to a concentration of 100 μM, and administered by local perfusion via the microdialysis probe. The final concentration of DMSO was 0.4%, which was the maximum concentration that could be used without having a major impact on the extracellular taurine output. It was consequently not possible to increase the DCPIB concentration. However, 100 μM DCPIB has previously been demonstrated to significantly reduce ischemia-induced glutamate and aspartate release using *in vivo* microdialysis (Zhang et al 2008).

CNO

Clozapine N-oxide dihydrochloride (CNO), a specific designer receptor exclusively activated by designer drug (DREADD) ligand used for activation of implanted DREADDs, was dissolved in 0.9% NaCl and systemically administrated 3 mg/kg, i.p., a dose within the suggested dose interval (Roth 2016).

Fluorocitrate

DL-Fluorocitrate (FC), a metabolic uncoupler specific for glial cells inhibiting the tricarbolic acid cycle/citric acid cycle, was dissolved in Ringer's solution, diluted to a concentration of 25 μM, and administered by local perfusion via the microdialysis probe. A previously performed dose-response study in our lab found 25 μM to reduce extracellular glutamine levels by about 60%, indicating desired effects on glial cells, and to be the highest possible dose without negative behavioral effects (data not shown).

Nicardipine

Nicardipine (NCD), a dihydropyridine and LTCC antagonist, was dissolved in pre-warmed (37°C) Ringer's solution, diluted to a concentration of 100 μM, and administered by local perfusion via the microdialysis probe after the solution reached room temperature. A previously performed dose-response

study found 100 µM to robustly suppress striatal dopamine release in male rats (Faro et al 2018, Kato et al 1992), but only partially in female rats (Costas-Ferreira et al 2023a, Costas-Ferreira et al 2023b).

Acamprosate

Calcium-bis(*N*-acetylhomotaurinate) (trade name Campral®; acamprosate; CaAcamp), suggested to restore excitatory and inhibitory imbalance induced by chronic alcohol intake, was, for the microdialysis studies (Paper II, III), dissolved in Ringer's solution, diluted to a concentration of 0.5 mM, and administered by local perfusion via the microdialysis probe or dissolved and diluted in 0.9% NaCl to a concentration of 200 mg/kg and administered systemically via an i.p. injection. The systemically administered dose of 200 mg/kg and the locally perfused dose of 0.5 mM have been demonstrated to elevate extracellular dopamine levels in the nAc to the same magnitude, to about 20-30% above baseline (Chau et al 2010b). For the ethanol consumption study (Paper III), rats were i.p. injected with 200 mg/kg acamprosate, which is a dose previously used to reduce ethanol intake in a robust manner (Chau et al 2010a, Lido et al 2012, Olive et al 2002).

Calcium chloride

Calcium chloride ($CaCl_2$) was, for the microdialysis studies (Paper II, III), dissolved in Ringer's solution, diluted to a concentration of 0.5 mM, and administered by local perfusion via the microdialysis probe or dissolved and diluted in 0.9% NaCl to a concentration of 73.5 mg/kg and administered systemically via an i.p. injection. For the ethanol consumption studies (Paper III, IV), rats were i.p. injected with 73.5 mg/kg calcium. The dose of 73.5 mg/kg calcium contains equal quantities of injected Ca^{2+} ions as 200 mg/kg acamprosate (0.499 mmol/kg).

Sodium Acamprosate

Sodium-*N*-acetylhomotaurinate (sodium acamprosate; NaAcamp), synthesized in order to investigate the mechanism of action of homotaurine/*N*-acetylhomotaurine, was, for the microdialysis studies (Paper II, III), dissolved in Ringer's solution, diluted to a concentration of 0.5 and 1 mM, and administered by local perfusion via the microdialysis probe or dissolved and diluted in 0.9% NaCl to a concentration of 200 mg/kg and administered systemically via an i.p. injection. Different concentrations of sodium acamprosate were administered by local perfusion in order to harmonize with the different elements of the acamprosate salt, which dissociates entirely into its components in aqueous solution. Sodium acamprosate at 0.5 mM contains

equal quantities of ions (Na^+ versus Ca^{2+}) as the same dose of regular acamprosate and calcium chloride, and 1 mM of sodium acamprosate contains equivalent amounts of the homotaurine molecule as 0.5 mM regular acamprosate. For the ethanol consumption studies (Paper III), rats were i.p. injected with 200 mg/kg sodium acamprosate. Here, the quantity of homotaurine in 200 mg/kg regular acamprosate and 200 mg/kg sodium acamprosate was almost similar differing with 1.46% (0.999 mmol/kg versus 0.984 mmol/kg respectively).

Strychnine

Strychnine, a GlyR antagonist, was dissolved in Ringer's solution, diluted to a concentration of 20 µM, and administered by local perfusion via the microdialysis probe. The dose of 20 µM has previously been demonstrated to be the highest concentration usable without having an impact on the extracellular accumbal dopamine output (Molander & Soderpalm 2005a).

3.3 IN VIVO MICRODIALYSIS

In vivo microdialysis was first described for neuroscience during the 1970s, and is a method that enables sampling and measurement of endogenous substances, such as neurotransmitters and amino acids, found in the extracellular space within a tissue (Ungerstedt & Pycock 1974). In the work forming this thesis, *in vivo* microdialysis has been used in all of the papers with a focus on sampling accumbal dopamine and taurine, as well as other amino acids when desirable. The concentration of dopamine and taurine in the dialysate collected from the tissue is determined by analyzing the samples using high performance liquid chromatography (HPLC), described in the following subchapter.

The probe used for *in vivo* sampling of extracellular fluid is fitted with a semipermeable membrane and is implanted into the brain region of interest using stereotactic surgery, ensuring high precision. The semipermeable membrane enables passive diffusion of analytes along the concentration gradient that exists between the artificial cerebrospinal fluid (Ringer's solution) perfused through the dialysis probe during the experiment and the extracellular space. In the papers constituting this thesis, the active space of the membrane, which is the part of the probe where the membrane is left exposed and where molecules diffuse across, is 2 mm (Fig. 6). This length is considered to cover the brain area of interest, the nAc, in a dorsal-ventral dimension and not reaching adjacent structures (Paxinos & Watson 2007). By

adjusting the length of the active space, if a different brain region is to be investigated, the recovery will also change. Further, the molecular cut-off of the membrane used is 20 kDa, precluding larger molecules from diffusing across the membrane and ending up in the perfusate. The cut-off of the membrane becomes a natural sample preparation and purification step, omitting proteins and peptides to cross the membrane, before placing the samples for analysis in the HPLC.

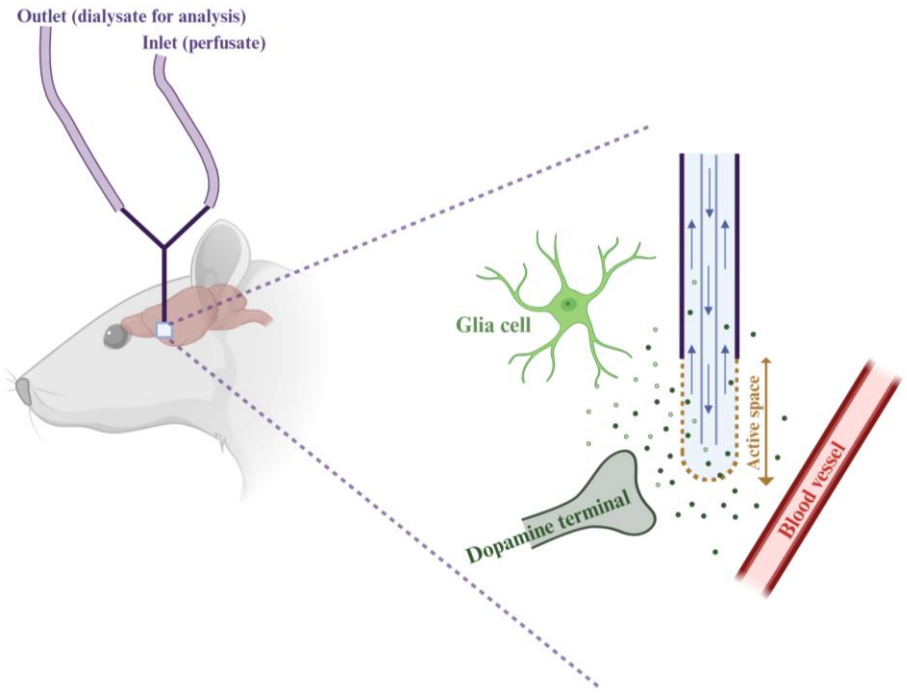

Figure 6. Descriptive illustration of the in vivo microdialysis probe set-up. *The probe, with the semipermeable membrane, is inserted into the brain region of interest. This allows molecules to diffuse across the active space of the semipermeable membrane along its concentration gradient, from the extracellular space into the probe or vice versa. The dialysis probe is continuously perfused by an artificial cerebrospinal fluid, allowing samples to be collected and analyzed. Adapted from (Loftén 2024). Image created using BioRender.com.*

By means of the method, pharmacological manipulations can be accomplished either by systemic or local administration of drugs. The advantage of using local administration, when the drug of interest is perfused through the dialysis probe to reach the surrounding tissue after diffusing over the semipermeable membrane, is that the drug exerts its effect locally within the brain region, thereby enabling manipulation of neuronal circuits and mechanistic studies in a highly site-specific way. In Paper I, II and IV, locally administrated drug and reversed *in vivo* microdialysis was used, while regular microdialysis was used in Paper I, III and IV. When employing reversed *in vivo* microdialysis, it is difficult to know which drug concentration that reaches the target tissue (excovery). Intrinsic properties of the drug, such as molecular weight, polarity, and shape, influence both the ability of the solute to cross the membrane and its propensity to bind the tubing material, and consequently the fraction reaching the tissue. Preliminary results from our lab have found that local administration of 300 mM ethanol through the dialysis probe leaves approximately 70 mM in the surrounding tissue nearby the probe, equal to an excovery of 24% (Loftén et al., unpublished). Another study suggests that approximately 10% of the concentration in the perfusion fluid reaches the tissue (Molchanova et al 2005).

The net fluid balance in the surrounding tissue of the probe is not altered using microdialysis, based on the passive diffusion phenomena, allowing sampling over a longer time period. In the experimental set-up used in this thesis, the collection rate of brain dialysate is set to every 20th minute. The rather slow sampling rate allows for sufficient sample volume and concentration of solute to accumulate in the dialysate needed for the HPLC analysis. This, however, leads to restricted temporal resolution, and fast changes or events like consequences of burst firing of dopamine neurons are not possible to measure, for which voltammetry or electrophysiological recordings are more appropriate. Increasing the flow rate to allow for a faster sampling rate will not improve the outcome, as a smaller fraction of the solute of interest will pass the membrane, requiring a more sensitive analytical method. In the case of this thesis, the neurochemical effects of interest are mainly changes over longer time periods, and thus, a result from bulk overflow from synaptic and non-synaptic sources rather than direct synaptic transmission. This makes *in vivo* microdialysis an ideal sampling method.

Following the insertion of the dialysis probe into the brain, tissue damage is unavoidably induced both in the brain region of interest and in structures passed (e.g. cortex) by the probe with gradual formation of gliosis around the

probe (Dykstra et al 1992, Norton et al 1992). In Paper I, microinjections of viral vectors encoding for DREADDs were performed before inserting the dialysis probe, meaning that two surgical procedures within the same structure were performed in these animals. This causes additional tissue damage and gliosis formation, and may partly influence local neurotransmission. Based on findings demonstrating no change in blood-brain barrier recovery between 24 and 48 hours following implantation of the microdialysis probe (Hammarlund-Udenaes 2017, Xie et al 2000) and the possible microglial activation and necrosis upon too long recovery (Plock & Kloft 2005), a recovery period of 48 hours was used for the animals in this thesis following the probe placement surgery.

3.3.1 HPLC ANALYSIS

High performance liquid chromatography is an analytical technique used for separation of molecules in a sample which could be of different origin. The HPLC is coupled to a detector, providing detection and quantification of the molecules in the sample and, in the case of this thesis, reflects the composition of the extracellular fluid collected by the *in vivo* microdialysis technique. The detectors used in this work are an electrochemical detector for dopamine quantification and a fluorescent detector for amino acid quantification. A total of three different HPLC-systems have been used for the results obtained in this thesis, two different systems run in parallel, operating for dopamine analysis and one system operating for amino acid analysis.

The general principle of HPLC involves high-pressure pumps that drive the sample through a column (stationary phase), densely packed with adsorbent material, meanwhile the sample is being diluted by a mixture of solvents (mobile phase). The molecules in the sample interacts with the adsorbent material differently, based on the intrinsic properties of each molecule, leading to retardation and different migration rates for the sample molecules. When the entire sample has been pumped through the column, the molecules are separated from each other and can be identified and quantified by the detector, generating a signal equivalent to the concentration of the specific molecule in the sample presented in a chromatogram. The HPLC system used for amino acid analysis relies on a reversed-phase system, meaning that the stationary phase is packed with a non-polar adsorbent, and the molecules elute in order of decreasing polarity. The two systems used for dopamine analysis run in parallel rely on the molecules being separated on either a reversed-phase column or an ion-exchange column. The latter is based on the attraction

between the ionic form of the molecules of interest and charged sites on the stationary phase, leading to different retention times. Further, external standards are used to be able to identify and quantify dopamine and the amino acids.

When an HPLC-system, with an appropriate method adjusted for your research question or purpose, is established, the system is delicate, and several parameters need to operate at constant conditions. The columns are expensive and the particles packed in the column can easily degrade by impurities in the sample or solvent, or by too large particles in the sample. As previously mentioned, the semipermeable membrane attached to the dialysis probe helps purify the sample from containing larger molecules. Temperature is another parameter influencing both the viscosity of the solvent and, if increased, it can cause degradation and a decreased lifetime of the column (Harris 2010). The composition (polarity) of the mobile phase will influence the rate of elution of the molecules, and in the reversed-phase system, the solvent is polar to avoid displacing the sample molecules in the column and consequently increase the retention time of the molecules.

3.4 VOLUNTARY ETHANOL CONSUMPTION

The extent and volume of ethanol consumption varies between humans beings, a phenomenon also observed in rats when they are presented with the option to consume ethanol voluntarily. The consumption pattern of problematic ethanol drinking is also variable, and individuals with AUD may engage in heavy drinking and/or relapse-like drinking. Animal models studying separate characteristics of voluntary ethanol consumption are of great value in disentangling diverse aspects of human ethanol consumption. This thesis models heavy drinking by using the intermittent ethanol consumption paradigm and relapse-like behavior by using an alcohol deprivation paradigm. Moreover, the models are considered to demonstrate high face and predictive validity for the behavior seen in humans (Spanagel 2017).

High levels of voluntarily consumed ethanol have been shown to be induced in outbred rats exposed to the intermittent ethanol access paradigm, as opposed to a continuous access paradigm (Wise 1973). The model has been demonstrated to generate blood alcohol concentrations being clinically relevant (Becker 2013, Carnicella et al 2014, Simms et al 2008), corresponding to approximately 10-50 mM (Carnicella et al 2009) (5-20 mM is the legal intoxication range for driving in many countries (Vengeliene et al 2008)). In

addition, the intermittent paradigm yields a pharmacologically significant extracellular dopamine increase in the nAc (Ericson et al 1998). The intermittent ethanol consumption model can be implemented to evaluate tentative effects of pharmacological drugs on ethanol intake and preference over water. In the experimental setting used in this thesis, animals were presented with free access to regular tap water, and food, and intermittently to a bottle containing ethanol solution (6% being increased to 12% (v/v) at early screening) for three 24-hour sessions a week. The intermittent paradigm was used for screening for ethanol intake in Paper III and IV, allowing the animals to reach a stable ethanol consumption.

In Paper III, when testing for pharmacological treatment effect, the intermittent paradigm was changed to a limited access paradigm. This means that the bottle containing ethanol was present each day, but only for six hours, and that the pharmacological treatment was administered directly before the addition of the ethanol bottles. The limited access paradigm has demonstrated escalated drinking in dependent subjects (Becker 2013) and is preferentially used to confirm that the treatment maintains its effect during the hours of drinking and to observe potential differences between treatment groups.

To engage consummatory behavior and facilitate high ethanol consumption among the rats, ethanol was presented at the beginning of the dark period as rodents are nocturnal. Further, to reach relevant blood ethanol levels, the concentration of the offered ethanol solution is of importance. Lower concentrations (8-12% (v/v)) are usually preferred to ensure a high intake of ethanol, as higher concentrations (20% (v/v)) tend to be favorable only for a particular group of individuals (Palm et al 2011). It is, however, difficult to measure the blood ethanol concentration, as the metabolism of ethanol in rats is rapid. In order to observe and measure the levels of ethanol drinking of each individual animal, single housing of the animals is necessary. This is a drawback of the method, as rats normally live in larger groups and single housing may have a negative impact on their wellbeing (Arakawa 2018). However, the animals are placed in the same room and can interact to some extent through ultrasonic vocalization and olfactory impressions. On the other hand, the social isolation, and the stress induced by social isolation, is a common feature of individuals with AUD and could reflect a feature of the addictive phenotype (Hosseinbor et al 2014).

3.4.1 ALCOHOL DEPRIVATION EFFECT

For individuals with AUD, the risk of relapse to drinking ethanol is a central dimension of the disorder. The alcohol deprivation effect (ADE) is a phenomenon seen in laboratory animals, and models relapse-like and uncontrolled drinking in the clinical situation (Spanagel & Hölter 1999). In voluntary ethanol consuming rats, which then are being deprived for a certain period of time, a temporary increase in ethanol intake is observed the first days after the ethanol solution is reintroduced compared to baseline drinking. This is referred to as the ADE, and was first described in rats during the 1960s (Sinclair & Senter 1967). The ADE is evaluated in Paper III and IV, which relied on different paradigms of the ADE. In Paper III, the ADE assessment followed in part as a continuation of the limited access paradigm to evaluate the effect of repeated drug treatment on relapse drinking, and in part as a continuation of the intermittent access paradigm to evaluate the effect of acute drug treatment on relapse-like behavior. In Paper IV, an ADE paradigm with repeated ethanol deprivation periods was used to induce a robust ADE (Spanagel & Hölter 1999) before assessing the effect of the pharmacological treatment. Each deprivation period, independently of the study, lasted for 14 days. It has been shown that 14 days of deprivation induces an actual effect of increased ethanol intake post-deprivation, even though an ADE also can be observed following shorter or longer deprivation phases (Hannigan et al 1999, Samson & Chappell 2001, Vengeliene et al 2014). Drugs found to suppress the ADE are promising in a clinical setting for treatment of AUD, since the model has proven face and predictive validity (Spanagel & Hölter 2000, Vengeliene et al 2014).

3.5 CHEMOGENETIC MANIPULATIONS

Chemogenetics is an approach where chemically engineered macromolecules can be modulated by otherwise inert small molecules. It was first applied during the 1990s (Coward et al 1998, Liu et al 1998, Strader et al 1991), and development has led to various chemogenetic technologies and strategies. Designer receptors exclusively activated by designer drugs are genetically modified G protein-coupled receptors (GPCRs), widely used in neuroscience for both *in vitro* and *in vivo* manipulation of neuronal and non-neuronal signal transduction in a cell-type-specific manner (Armbruster et al 2007, Roth 2016). The DREADD is generally based on human muscarinic acetylcholine receptors and is insensitive to the endogenous ligand acetylcholine, but has affinity for the biologically inert ligand CNO activating the receptor.

In this thesis, the DREADD technique was used in Paper I to selectively influence the activity of nAc astrocytes to define their role in ethanol-induced taurine increase with more specificity, and was combined with *in vivo* microdialysis. The animals were transfected with inhibitory (Gi) or excitatory (Gq) DREADDs, alternatively a control plasmid (sham), in the nAc. For selective astrocyte expression, the viral vectors containing DREADD were expressed under the control of the glial fibrillary acidic protein (GFAP) promoter, a protein expressed by astrocytes providing mechanical strength and cell shape. The viral vectors were administered unilaterally using stereotactic surgery for accuracy, and spatial specificity, and an injection cannula was coupled to a micropump ensuring a low flow rate to avoid inducing sudden volume expansion causing tissue damage.

The DREADD technique offers the possibility to interfere with endogenous neuronal and/or astrocytic signaling for modulation and/or identification of different cellular circuits within the CNS (Roth 2016). Due to the technique being relatively new, there are challenges to consider. One concern is that the expression density of the DREADDs in the target area may vary among different astrocytes (our target cells), leading to unpredictable activation of the receptors and, consequently, the downstream effects of the targeted cells. In addition, the injection of the viral construct allowing for DREADD expression is performed before inserting the microdialysis probe. Thus, the probe is possibly not implanted exactly in the site of where the DREADDs are expressed, as the receptors are expressed in a restricted area, leading to the effect of the receptor activation using CNO being missed or lost. Insertion of the dialysis probe also causes additional gliosis and necrosis formation, influencing variable output from different transfected animals. The majority of studies using the DREADD technology have investigated DREADD expression in neurons. Activation of Gq-coupled DREADDs leads to neuronal excitation, as a result of depolarization, and activation of Gi-coupled DREADDs leads to attenuation of neuronal firing (Armbruster et al 2007, Durkee et al 2019). The functional effects in astrocytes are, in contrast, less clear. This is based on the fact that it has been difficult to establish whether DREADD expression in astrocytes has the ability to accurately mimic endogenous GPCR activity (Lee et al 2023), and whether it, in the strictest sense, is possible to activate or deactivate astrocytes through GPCR activation (Durkee et al 2019, Lee et al 2023, Shen et al 2021). Another thing to consider is the specificity of CNO, reported to possibly be metabolized back to clozapine though to a very small extent (Roth 2016). However, if administered within the recommended dose interval (0.1-3 mg/kg, i.p.), as in Paper I, it is

supposed to be pharmacologically and behaviorally inert in rodents (Roth 2016).

3.5.1 IMMUNOFLUORESCENCE

Immunofluorescence is a method used to detect cellular components in tissue samples, such as proteins, by utilizing antibody-antigen interaction. To enable visualization using confocal microscopy, the antibodies and/or the viral constructs are conjugated with a fluorophore. Immunofluorescence is an example of immunohistochemistry. The technique was utilized to verify the astrocyte-specific DREADD transfection and to confirm that the DREADDs were expressed and localized on accumbal astrocytes. Although both the viral constructs encoding for the Gi- and Gq-coupled DREADD are provided with a fluorophore for visualization, mCherry and mCitrine, respectively, antibodies conjugated to a fluorophore were used in order to amplify the signal for the Gq-coupled DREADD construct. Beyond visualization of the DREADDs, antibodies targeting NeuN, marking neurons, and GFAP, marking astrocytes, were used.

Proper preparation of the specimen is crucial for good visualization of the cellular structures and for obtaining high-quality images. Several steps in the sample preparation are critical to achieve a reliable result, such as accurate tissue collection, fixation, and sectioning, in order to avoid weak and/or unspecific binding of antibodies and autofluorescence. In this thesis, this was achieved by dehydrating the tissue using increasing concentrations on sucrose solutions, followed by the brain being snap frozen using cold isopentane on dry ice to prevent ice crystal formation in the tissue. Further, the conditions of the cryostat are crucial for proper sectioning of the brain. Using a blocking buffer minimizes reactive sites that the antibodies may bind to in the tissue slices, even though some antibodies still can bind undesired epitopes. There is also a risk that the antibodies used are not as specific as claimed by the manufacture.

3.6 STATISTICAL ANALYSIS

In the present thesis, the data was statistically analyzed using the software Graphpad Prism version 8-10 for Windows (Graphpad Software, San Diego, CA, USA). All data are expressed as means ± standard error of the mean and statistical significance considered a probability value (p) less than 0.05.

In Paper I-IV, in order to analyze the difference between the mean of data from two, or more, groups being dependent on two categorical variables, a two-way analysis of variance (ANOVA) with repeated measures was used. The two-way ANOVA estimates the main effect of each independent variable, which in the case of the data presented in this thesis was treatment and time, and if any interaction between them was present. In Paper I-IV, the two-way ANOVA was used for assessing the change over time of the dopamine and taurine content in microdialysis samples, and for the ethanol and water consumption as well as ethanol preference in Paper III. For the analysis of data being dependent on one categorical variable, originating from two or more groups, one-way ANOVA with repeated measures was used. In Paper I-IV, one-way ANOVA, with treatment as a variable, was used for analysis of the area under the curve (AUC) of dopamine and taurine content (Paper I, II), the ADE (Paper III), and the dopamine and taurine content at specifically stated time-points (Paper IV). Tukey's or Dunnet's post hoc test were used for multiple comparisons when appropriate.

In order to compare the difference between the mean values of two groups, the t-test was applied. An unpaired t-test was performed when the data sets were independent of each other, as done in Paper IV for the comparison of absolute values of dopamine content. Paired t-test, on the other hand, was used when data sets were matching and dependent on each other, as in Paper III and IV when the ADE was analyzed before and after the deprivation period within the same animal.

The different tests used for the analysis of data in the present thesis were all parametric tests. The use of parametric tests, compared to non-parametric tests, requires data to be normally distributed, meaning the data follows a bell-shaped curve when manually plotted on a graph. Beyond testing for normal distribution by plotting the data, a normality test can be performed where a resulting p-value gives an indication of normally distributed data or not. Large sample sizes are needed to obtain a conclusive suggestion of whether the data is normally distributed. However, in this thesis, small sample sizes were used, as animal experiments aspire to use as low numbers of animals as possible. Instead, normal distribution is assumed from which the sample is drawn, i.e. outbred rats that bring genetic variance within the population, and based on considerable experience of the methods used.

4 RESULTS AND DISCUSSION

This section comprises the presentation of the results from the four papers included in this thesis, and a discussion of the findings. Detailed statistical information is to be found in the individual papers.

4.1 ETHANOL-MODULATING EFFECTS OF ACCUMBAL TAURINE

Endogenous taurine levels in the nAc are increased upon exposure to ethanol, indicated by several *in vivo* microdialysis studies (Adermark et al 2011, Dahchour et al 1994, Dahchour et al 1996, Ericson et al 2011, Ericson et al 2020, Loftén et al 2023). Further, taurine is likely involved in the rewarding effects of ethanol, as demonstrated by the elevated accumbal levels during operant ethanol self-administration, where the amount of ethanol consumed correlated with the magnitude of taurine increase (Li et al 2008). Thus, taurine, like dopamine (Di Chiara & Imperato 1988, Weiss et al 1993), appears to be closely related to ethanol-induced effects in the mesolimbic dopamine system. However, the mechanism underlying the extracellular taurine increase following ethanol exposure is not clear. A hypothesis was established based on the fact that taurine is recognized as an osmoregulator, thus released into the extracellular space from both neurons and astrocytes to counteract cell swelling caused by reduced external osmolarity (Huxtable 1992, Pasantes-Morales et al 1993, Solís et al 1988, Vitarella et al 1994), and that ethanol is known to induce cell-swelling of astrocytes in primary culture (Adermark et al 2011, Allansson et al 2001, Kimelberg et al 1993). More specifically, the hypothesis was that nAc taurine levels are increased upon ethanol exposure to compensate for ethanol-induced cellular swelling and is released from accumbal astrocytes. To investigate the mechanistic principles of ethanol-mediated accumbal taurine elevation, both pharmacological and chemogenetic manipulations of the rat nAc were implemented (Paper I).

4.1.1 INVOLVEMENT OF ASTROCYTES (OR NEURONS)

The swelling-activated channel VRAC is central for the osmotically-stimulated release of taurine in cultured astrocytes (Brès et al 2000, Deleuze et al 1998, Jackson & Strange 1993), and the VRAC inhibitor DCPIB (100 μM) was therefore locally applied into the nAc to investigate mechanisms behind

ethanol-mediated taurine increase. The basal taurine levels were not affected by DCPIB-treatment (Fig. 5B-C, Paper I). Further, the ethanol-induced taurine elevation was not significantly reduced by DCPIB, but not significantly increased either (Fig. 7A-B; see also Fig. 5A-D, Paper I), suggesting that VRACs may potentially be involved in regulating taurine elevation after ethanol challenge.

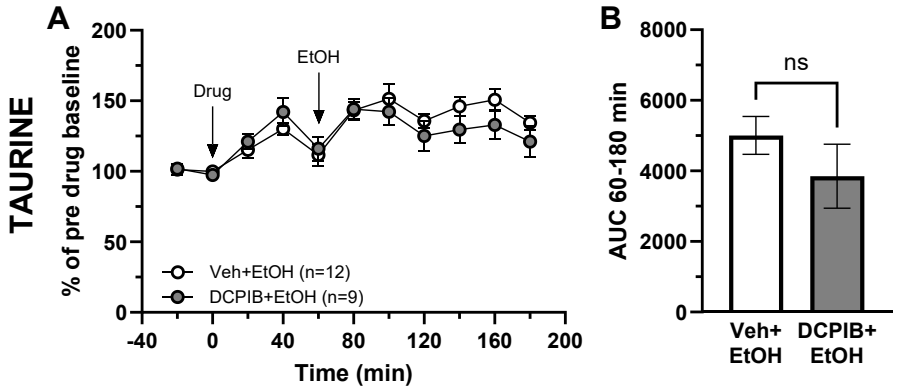

Figure 7. Ethanol-induced taurine increase following DCPIB pre-treatment. *A) Time-course graphs of nAc taurine levels after nAc ethanol (300 mM) perfusion alone or after pre-treatment with DCPIB (100 µM) presented as % of baseline. B) AUC of the time following ethanol administration showed no significant reduction in taurine output in rats pre-treated with DCPIB. Arrows in A) indicate the start of vehicle or DCPIB perfusion at time-point 0 and the addition of ethanol at time-point 60. AUC=area under the curve, DCPIB=oxybutyric acid, EtOH=ethanol, Veh=vehicle. Adapted from Paper I.*

However, in the experimental set-up used in this thesis, it is not possible to determine if it is inhibition of VRACs located on astrocytes or neurons that is responsible for the results observed, as the *in vivo* microdialysis technique yields a bulk overflow of neurochemicals and cannot distinguish from which source the analytes are originating. In the supraoptic nucleus, studies have shown that neurons within this region do not accumulate taurine or express VRACs (Decavel & Hatton 1995, Deleuze et al 1998), proposing that astrocytes exclusively mediate the taurine release following DCPIB treatment (Choe et al 2012). Further, comparison of VRACs located on astrocytes and neurons shows differences in their pharmacological profile, as neuronal VRACs are insensitive to the potent VRAC inhibitor tamoxifen (Deleuze et al

1998, Leaney et al 1997, Mongin 2016). Additionally, even though DCPIB is supposed to be the most selective blocker of VRACs, off-target actions have been reported (Afzal et al 2019, Bowens et al 2013). Thus, based on the fact that contradictive results have been found with regards to hypoosmotically-induced taurine release *in vivo* using microdialysis compared to cultured astrocytes (Haskew-Layton et al 2008), and the likelihood of regional differences in subunit composition of VRACs, it is difficult to determine whether taurine elevation after ethanol exposure derives from accumbal astrocytes or neurons when interpreting these results.

To explore the involvement of the TauT in the taurine increase after ethanol challenge, the TauT antagonist GES (5 mM) was used. When the TauT is blocked by GES, or when using TauT knockout-mice, cells become taurine-depleted and the clearance of taurine from the extracellular space is impaired (Huxtable 1989, Huxtable 1992, Molchanova et al 2004). The basal taurine levels were substantially increased by GES treatment (Fig. 4B-C, Paper I). Further, the ethanol-induced taurine increase was prevented, an effect presumably due to a ceiling effect rather than an inhibition of cellular release from neurons and/or astrocytes (Fig. 4A-D, Paper I). It has been shown that hypoosmotically-induced taurine release is not prevented by TauT blockade, leading to the suggestion that the basal taurine release possibly relies on both an osmolarity-dependent and -independent mechanism (Brès et al 2000). For the ethanol-induced taurine increase, an osmolarity-dependent mechanism has been observed when ethanol diluted in a hypertonic saline solution (3.6% NaCl) prevented ethanol from elevating nAc taurine levels (Ericson et al 2011, Quertemont et al 2003). An osmolarity-independent mechanism is difficult to evaluate, based on the fact that ethanol exposure changes the extracellular milieu and induces astrocytic cell-swelling (Adermark et al 2011, Allansson et al 2001, Kimelberg et al 1993). Here, the response to TauT inhibition suggests that the transporter is involved in regulating basal taurine levels in the nAc, whereas it is not participating in accumbal taurine increase after ethanol challenge.

To further address the role of accumbal astrocytes in ethanol-induced taurine increase, chemogenetic and metabolic manipulations were performed. Gi- and Gq-coupled DREADDs were expressed under the astrocyte-specific GFAP promoter. Both Gi- and GqDREADD-expressing animals receiving accumbal administration of ethanol (300 mM) demonstrated elevated taurine levels compared to their respective vehicle-treated Gi- and Gq-control (Fig. 6F-H, N-P, Paper I). Interestingly, following ethanol perfusion, a significant increase in

extracellular taurine levels between rats with activated Gi-coupled receptors and the sham-treated counterpart was seen (Fig. 6G-H, Paper I). This effect was not observed in rats transfected with GqDREADDs (Fig. 6O-P, Paper I). To disrupt the astrocytic function, fluorocitrate (25 µM) was applied, leading to metabolic inhibition of astrocytes when the tricarboxylic acid cycle is specifically interrupted (Adermark et al 2022, Fonnum et al 1997). While the basal taurine levels increased following fluorocitrate administration, the taurine response to ethanol showed a trend towards elevated levels in fluorocitrate-pretreated rats compared to ethanol-treated controls (Fig. 7A-D, Paper I). Even though the DREADD technology is recognized with robust effects upon transfecting neurons (Durkee et al 2019, Roth 2016, Shen et al 2021), there are concerns whether activation of DREADDs expressed in astrocytes might not accurately mimic endogenous GPCR activity based on the emersion of varying results hitherto (Lee et al 2023). Moreover, the functional aspects of activating or deactivating astrocytes by GPCR-evoked dynamics are debated (Lee et al 2023, Shen et al 2021). Thus, the consequences of DREADD activation in astrocytes must be carefully assessed. This especially applies to the inhibitory Gi-coupled DREADDs, as the excitatory Gq-DREADDs are recognized with a higher level of similarity between DREADD-mediated and endogenous GPCR effects (Lee et al 2023), which may consequently be a potential reason for the augmentation in the elevation of taurine after ethanol exposure observed in this thesis. In addition, the specificity of fluorocitrate may be questioned (Adermark et al 2024, Fonnum et al 1997). Taken together, modulation of ethanol-evoked taurine levels in the nAc by chemogenetic or metabolic inhibition of astrocytes rather enhanced than suppressed the output. This suggests that astrocytes may not be the primary cell type responsible for the increase of extracellular taurine levels following ethanol exposure within the nAc. Here, it should be noted that astrocytes are highly interconnected through gap-junction channels allowing astrocytic network communication, which could be a contributing cause for the presence of an intact ethanol-induced taurine elevation. In addition, astrocytes are recognized to be a more heterogeneous group of cells than previously estimated (McNeill et al 2021, Zhang & Barres 2010), leading to astrocytes in different brain regions, and even within the same region, to display functional diversities. Thus, astrocytes are not behaving as neurons, complicating the interpretation of the results.

4.1.2 INVOLVEMENT OF NEURONS (OR ASTROCYTES)

Action potential mediated exocytosis is central for neurotransmitter release, which is blocked by TTX when voltage-gated sodium channels and neuronal depolarization are inhibited. Local nAc perfusion with TTX (1 μM) increased not only the basal taurine levels, but also further increased taurine levels after ethanol administration (Fig. 8A-B; see also Fig. 2A-C, Paper I). This suggests that taurine release is under the control of action potential firing at normal conditions, which applies both to the basal release and ethanol-induced increase, and consequently could originate from a non-neuronal source or from neurons via a non-synaptic and non-exocytotic mechanism. Worth noting, voltage-gated sodium channels are also expressed in astrocytes, but these channels are not responsible for action potential generation in astrocytes (McNeill et al 2021).

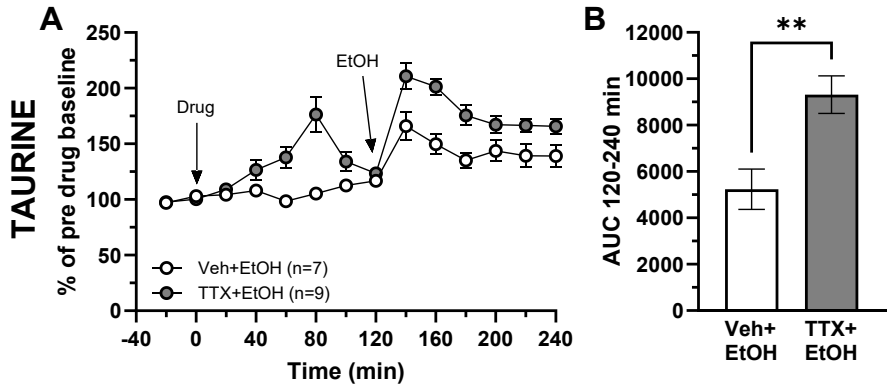

Figure 8. Ethanol-induced taurine increase following TTX pre-treatment. *A) Time-course graphs of nAc taurine levels after nAc pre-treatment with TTX (1 μM) and ethanol (2.5 g/kg) injection presented as % of baseline. B) AUC of the time following ethanol administration displayed a significant increase in taurine output in rats pre-treated with TTX. Arrows in A) indicate start of vehicle (Ringer) or TTX perfusion at time-point 0 and addition of a systemic injection of ethanol at time-point 120. **p<0.01. AUC=area under the curve, EtOH=ethanol, TTX=tetrodotoxin, Veh=vehicle. Adapted from Paper I.*

Basal taurine levels were also found to be tonically inhibited by NMDA receptor activation at normal conditions, as memantine (100 μM)

administration increased baseline taurine output (Fig 3B-C, Paper I). In the memantine-pretreated rats, the taurine response to ethanol showed a trend towards increased levels compared to ethanol-treated controls (Fig. 3A-D, Paper I), suggesting the involvement of NMDA receptors in ethanol-induced taurine release less likely. The NMDA receptor has previously been suggested to play a role in controlling extracellular taurine when the NMDAR antagonist MK801 blocked taurine release in hippocampus after ethanol challenge (Lallemand et al 2000). The observed differences between memantine and MK801 on ethanol-induced taurine output may however originate from slightly different pharmacological profiles combined with region specific effects (Duda et al 2016, Frankiewicz et al 1996, Song et al 2018). TTX has also been found to produce region specific effects *in vivo*, as TTX administration elevates basal taurine levels in the striatum (Molchanova et al 2004), while the taurine output in hypothalamus (Singewald et al 1993) and substantia nigra (Bianchi et al 1999) is reported to be reduced. The lack of effect of TTX on ethanol-induced taurine release rules out the contribution of exocytotic neurotransmitter signaling, but does not dismiss potential involvement of other neuronal mechanisms. This is supported by other findings suggesting that ethanol-mediated taurine elevation may be related to neuronal activity in both the dorsal and ventral part of the striatum (Smith et al 2004), as well as the observation that depolarization of neuronal populations in the cortex (Collins & Topiwala 1974, Kaczmarek & Davison 1972) and cerebellum (Schousboe et al 1990) produces taurine release and thus regulates baseline levels.

To further assess the role of voltage-gated ion channels, voltage-gated calcium channels and the involvement of calcium on ethanol-induced taurine elevation in the nAc was investigated. Generally, voltage-gated calcium channels are involved in generating action potentials in neurons, and other excitable cells such as muscle cells, but they are however also located on astrocytes and have other important roles beyond initiation of action potentials in these cells (D'Ascenzo et al 2004, Latour et al 2003, Verkhratsky & Steinhäuser 2000). Local accumbal pre-treatment using the dihydropyridine and LTCC antagonist nicardipine (100 μM) prevented ethanol-evoked taurine elevation compared to naïve ethanol-treated rats, while the basal taurine levels were unaffected (Fig. 9A-B; see also Fig. 8A-D, Paper I). This suggests that LTCCs are involved in nAc taurine elevation after ethanol exposure and that the mechanism is calcium dependent, while the basal taurine levels do not seem to be regulated by LTCCs. Dihydropyridines are demonstrated to inhibit taurine release evoked by hypoosmolarity both in astrocytic (Li et al 2002, Sánchez-Olea et al 1995)

and neuronal cultures (Philibert & Dutton 1989, Philibert et al 1989), whereas basal, isosmotic, efflux levels are unaffected in both cell types (Philibert & Dutton 1989, Sánchez-Olea et al 1995). The mechanism by which LTCC blockade prevents ethanol-mediated taurine response remains to be delineated, and whether it is LTCCs predominantly located on neuronal cells or astrocytes that are involved in the observed effects.

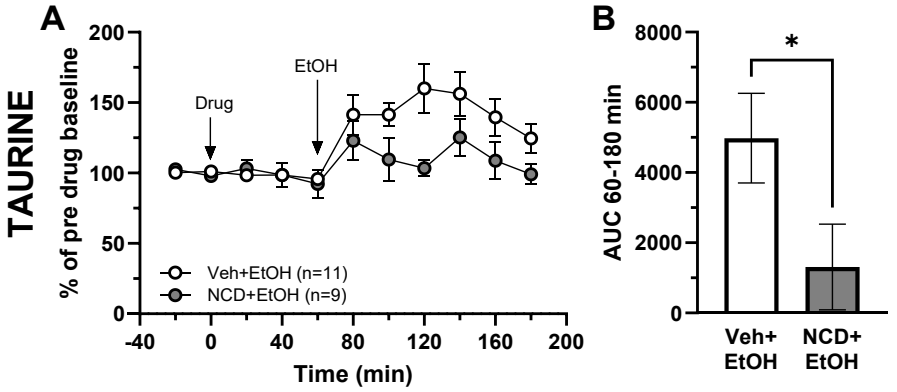

Figure 9. Ethanol-induced taurine decrease following nicardipine pre-treatment. *A) Time-course graphs of nAc taurine levels after nAc ethanol (300 mM) perfusion alone or following pre-treatment with nicardipine (100 µM) presented as % of baseline. B) AUC of the time following ethanol administration revealed a significant reduction in taurine output in rats pre-treated with nicardipine. Arrows in A) indicate start of vehicle (Ringer) or nicardipine perfusion at time-point 0 and addition of ethanol at time-point 60. *p<0.05. AUC=area under the curve, EtOH=ethanol, NCD=nicardipine, Veh=vehicle. Adapted from Paper I.*

By chemogenetically or metabolically inhibiting astrocytes, we found low evidence for astrocytes mediating the ethanol-induced taurine elevation but that they are important for regulating baseline levels of extracellular taurine. Thus, a neuronal mechanism that does not require action potential mediated exocytosis appears to mediate the effect. Supporting the role for neuronal origin of accumbal taurine after ethanol challenge is the observation that ablation of CINs prevents ethanol-evoked taurine increase, whereas the basal taurine levels were left unaltered (Loftén et al 2023). This is further supported, albeit only to some extent since the study was performed in astrocytes, by the suggestion that different mechanisms may control efflux of taurine in

isoosmotic and hypoosmotic environments (Sánchez-Olea et al 1995). To be noted, ethanol is recognized to inhibit LTCCs by reducing the open probability of the channels, primarily by shortening the open kinetics of the channel (Wang et al 1994). These findings are mainly found in cell cultures using electrophysiological recordings (Mullikin-Kilpatrick et al 1995, Mullikin-Kilpatrick & Treistman 1995, Wang et al 1994, Wang et al 1991), reflecting a manipulated system and consequently may not accurately mimic an intact *in vivo* system. Thus, this is less likely to have a major impact in the *in vivo* experimental set-up used in this thesis and on the results presented in Paper I, as ethanol-induced taurine increase is prevented upon inhibition of LTCCs and present at normal channel function. However, ethanol-modulated elevation of accumbal taurine is a complex array of several mechanisms, and the exact underpinnings involved are not possible to disentangle without using methods other than those used in this thesis.

4.2 REGULATION OF ACCUMBAL DOPAMINE AND TAURINE BY ACAMPROSATE-RELATED COMPOUNDS

Ethanol- or taurine-induced dopamine elevation in the nAc involves GlyRs in the nAc and nAChRs in the VTA, either directly or indirectly. Similar observations are found for acamprosate (Chau et al 2010b), the pharmacological drug used for treatment of AUD through its anti-relapse properties. However, the complete mechanistic principles behind the clinical effect in man are, and have been since its first use in the 1980s, obscure and controversial. To further outline the mechanism of action of acamprosate, the different components of acamprosate, calcium and *N*-acetylhomotaurine, were locally applied into the nAc using reversed *in vivo* microdialysis (Paper II). Using the same experimental design, systemically administrated drug was also examined for functional relevance (Paper III). The results revealed a significant increase of extracellular dopamine levels by regular acamprosate following local (0.5 mM) perfusion, while a trend towards elevation was found following systemic (200 mg/kg) administration (Fig. 2A, Paper II; Fig. 3A, Paper III). To explore the involvement of calcium in the observed effect, calcium chloride was used. Local calcium chloride (0.5 mM; calcium chloride referred to as calcium) administration elevated nAc dopamine levels (Fig. 10A), whereas the systemic (73.5 mg/kg) injection resulted in a trend towards an increase (Fig. 10B). Further, treatment with the sodium salt of acamprosate, sodium acamprosate (interchangeably referred to as sodium acamprosate and *N*-acetylhomotaurine), was found not to influence accumbal dopamine levels alone following neither local (1 mM; Fig. 10A) nor systemic (200 mg/kg; Fig. 10B) administration, supporting the proposal that the *N*-acetylhomotaurine part of acamprosate to be biologically inactive (Melugin et al 2022, Pradhan et al 2018, Spanagel et al 2014). However, when calcium and sodium acamprosate were administrated in combination, the dopaminergic output was enhanced and prolonged compared to either drug given alone, following both routes of administration (Fig. 10A-B). This demonstrates that the combination of calcium and *N*-acetylhomotaurine has potentiating effects on the dopamine output in the nAc, and suggests that *N*-acetylhomotaurine is not biologically inert.

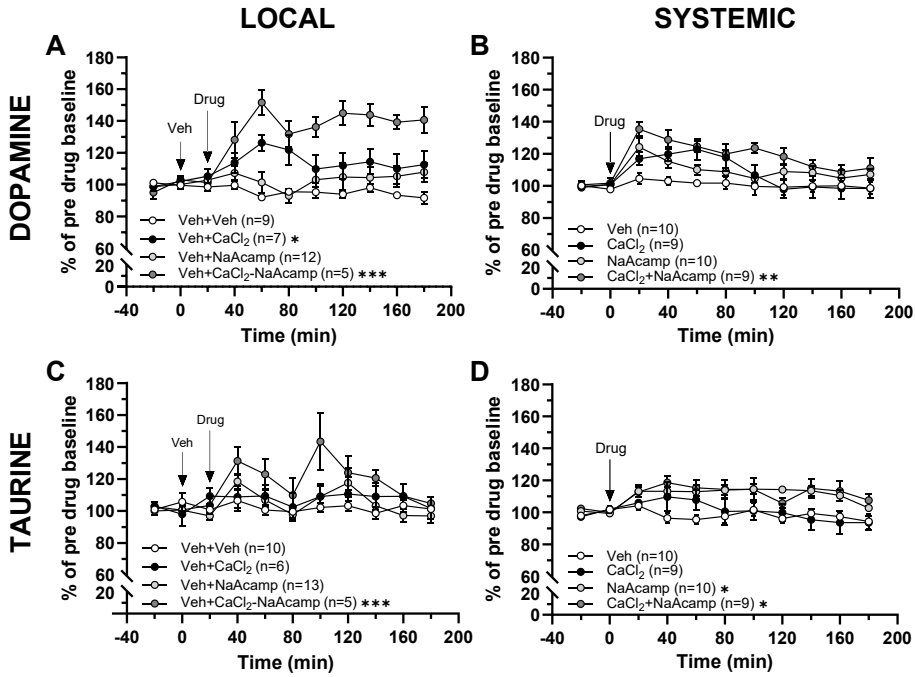

Figure 10. Dopamine and taurine release following administration of the components constituting regular acamprosate. *Time-course graphs of the nAc dopamine and taurine levels presented as % of baseline. Arrows in A) and C) indicate start of vehicle (Ringer) perfusion at time-point 0 and addition of drug at time-point 20. The reason for start of drug-perfusion at time-point 20 depends on appropriate comparison between treatments where strychnine was included (data not shown in the thesis, but are to be found in Paper II). Arrows in B) and D) indicate drug injection at time-point 0. A) $CaCl_2$ (0.5 mM) alone and when combined with NaAcamp (1 mM) elevated nAc dopamine levels following local drug administration into the nAc. B) Only the combination of $CaCl_2$ (73.5 mg/kg) and NaAcamp (200 mg/kg) increased nAc dopamine levels after systemic administration. C) Accumbal taurine output was increased following local nAc administration of $CaCl_2$ and NaAcamp in combination. D) NaAcamp alone and when combined with $CaCl_2$ elevated nAc taurine levels following systemic administration. *p<0.05, **p<0.01, ***p<0.001. $CaCl_2$=calcium chloride, NaAcamp=sodium acamprosate/the sodium salt of N-acetylhomotaurine, Veh=vehicle. Adapted from Paper II, III.*

Supporting the fact that calcium and *N*-acetylhomotaurine act in concert on a neurochemical level, is the finding that the drug combination elevated extracellular taurine levels in the nAc following both local and systemic administration (Fig. 10C-D). In addition, regular acamprosate elevated accumbal taurine levels following both routes of administration (Fig. 6A, Paper II; Fig. 3B, Paper III), and taurine output was also enhanced following systemic treatment with sodium acamprosate (Fig. 10D). Calcium alone did not influence extracellular taurine levels in the nAc. The main findings from the *in vivo* microdialysis studies investigating regular acamprosate, calcium and sodium acamprosate are summed up in table 3 in the chapter covering thesis summary and concluding remarks of the thesis. The results demonstrate that the *N*-acetylhomotaurine molecule of acamprosate has a minor impact on accumbal dopamine levels, while the calcium moiety appears to affect dopaminergic transmission to a larger extent. In contrast, the extracellular taurine levels were not stimulated by calcium, whereas *N*-acetylhomotaurine increased accumbal taurine signaling. This suggests that calcium and *N*-acetylhomotaurine have separate dopamine- and taurine-modulating properties, which act in a potentiating manner following co-administration and may indicate a likelihood of multiple mechanisms contributing to the dopamine- and taurine-elevating effect of regular acamprosate. Dopamine is important for the ethanol reward process, but taurine is also associated with the rewarding properties of ethanol (Li et al 2008). As acamprosate has both dopamine- and taurine-elevating properties, the ethanol-intake decreasing effect of the drug may be related to either acamprosate-induced dopamine or taurine levels or both. To be noted, the taurine elevation following *N*-acetylhomotaurine, or regular acamprosate, administration is not originating from acetylhomotaurine being metabolized to taurine, as the majority of acetylhomotaurine is found unchanged in urine (Saivin et al 1998).

The existence of functional GlyRs in the nAc are reported (Förstera et al 2017, Martin & Siggins 2002) and are known to be involved in maintaining the baseline dopamine tone in this area (Molander & Soderpalm 2005b). Recently, it was suggested that these GlyRs, regulating basal dopamine levels, are not located on CINs (Loftén et al 2023), but rather tentatively on projecting MSNs (Förstera et al 2017, Muñoz et al 2018). Interestingly, beyond previous observations that acamprosate-induced dopamine elevation seems to depend on activation of the GlyR (Chau et al 2010b) and that antagonism of the GlyR reverses the ethanol-intake reducing effect of acamprosate (Chau et al 2010a), local pre-treatment using the GlyR antagonist strychnine (20 μM) blocked the calcium-induced dopamine increase (Fig. 3B, Paper II). This suggests that

GlyRs also play a role for elevated dopamine levels in the nAc after calcium challenge and that GlyRs are important for the dopamine-modulating properties of calcium. In addition, the indication for the involvement of GlyRs in dopamine increase after local exposure to acamprosate was here independently repeated (Fig. 2B, Paper II). However, whether the interaction is direct or indirect is not possible to determine using the experimental set-up used in the present thesis. For acamprosate, evidence support an indirect, rather than direct, interaction possibly through the release of taurine, as acamprosate has been found not to interact directly with GlyRs containing the α1 subunit (Reilly et al 2008). There is a possibility of a direct interaction with α2- or α3-containing GlyRs. However, at least in the mouse nAc, the α1 subunit is expressed at higher levels compared to α2 and α3 (Förstera et al 2017), and a direct link between acamprosate and α2 or α3 containing GlyRs is perhaps less likely. On the contrary, calcium did not elevate nAc taurine levels, thus no firm speculations can be made regarding calcium influencing the GlyR directly or indirectly for a dopaminergic elevation. Nevertheless, it seems likely that the accumbal GlyR is contributing to the dopamine increase observed following both acamprosate and calcium administration, suggesting that the dopamine-elevating property of the drugs is mediated via local microcircuits in the nAc.

In all Papers (I-IV) constituting this thesis, drugs were administered both locally and systemically in the *in vivo* microdialysis studies. Different routes of administration are primarily used depending on the research question. Local drug perfusion in the nAc using reversed microdialysis allows for the exploration of mechanistic principles, while systemic administration of drug has a physiological approach and a higher clinical relevance. In Paper II and Paper III, different routes of administration were used using the same array of drugs, to provide a wider perspective of the drug effects and, although somewhat different results were obtained, consequently be able to draw more likely conclusions regarding the effects on the mesolimbic dopamine system.

4.3 REGULATION OF ACCUMBAL DOPAMINE BY ACUTE AND SUB-CHRONIC CALCIUM TREATMENT

Acamprosate activates the mesolimbic dopamine system leading to increased extracellular dopamine and taurine levels in the nAc, as shown in the present thesis, which also is a robust phenomenon following administration of ethanol. Elevations in calcium levels have been shown to mimic the effects of acamprosate and is believed to be the active component of the drug (Melugin et al 2022, Pradhan et al 2018, Spanagel et al 2014), even though the mechanisms of action remain controversial (Mann et al 2016, Spanagel et al 2016). To investigate if calcium-induced dopamine elevation may interfere with ethanol-induced dopamine elevation, an effect previously observed using acamprosate (Chau et al 2018), *in vivo* microdialysis was used (Paper IV). Local perfusion of calcium (0.5 mM) in the nAc increased extracellular dopamine levels compared to vehicle treatment (Fig. 11A), in line with observations in Paper II. When adding ethanol (300 mM) to the perfusion solution, no further increase in dopaminergic output was detected (Fig. 11A; see also Fig. 2B-C, Paper IV). Consequently, acute calcium administration prevents ethanol from further elevating accumbal dopamine levels, in congruence with acamprosate, supporting the suggestion that calcium plays a role in the mechanistic principles of acamprosate.

To further address the role of calcium on mesolimbic dopamine, rats were sub-chronically treated with calcium or vehicle for ten days to evaluate if long-term calcium (73.5 mg/kg) administration may influence the dopaminergic output differently. On the day of the microdialysis experiment, all animals received a calcium injection (73.5 mg/kg), and the results showed that calcium was only able to mediate an extracellular dopamine increase in the nAc of calcium naïve animals (Fig. 11B), in line with the results in Paper III. Thus, the rats repeatedly exposed to calcium demonstrated a significantly blunted dopamine response to the acute calcium injection (Fig. 11B), suggesting that the dopamine-elevating property of calcium is lost, and hence that tolerance has developed to this effect of calcium. This is in support of the same phenomenon observed in long-term acamprosate-treated rats, where acamprosate-induced dopamine elevation is abolished following sub-chronic treatment (Chau et al 2018, Cowen et al 2005), indicating that calcium is the component of acamprosate to which tolerance develops within the mesolimbic dopamine system upon sub-chronic

treatment. To be noted, basal dopamine transmission was not altered by sub-chronic calcium treatment (Fig. 5A, Paper IV).

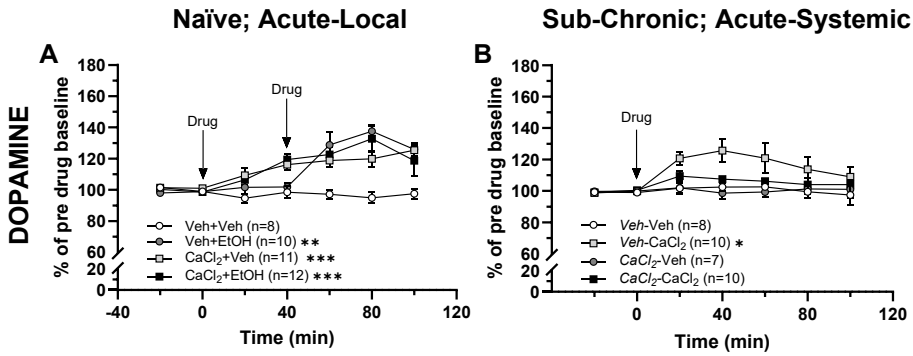

Figure 11. Dopamine release following acute or sub-chronic calcium administration. *Time-course graphs of the nAc dopamine levels presented as % of baseline. Arrows in A) indicate start of vehicle (Ringer) or $CaCl_2$ perfusion at time-point 0 and addition of ethanol (300 mM) perfusion at time-point 40. Arrow in B) indicates systemic $CaCl_2$ injection at time-point 0. A) Accumbal dopamine levels in drug naïve rats were increased following acute local drug administration into the nAc in all treatment groups, and $CaCl_2$ prevented ethanol from further increasing dopamine. B) In rats sub-chronically treated with systemic $CaCl_2$ for ten days, dopamine levels were only significantly elevated in $CaCl_2$ naïve rats receiving an acute injection of $CaCl_2$. The legend in italics in B) represents the pre-treatment that each group received. *p<0.05, **p<0.01, ***p<0.001. $CaCl_2$=calcium chloride, EtOH=ethanol, Veh=vehicle. Adapted from Paper IV.*

The findings on acute and long-term effects of calcium on mesolimbic dopamine output suggest that the dopamine response in nAc depends on the length of calcium treatment. Acute administration elevates dopamine levels, which appears to be a robust phenomenon as separate experiments using different batches of animals and different routes of administration show the same results (Paper II, Paper IV), and prevents ethanol from further increasing the dopamine levels. However, following sub-chronic treatment, there is a blunted response of the dopamine system to calcium. An altered activity of the mesolimbic dopamine system has previously been suggested to play a prominent role in the development of tolerance to the effects of acamprosate (Cowen et al 2005). In the acute stage, acamprosate was demonstrated to

increase the dopamine transporter density and decrease the D2-receptor density in the nAc, thus indicative for an altered dopaminergic tone, whereas these markers of the dopamine system returned to steady-state levels following repeated administration (Cowen et al 2005). Consequently, it is reasonable to speculate that the same phenomenon applies to acute and repeated administration of calcium. However, firm conclusions cannot be drawn, and further mechanistic investigations are needed to address whether calcium is the component of regular acamprosate responsible for the tolerance development observed within the mesolimbic dopamine system.

4.4 REGULATION OF VOLUNTARY ETHANOL INTAKE BY ACAMPROSATE-RELATED COMPOUNDS

The acute reinforcing properties of ethanol involve enhanced dopamine activity in the nAc (Di Chiara & Imperato 1988, Imperato & Di Chiara 1986), and chronic consumption is characterized by abnormal activity of the mesolimbic dopamine system in both humans and animals including a low dopamine tone (Diana et al 1993, Koob & Volkow 2016, Volkow et al 2007). Acamprosate has been suggested to reduce ethanol intake through its dopamine-elevating properties (Chau et al 2018), thereby partly substituting for the dopamine-releasing effect of ethanol on a neurochemical level. In order not to disregard other findings, acamprosate is proposed to reduce relapse-like drinking by normalizing the hyperglutamatergic state appearing during withdrawal (al Qatari et al 1998, Kiefer & Mann 2010, Mann et al 2008) and/or through the elevated calcium levels brought about by acamprosate administration (Schuster et al 2017, Schuster et al 2021, Spanagel et al 2014). To be noted, acamprosate specifically affects ethanol-related behaviors, and does not alter sucrose-, water-, or food-related behaviors (Czachowski et al 2001). In the neurochemical studies using *in vivo* microdialysis in the nAc, the addition of *N*-acetylhomotaurine was found to enhance the effect of calcium-induced dopamine and taurine increase (Paper II, Paper III). Consequently, it was hypothesized that these potentiated neurochemical effects may also be functionally relevant in terms of decreasing voluntary ethanol intake in the rat. To examine this hypothesis, a voluntary ethanol consumption model with a two-bottle choice paradigm was used (Paper III). In addition, a subset of ADE paradigms, evaluating both acute and repeated drug administration to investigate development of tolerance, were applied (Paper III, Paper IV).

4.4.1 VOLUNTARY ETHANOL CONSUMPTION MODULATED BY CALCIUM AND SODIUM ACAMPROSATE

Following intermittent access to ethanol during the screening phase, the paradigm was interchanged to limited access during the treatment phase. Rats treated with the combination of calcium and sodium acamprosate (73.5 mg/kg + 200 mg/kg) reduced ethanol intake during several more days compared to calcium (73.5 mg/kg) treatment alone (Fig. 5A, Paper III). However, adding sodium acamprosate in order to enhance the ethanol-reducing effect of calcium

had no significant effect on the amount of ethanol consumed over time. Thus, the combination of calcium and sodium acamprosate produce potentiated effects with respect to dopamine and taurine output, while this does not appear to translate into potentiated reduction of the voluntary ethanol intake, in the paradigm used in this thesis. It is reasonable to speculate that differences in treatment response may arise from the fact that the animals used in the neurochemical study were ethanol naïve, while the animals consuming ethanol over a period of at least eight weeks were influenced by ethanol-induced alterations. However, this appears less likely, as acamprosate was shown to elevate accumbal dopamine in animals consuming ethanol over two months, indicating no ethanol-evoked adaptations (Chau et al 2018). Notably, regular acamprosate is recognized with reducing ethanol intake only for a few couple of days when used in voluntary ethanol consumption paradigms, an effect associated with tolerance development (Chau et al 2018, Cowen et al 2005, Lido et al 2012, Vengeliene et al 2010). This loss of effect over time was, however, not observed using calcium and sodium acamprosate in combination, as a treatment effect of the combination lasted until the final day of treatment (Fig. 5A, Paper III). To be noted, when the rats were divided into high- and low-consuming groups according to their ethanol intake, interesting observations were made following both calcium treatment alone and when calcium was combined with sodium acamprosate. In the high-consuming animals, a robust decrease in ethanol intake was found following both treatments that lasted over the entire treatment period, whereas no ethanol-intake reducing effect by treatment was observed in the low-consuming animals (unpublished data). In the vehicle-treated controls, ethanol intake was not altered over time. Studies have shown that a low endogenous dopaminergic tone, innate or as a result of chronic ethanol consumption when there is a significant reduction in D2-receptor availability in the striatum (Volkow & Fowler 2000, Volkow et al 2006), is related to a high ethanol consumption in both humans and animals (Diana et al 1993, Ericson et al 2020, Volkow et al 2006, Volkow et al 2007). Thus, the high-consuming animals, presumably with a low dopaminergic tone, decrease their drinking upon pharmacological treatment, as a treatment-induced dopamine elevation is obtained reducing the motivation for consuming. However, this can only be speculated in, as the dopamine levels in these animals were not monitored simultaneously with their ethanol consumption.

4.4.2 THE ALCOHOL DEPRIVATION EFFECT FOLLOWING ACUTE AND REPEATED DRUG TREATMENT

The outcome in the ADE paradigm has in several studies appeared to be associated with dopamine mechanisms (Loftén 2024, Söderpalm et al 2020), including for acamprosate (Lido et al 2012). The effect of acamprosate on the ADE has however not been consistent, as shorter treatment periods demonstrate drug-reduced relapse-like drinking (Heyser et al 1998, Spanagel et al 1996, Spanagel et al 2014), while repeated treatment before testing for ADE shows no clear inhibition (Lido et al 2012) and has been associated with tolerance development (Chau et al 2018, Lido et al 2012). To investigate acute or repeated drug-treatment on relapse-like drinking, and evaluate if rats develop tolerance to the effects of acamprosate or its different components, a subset of ADE paradigms were performed (Paper III, Paper IV).

Regular acamprosate (200 mg/kg) and the combination of calcium and sodium acamprosate (73.5 mg/kg + 200 mg/kg), both shown to induce dopamine increases in the nAc using *in vivo* microdialysis (Paper II, Paper III), were administered acutely in drug naïve rats following two weeks of ethanol deprivation and both treatments were found to abolish the ADE (Fig. 12B for the combination treatment; see also Fig. 4B-C for acamprosate, Paper III). Further, the ADE was assessed in animals previously exposed to drug treatment in the voluntary ethanol consumption study during the limited access paradigm. Repeated drug administration of calcium (73.5 mg/kg) and calcium combined with sodium acamprosate, before the ethanol deprivation period and the subsequent ADE-testing, did not suppress the ADE (Fig. 12C for the combination treatment; see also Fig. 6B-C for calcium alone, Paper III). This implies that the combination of calcium and sodium acamprosate loses some ethanol-intake modulating effect over time, indicative of tolerance development.

In long-term ethanol drinking rats that undergo several deprivation periods, compulsive drinking, loss of typical circadian drinking patterns, and insensitivity to taste adulteration with quinine are typical characteristics during relapse-like drinking and resemble the clinical situation (Vengeliene et al 2014, Vengeliene et al 2013). Thus, acute or repeated administration of calcium (73.5 mg/kg) were evaluated using the ADE paradigm with repeated deprivation phases, to ensure a robust ADE, in order to investigate if calcium is the component contributing to tolerance development to acamprosate. Calcium

administrated to treatment naïve rats suppressed the ADE, whereas rats injected with calcium for ten consecutive days prior to ADE-testing displayed an ADE (Fig. 12E-F; see also Fig. 6B-C, Paper IV).

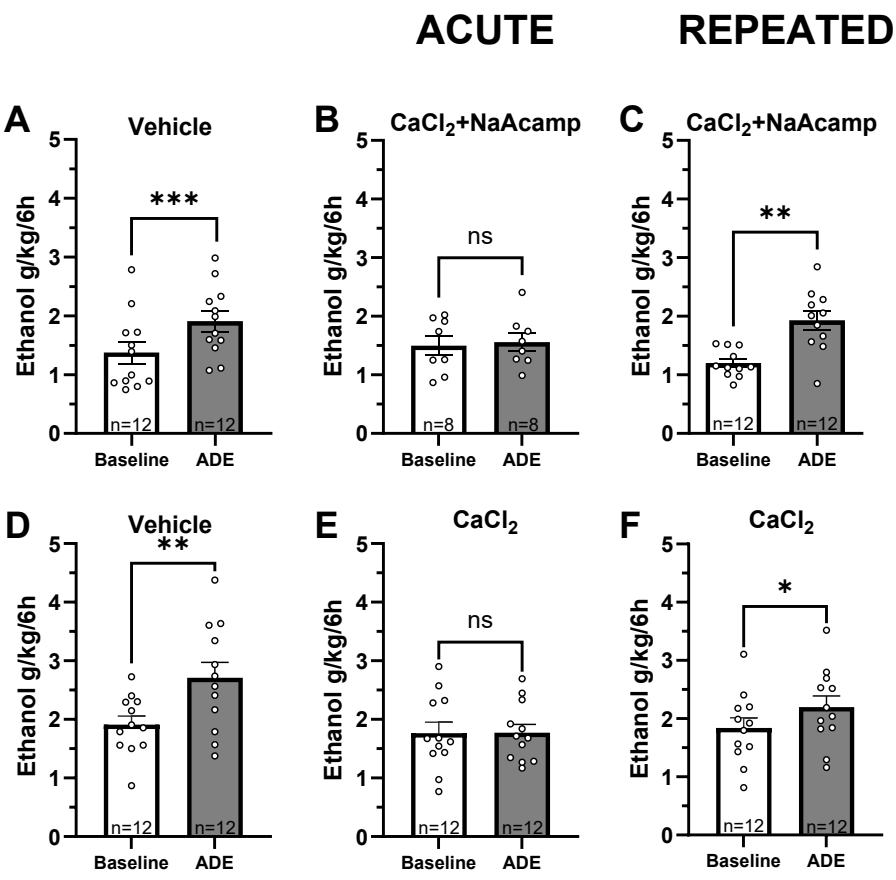

Figure 12. The alcohol deprivation effect is abolished in drug naïve rats, but present in rats previously exposed to drug. *A, D) An ADE was present in vehicle (0.9% NaCl) treated rats. B, E) In drug naïve rats, acute treatment suppressed the ADE following treatment with $CaCl_2$ in combination with NaAcamp (73.5 mg/kg + 200 mg/kg) and $CaCl_2$ alone. C, F) In rats previously exposed to drug treatment, an ADE was not prevented and a significant increase in ethanol intake was observed in both treatment groups. *p<0.05, **p<0.01, ***p<0.001. ADE=alcohol deprivation effect, $CaCl_2$=calcium chloride, NaAcamp=sodium acamprosate. Adapted from Paper III, IV.*

These results are in congruence with the findings investigating the ADE in Paper III, suggesting that the observed differences between acute and repeated drug treatment on ADE outcome are unlikely to depend on different treatment regimens used, rats exposed to a single deprivation period versus rats exposed to repeated deprivation phases, but rather to an association between long-term drug treatment and the development of tolerance. This is supported by the fact that rats sub-chronically treated with calcium show loss of calcium-induced dopamine elevation in the nAc (Paper IV), further supported by the same phenomenon observed for acamprosate (Chau et al 2018). Consequently, there is an indication of tolerance development to both the dopaminergic and behavioral ethanol-related effects of calcium, mimicking previously observed outcomes for acamprosate, and suggesting that calcium may be the component of acamprosate responsible for the tolerance development of the same drug. This also argues for that calcium may be the part of acamprosate responsible for the hypothesized substitution for ethanol-induced dopamine elevation in the nAc and the reduced ethanol intake, before this effect is lost with the onset of tolerance. Whether this holds true also for the human situation, or if there is a difference between species with regards to tolerance development to the effects of calcium and acamprosate on mesolimbic dopamine and ethanol intake, is difficult to assess. It is known that successful treatment with acamprosate appears to be associated with compliance and motivation for complete abstinence (Koeter et al 2010), but if tolerance is the reason for lack of compliance or the treatment being ineffective in a subset of individuals is unknown. In addition, even though acamprosate and ethanol have resembling neurochemical profiles, acamprosate has never been reported to display addictive properties, neither in clinical nor in preclinical studies.

Some aspects of the results obtained from the different drinking models need to be highlighted. First, it has been observed that a drug effect found in, for instance, the voluntary ethanol consumption model, does not always generate a treatment effect in, for instance, the ADE paradigm, and vice versa. This has been observed for glycine reuptake inhibitors being effective in reducing voluntary ethanol intake but not influencing the ADE ((Lido et al 2012, Molander et al 2007); but see however (Vengeliene et al 2010)), while the combination of varenicline and bupropion abolishes the ADE but does not reduce intermittent ethanol drinking (Söderpalm et al 2020). The results presented in this thesis (Paper III, Paper IV) do not apply to this fact, as a drug-induced effect was found both in the voluntary ethanol drinking model and in the ADE paradigm. However, rats only displayed a treatment response following acute drug administration in the ADE model, and not after repeated

drug exposure, probably due to the onset of tolerance. Second, the genetic background of rats is decisive for ethanol intake. In various alcohol-preferring rat lines, genetically bred for high consumption, all strains drink high amounts of ethanol in an intermittent two-bottle choice model, while not all strains show an ADE even after repeated deprivation phases (Ciccocioppo 2013, Vengeliene et al 2014). The outbred Wistar rat used in this thesis generally displays a high ethanol intake, and since the same strain is used throughout the studies of ethanol consumption, although different batches of animals, the results presented rather depend on a real drug effect, and loss of effect, than the animals not responding in the ADE model.

5 THESIS SUMMARY AND CONCLUDING REMARKS

Along with the global burden of disease, the complexity of alcohol use disorder, comprising altered neurochemical function and manifest neuroadaptations, emphasizes the requirement of improved pharmacotherapies to be developed to complement the current arsenal of low-efficacy treatments. In fact, as AUD often occurs together with other psychiatric disorders, emerging treatments would preferably target and address both or several conditions. To enable the development of novel pharmacological interventions, the insight of basic biochemical mechanisms associated with AUD, including a deeper understanding of events within the mesolimbic dopamine system and enhanced knowledge of how existing drugs interact with the brain reward system at a neurochemical level, needs to be improved. This thesis focused largely on the role of taurine-related compounds on mesolimbic dopamine and taurine levels and the effects of and on ethanol and ethanol intake, respectively. Overall, the results show that the mechanism(s) underlying ethanol-induced taurine release in the nAc is multidimensional. In addition, the results indicate an important role of the acute effects of acamprosate, and its components, on dopamine signaling and behavioral ethanol-related effects.

Elevated accumbal taurine levels following ethanol administration is a robust phenomenon. Antagonizing VRACs, channels important for osmoregulation, appeared not to reduce ethanol-induced taurine levels, even though an insignificant trend was observed, questioning the role of astrocytes in ethanol-evoked taurine increase. By selectively targeting accumbal astrocytes, using astrocyte specific Gi- and Gq-coupled DREADDs and fluorocitrate, an intact ethanol-induced accumbal taurine increase was present, suggesting astrocytes not to be the main cell type important for evoked taurine release. Administration of TTX mediated elevated taurine levels after ethanol exposure in the nAc, demonstrating that the ethanol-induced taurine elevation does not rely on action potential firing. Blockade of LTCCs attenuated the taurine-elevating property of ethanol, suggesting that activation of LTCCs and calcium flux to play a key role in the mechanisms underlying ethanol-induced taurine increase. The main findings from Paper I are provided in table 2.

Table 2. *Summary of how different manipulations (treatments) affect accumbal taurine levels and ethanol-induced taurine levels. Comparison is made between manipulation and vehicle (column 1), manipulation+EtOH and treatment (column 2) and manipulation+EtOH and vehicle+EtOH (column 3). EtOH=ethanol, FC=fluorocitrate, GES=guanidinoethyl sulfonate, DREADDs=designer receptors exclusively activated by designer drugs, LTCCs=L-type calcium channels, VRAC=volume regulated anion channels, TTX=tetrodotoxin. ↔ signify a non-significant result. N/A=experiment not assessed.*

Manipulation	**Effect on taurine**		
	Manipulation vs. Vehicle	Manipulation +EtOH vs. Treatment	Manipulation +EtOH vs. Vehicle +EtOH
EtOH *Local administration*	↑	N/A	N/A
EtOH *Systemic administration*	↑	N/A	N/A
TTX *Action potential inhibition*	↑	N/A	↑
Memantine *NMDAR inhibition*	↑	↑	↔
GES *Taurine transporter inhibition*	↑	↔	↑
DCPIB *VRAC inhibition*	↔	↔	↔
Gi-DREADDs (inhibitory) *Activation of inhibitory receptors on astrocytes*	N/A	↑	↑
Gq-DREADDs (excitatory) *Activation of excitatory receptors on astrocytes*	N/A	↑	↔
FC *Astrocyte TCA-cycle inhibition*	↑	↑	↔
NCD *LTCCs inhibition*	↔	↔	↓

Although both astrocytes and neurons express several of the channels and receptors investigated, the results indicate that astrocytes appear to play an important role in regulating basal taurine levels, while ethanol-induced taurine increase may originate from and is associated with spontaneous, action potential-independent, release from neuronal cells. The exact mechanisms

involved are not possible to outline with the experimental set-up used in this thesis, and a firm conclusion of the ethanol-induced taurine increase cannot be drawn. In fact, the cellular localization and accumulation of taurine is diverse throughout the brain (Decavel & Hatton 1995, Hussy et al 2000, Madsen et al 1985), not facilitating the interpretation of the results. It is highly warranted to further disentangle the neuronal and/or astrocytic origin of ethanol-induced taurine release in an *in vivo* system, as *ex vivo* settings not accurately mimic an intact system due to loss of reciprocal connectivity. Based on the regional diversity of astrocyte physiology and the ability of astrocytes to form networks of interconnected cells, appropriate decisions regarding experimental design have emerged to be critical in order to obtain reliable results in these cells. Both optogenetics and chemogenetics are useful techniques, but the heterogeneous nature of astrocytes needs to be considered as is done for corresponding studies involving neurons where it is possible to specifically target dopaminergic or glutamatergic neurons for instance. Fiber photometry is another method available to use for investigation of neuronal and non-neuronal activities, but requires specific biosensors currently not available for taurine assessment.

GlyRs in the nAc have a central role in modulating mesolimbic dopamine activity. Administration of acamprosate, the calcium salt of *N*-acetylhomotaurine, and calcium increased accumbal dopamine levels, while local pre-treatment with strychnine blocked this effect. This suggests that the extracellular dopamine increase produced by these drugs in the nAc is GlyR dependent either by direct or indirect interaction, possibly through the involvement of a simultaneous taurine elevation within the same area. At least for acamprosate, since a calcium-induced taurine elevation does not occur. Calcium administrated in combination with the sodium salt of *N*-acetylhomotaurine potentiated the effects on dopamine output, increased accumbal taurine levels, robustly reduced ethanol consumption, and abolished the ADE in drug naïve rats, indicating that the combination outperforms the effect of either drug component alone. These findings suggest that both components of regular acamprosate have pharmacological activity and possibly that dual mechanistic effects underlie the clinical outcome in humans. However, the observed effects appear to be limited to acute drug treatment, as repeated drug exposure, of both calcium alone and when combined with sodium acamprosate, was associated with tolerance development as previously observed with acamprosate. The main findings from Paper II-IV are provided in table 3.

Table 3. *Summary of how different manipulations (treatments) affect accumbal dopamine (column 1) and taurine (column 2) levels, and how voluntary ethanol consumption (column 3) is affected by treatment where applicable. ADE=alcohol deprivation effect, i.p.=intraperitoneal, corresponding to systemic drug administration, nAc=nucleus accumbens, corresponding to local drug administration. ↔ signify a non-significant result. N/A=experiment not assessed.*

Manipulation	**Effect on dopamine**		**Effect on taurine**		**Effect on ethanol consumption**		
	nAc	i.p.	nAc	i.p.	Voluntary	ADE	
						Acute	Repeated
Acamprosate	↑	↔	↑	↑	N/A	↔	N/A
Calcium	↑	↔	↔	↔	↓ initially	↔	↑
Sodium acamprosate	↔	↔	↔	↑	N/A	N/A	N/A
Calcium + sodium acamprosate	↑	↑	↑	↑	↓ initially	↔	↑

Ethanol acts on a multitude of targets in the brain, as declared in the introduction of this thesis, and it is reasonable to believe that the activity of acamprosate also is attributed to the interaction with several targets, presumably not through action at a specific molecular site as this may simply not exist. Calcium appears central for the effects of acamprosate, as the ethanol-induced dopamine increase is blocked by acute calcium treatment and the ADE is abolished, while repeated calcium exposure attenuates the dopamine response, and the ADE is not suppressed. All these observations mimic effects seen following acute and long-term acamprosate treatment, suggesting calcium as the component of acamprosate responsible for tolerance development. To investigate the effects of *N*-acetylhomotaurine alone on these parameters is needed and experiments exploring this should be performed, to confidently determine which part of acamprosate that is in control of the development of tolerance in rats. In addition, to verify whether acamprosate mainly interferes with the dopamine- or taurine-elevating effects of ethanol, it would be desirable to examine if acamprosate prevents ethanol from further raising accumbal taurine levels and if this effect possibly is lost over time as observed for dopamine. This may provide information on whether an intact taurine increase is necessary for the acamprosate-induced dopamine release or not, in the acute and long-term perspective, and consequently if it is when acamprosate no longer increases accumbal dopamine or simultaneously no

longer elevates dopamine and taurine that the compound loses its ethanol intake-reducing effect. Thus, it would be valuable to gain knowledge of whether it is the loss of dopamine- or dopamine and taurine-related effects that underlies the tolerance development, in order to be able to refine the acamprosate treatment in a future perspective.

In this thesis, *N*-acetylhomotaurine is proven not to be biologically inert. On the contrary, it appears to enhance the neurochemical effects of calcium within the mesolimbic dopamine system although it did not significantly add on to the reduced ethanol intake produced by calcium alone. Calcium elevates accumbal dopamine levels and inhibits further dopamine elevation by ethanol, while taurine levels are increased following *N*-acetylhomotaurine treatment. The two components of acamprosate contribute together with various important properties of regular acamprosate, making acamprosate resembling the effects of ethanol and consequently acting as a neurochemical substitution for ethanol and its positive reinforcing effects within the mesolimbic dopamine system until tolerance has developed.

6 FUTURE PERSPECTIVES

Lifting the view from the preclinical to the clinical perspective, and bearing in mind the heterogeneity of the disorder and the fact that available pharmacological AUD treatments possess limited efficacy, identification of pharmacogenetic and pharmacometabolomic predictors would benefit personalized treatment regimens to avoid one-size-fits-all and improve treatment outcome. Studies using pharmacometabolomics found higher baseline serum glutamate levels as a biomarker for treatment response of acamprosate in AUD patients (Mason & Heyser 2021). Precision medicine would likely advance the field of AUD treatment in combination with the development of new effective compounds and the use of repurposed drugs alone or in combination based on the clinical presentation. Based on alterations in several integrating neurocircuitries due to neuroadaptations, combinations of drugs might provide AUD patients with a long-lasting and enhanced effect when multiple systems are modulated in parallel.

In the light of calcium as the active component of acamprosate, calcium as a pharmacological intervention for AUD needs to be discussed. Calcium was already during the 1950-60s shown to reduce physical withdrawal symptoms in individuals with AUD receiving a high load of intravenously administrated calcium (O'Brien 1952, O'Brien 1964). However, calcium as therapy for AUD has not been evaluated until recently, where oral calcium administration was demonstrated to reduce craving and decrease the intensity of withdrawal symptoms in alcohol-dependent individuals during inpatient detoxification treatment (Schuster et al 2021). Furthermore, lowered plasma calcium concentration has been linked to increased craving and as a risk factor for relapse (Bach et al 2022, Schuster et al 2017). In acamprosate treated abstinent alcohol-dependent individuals, plasma calcium levels have been found both to be correlated to reduced relapse susceptibility (Spanagel et al 2014, Spanagel et al 2016) and not to correlate with the propensity to relapse (Mann et al 2016, Mann et al 2013). Interestingly, it was recently suggested that peripheral calcium activates brain areas engaged in inhibition of craving (Bach et al 2022). However, the neurobiological basis of the findings is to be elucidated. At a healthy physiological state, the levels of calcium in plasma are tightly regulated primarily by the kidneys, bone, and parathyroid hormone (Blaine et al 2015, Fleet & Schoch 2010). Therefore, using calcium as a treatment may appear temporary. However, calcium supplements, such as calcium carbonate, are frequently used for other indications and are harmless up to 2500 mg

calcium daily (FASS 2024, German Nutrition Society 2013). Consequently, it may be clinically safe to use a dosage regimen already used for such calcium supplements, if calcium appears as a pharmacological treatment option for AUD and evidence demonstrates clinical relevance. At the same time, our data, in rats, indicate that calcium is associated with a gradual decrease in efficacy on alcohol intake, as reduced voluntary alcohol intake only persists the initial days of treatment and that an ADE is present following repeated calcium treatment due to tolerance development. However, long-term treatment may not be associated with tolerance development in humans and could be an important treatment supplement to the most vulnerable group of AUD patients. In addition, based on the difficulties in developing mechanistically novel medications the last decades, the use of repurposed drugs should be encouraged. LTCC blockers, where several of them penetrate into the brain, are associated with a decreased occurrence of some neuropsychiatric disorders (Colbourne & Harrison 2022). Whether LTCC antagonists are effective in decreasing alcohol intake, the risk of relapse, or even lowering the incidence of AUD would be interesting to evaluate. To initiate this investigation, a first step may be to examine the prevalence of AUD among patients receiving LTCC blockers for other indications, to gain insight into whether LTCCs may possibly be a target for some individuals who are at risk of developing AUD.

ACKNOWLEDGEMENT

Throughout my PhD project, I have been fortunate to work with and receive support from several incredible and hardworking people, and to have a wonderful group of friends and family by my side. I would like to express my deepest gratitude to:

First and foremost, **Mia**, my main supervisor, for replying the e-mail I sent to you asking whether I could perform my master thesis project within the group. Your encouragement and endless support have been a security along the way, and your pedagogic guidance has navigated me in the right direction when necessary. Thank you for always having your door open being available for questions, no matter whether they have been research-related or not, despite your permanently full agenda. Your enormous knowledge, and wisdom, is truly admirable, and if I could achieve just a fraction of it, my future self would be very grateful.

Louise, for always being truly engaged and helping out regardless of the current status of your workload. Your feedback, whether oral or written, has greatly improved my ability to look at data and to write manuscripts. The enthusiasm and sharp scientific mind you possess provides a huge inspiration.

Bosse, for your unlimited knowledge and supportive engagement. Thank you for providing me, as a pharmacist, with a broader insight and perspective of the clinical picture and for sharing all your experience with kindness. Your help have been invaluable and you never fail to inspire.

Rosita, you know that without all your support, knowledge and vast technical skills, there would have been no data. Thank you for your guidance in the lab, but also for all the laughs and conversations about everyday life such as sports and training, trips to Skåne, parenthood, and for you always being available as a listener.

My dearest co-PhD colleagues and friends, **Klara**, **Anna**, **Oona** and **Yasmin**, for your crazy open-mindedness and willingness to always help out and discuss science and everything beyond. Thank you for our everyday laughs and for all that we have shared throughout the years. It has been a privilege to learn from you and work by your side. I also want to thank **Lisa**, former PhD-student, for teaching and guiding me during my master thesis project and pre-PhD year.

Ana, **Erika**, **Davide** and **Johanna**, members of Adermark's lab, for your curiosity and kindness, and for contributing with Italian intelligence to the work environment. The clinical part of the group, **Barbro**, **Cecilia**, **Andrea**, although we seldom meet, for gatherings and meetings. I also want to thank all past members and students of the Addiction Biology Unit, especially **Mathilda**, for being part of one of my projects.

Johanna and **Jenny**, for being by my side since we were all playing around in the pool, believing that was the most important thing in life. Thank you for your patience through the years and for always putting effort in supporting and encouraging me. We may be far apart, but you are always with me. **Josefin**, for our friendship that began during our university studies and for our sometimes odd humor. Thank you for your bright inputs in everyday life. There is definitely no one like you.

Sven-Göran & **Helen**, **Lovisa** & **Andreas**, **Karolina**, **Ellika** & **Jack**, **Stefan**, **Fredrik** & **Pernilla** and **Wiveca**, for all kindness, laughs, and happiness you give me. **Anna**, my mother-in-law, for your availability and the support you give our little family.

Mamma, without your endless love and support you always have given me, nothing in life would have been possible. Thank you for always being there, for taking care of and serving me and my family through ups and downs. Thank you for everything. **Anna**, for being my best sister. For your love and care. Thank you for supporting and believing in me, always. **Pappa**, for being curious and caring. For all your stupid (!?) questions you never got answers to. I am sorry for you never had the opportunity to read this thesis. I miss you!

My own family. **Alexander**, for believing in me and encouraging me since the first day we met. Without all your support, there would be not thesis. Thank you for calming me down and for making me feel safe and loved. **Iris**, for all the joy and meaningfulness you bring to my life, being the most sweetest and clever little girl. Thank you, and thank you to your currently growing sibling, for being the best part of me. Together the three of you are filling my life with endless love and laughter. Love you ♥

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