Taurine and dopamine-related effects of ethanol

- an experimental study in rodents

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To my family

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ABSTRACT

The reinforcing properties of alcohol (ethanol) are associated with activation of the mesolimbic dopamine system and the concomitant increase in dopamine in the nucleus accumbens (nAc). Changes in this system are thought to be a predominant underlying factor in promoting excessive alcohol intake and alcohol use disorder. We have previously shown that a simultaneous increase in endogenous taurine is required in order for ethanol to increase nAc dopamine levels, and hypothesize that taurine, which acts as an osmoregulator, is released in order to re-equilibrate the osmotic pressure. The intake of taurine has escalated over the last decade due to consumption of taurine-containing energy drinks, but whether a long-term intake of taurine induces adaptations influencing ethanol-induced dopamine elevation is not clear. Thus, the overall aim of this thesis was to investigate correlations between taurine and dopamine during ethanol exposure, with special focus on the nAc. To this end, behavioral tests were combined with neurochemical measurements and gene expression analysis performed in rodents. Our data show that systemically administrated taurine enters the CNS, a process that is not influenced by sub-chronic taurine treatment. Even though acute exposure does not increase locomotion, repeated exposure leads to behavioral sensitization to the drug, and taurine combined with caffeine potentiates ethanol-induced locomotion, a phenomenon previously linked to the reinforcing properties of the drug. By means of *in vivo* microdialysis we show that rats consuming high levels of ethanol respond with a blunted taurine elevation in response to acute ethanol treatment, and exhibit a lower dopamine tone compared to rats consuming low amounts of ethanol. At the same time, repeated taurine exposure does not influence the dopamine elevating properties of ethanol. By combining microdialysis with pharmacological and chemogenetic manipulations, we found that ethanol-

induced taurine release is not action potential dependent and may involve astrocytes and volume regulated anion channels (VRACs). In conclusion, we suggest that increased nAc taurine levels following ethanol exposure mainly derives from astrocytes and involves VRACs, supporting an osmoregulatory role of taurine. Even though ethanol-induced dopamine release is not influenced by sub-chronic taurine exposure, taurine could contribute to the increase in alcohol consumption seen in humans drinking alcohol mixed with energy drinks.

SAMMANFATTNING PÅ SVENSKA

Taurin- och dopaminrelaterade effekter av etanol – en experimentell studie på gnagare

Alkohol har konsumerats i årtusenden världen över. Idag utgör konsekvenser av alkoholkonsumtion ungefär 5% av den globala sjukdomsbördan och 3 miljoner människor dör varje år på grund av alkoholrelaterade skador. De skador som alkoholen medför är inte enbart begränsade till den enskilda individen utan även familj, vänner och samhället påverkas negativt. När konsumtionen blir skadlig och ett antal diagnoskriterier uppfylls används sjukdomstermen Alcohol Use Disorder (AUD). Sjukdomen är ett kroniskt tillstånd som orsakar psykisk så väl som fysisk ohälsa och samhällskostnaderna för alkoholrelaterade sjukdomar är enorma både i Sverige och globalt. De farmakologiska behandlingar som finns tillgängliga för AUD-patienter idag har begränsade effekter och många återfaller i alkoholmissbruk. För att på sikt kunna ta fram en förbättrad läkemedelsbehandling behöver vi ökade kunskaper om de mekanismer som ligger till grund för utvecklingen av AUD. Alkohol (etanol) och andra beroendeframkallande droger aktiverar det mesolimbiska dopaminsystemet, som är en del av hjärnans belöningssystem. Denna aktivering resulterar i ökade dopaminnivåer i nucleus accumbens (nAc), vilket ger en känsla av välbehag. Vår forskning har tidigare visat att för att etanol ska kunna aktivera det mesolimbiska dopaminsystemet så krävs en frisättning av den kroppsegna aminosyran taurin. Det övergripande syftet med denna avhandling var att kartlägga interaktionen mellan taurin och dopamin vid etanol-inducerat beteende och neurotransmission hos obehandlade så väl som etanol- eller taurin-förbehandlade djur.

Då förändringar i det mesolimbiska dopaminsystemet har föreslagits ligga till grund för en överdriven alkoholkonsumtion ville vi i den första studien (paper I) undersöka om den etanolinducerade taurin/dopaminfrisättningen i nAc var förändrad efter sju veckors frivilligt alkoholintag. Genom att genomföra *in vivo* mikrodialys studier på Wistar råttor fann vi att de råttor som valde att konsumera stora mängder etanol hade en lägre och långsammare ökning av taurin när de exponerades för etanol. Dessa djur hade också lägre dopaminnivåer i nAc jämfört med djur som valt att dricka små mängder etanol. Om dessa förändringar är en konsekvens av alkoholintaget, eller om genetiska skillnader från början påverkat konsumtionen, är i nuläget inte känt. Framtida studier får utvisa om det finns ett kausalt samband mellan ett dämpat etanolinducerat taurinsvar och en låg dopaminerg ton.

Då intaget av taurin har ökat kraftigt det senaste årtiondet, mycket på grund av konsumtion av taurininnehållande energidrycker, ville vi i den andra studien (paper II) undersöka om den etanolinducerade taurin-/dopaminfrisättningen i nAc var förändrad efter långvarig exponering för höga doser av taurin. Vi fann att en längre tids behandling med taurin inte påverkar dess förmåga att passera från blodet till hjärnan eller taurinnivåerna i nAc. Även om upprepad behandling med taurin leder till en ökad känslighet för ämnets lokomotorstimulerande egenskaper, ett beteende som tidigare kopplats samman med en ökad dopaminaktivitet i det mesolimbiska dopaminsystemet, så påverkar upprepad exponering av taurin inte dopaminfrisättningen efter en akut injektion med etanol. En av taurinmolekylens viktigaste egenskaper i kroppen är att fungera som en osmoreglerare. Vi tror därför att alkohol ger en frisättning av taurin för att balansera vätsketrycket över cellmembranet och på så sätt motverka cellsvullnad. I den tredje studien (paper III) ville vi definiera från vilken typ av celler som taurin frisätts efter etanolexponering och hur denna frisättning sker. I ett flertal mikrodialysstudier blev råttorna lokalt behandlade med nervcells- eller astrocythämmande substanser och därefter etanol. Sammantaget drog vi slutsatsen att den etanolinducerade taurinökningen kommer från astrocyter, inte nervceller, och att frisättningen framförallt medieras via volymreglerande kanaler (VRACs).

I energidrycker förekommer förutom koffein ofta stora mängder taurin, och intag av energidrycker i kombination med alkohol har visats öka risken för en skadlig alkoholkonsumtion och även AUD. Individer som konsumerar kombinationen uppvisar ett försämrat omdöme, vilket medför att de utsätter både sig själva och andra för risker. För att studera om detta beror på en farmakologisk interaktion gavs möss en akut injektion med olika koncentrationer och kombinationer av etanol, taurin och koffein, och djurens lokomotoraktivitet registrerades. Administrering av etanol eller koffein gav upphov till en signifikant ökning av lokomotionen, men inte taurin. Även om taurin inte påverkade etanol-inducerad lokomotion i sig själv, sågs en potentiering vid administrering tillsammans med koffein och etanol vid specifika doskombinationer (paper IV). Ökad lokomotion är ett fenomen som tidigare har associerats till drogers positivt förstärkande egenskaper. Detta skulle därför kunna innebära att taurin, troligen tillsammans med koffein, bidrar till den ökade alkoholkonsumtion som rapporterats hos människor som förtär alkohol i kombination med energidrycker.

Sammanfattningsvis tyder fynden i denna avhandling på att de ökade taurinnivåerna i nAc efter exponering för etanol är ett robust fenomen, och att denna taurinfrisättning huvudsakligen härrör från astrocyter och inbegriper

volymreglerande kanaler. Resultaten tyder också på att de kroppsegna nivåerna av taurin i hjärnan är hårt reglerade och inte påverkas av upprepad exponering för etanol eller taurin. Vidare föreslår vi att systemisk administrering av taurin kan ha långvariga effekter i hjärnan, som i den aktuella avhandlingen visas som beteendemässig sensitisering, men att upprepad exponering för taurin inte påverkar hur eller i vilken grad etanol ökar dopamin. Slutligen, det är möjligt att en interaktion mellan taurin, koffein och etanol kan orsaka ett ökat alkoholintag hos personer som blandar energidrycker med alkohol.

LIST OF PAPERS

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

I.   Ericson M, **Ulenius L**, Andrén A, Jonsson S, Adermark L, Söderpalm B. *Different dopamine tone in ethanol high- and low-consuming Wistar rats*
Addiction Biology 2019; doi: 10.1111/adb.12761

II.   **Ulenius L**, Andrén A, Adermark L, Söderpalm B, Ericson M. *The influence of sub-chronic taurine administration on locomotor activity and nucleus accumbens dopamine following ethanol*
Submitted 2019

III.   **Ulenius L**, Adermark L, Andrén A, Ademar K, Söderpalm B, Ericson M. *The role of astrocytes in regulating taurine and dopamine interactions during ethanol exposure*
Manuscript

IV.   **Ulenius L**, Adermark L, Söderpalm B, Ericson M. *Energy drink constituents (caffeine and taurine) selectively potentiate ethanol-induced locomotion in mice*
Journal of Pharmacology, Biochemistry and Behavior 2019; doi: 10.1016/j.pbb.2019.172795

CONTENT

ABBREVIATIONS

5-HT$_3$R	5-hydroxytryptamine subtype 3 receptor
5-HT	5-hydroxytryptamine (serotonin)
A$_2$R	Adenosine subtype 2 receptor
ACh	Acetylcholine
AmED	Alcohol mixed with energy drink
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUD	Alcohol use disorder
BBB	Blood brain barrier
BCSFB	Blood-CSF-barrier
cDNA	Complementary DNA
CE-LIF	Capillary electrophoresis coupled with laser-induced fluorescence detection
CNO	Clozapine-N-oxide
CNS	Central nervous system
CSF	Cerebrospinal fluid
D1 receptor	Dopamine receptor type 1
D2 receptor	Dopamine receptor type 2
D3 receptor	Dopamine receptor type 3
D4 receptor	Dopamine receptor type 4
D5 receptor	Dopamine receptor type 5
DCPIB	(4-(2-Butyl-6-7-dicholro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DREADDs	Designer receptor exclusively activated by designer drugs
DS	Dorsal striatum
DSM-5	Diagnostic and statistical manual for mental disorders-5
GABA	γ-aminobutyric acid
GABA$_A$R	GABA receptor type A
GABA$_B$R	GABA receptor type B
Gi-DREADD	Inhibitory DREADD
GlyR	Glycine receptor
Gq-DREADD	Excitatory DREADD
HPLC	High performance liquid chromatography
LDTg	Laterodorsal tegmental nucleus
mGluR	Metabotropic glutamate receptor
mRNA	Messenger RNA
MSNs	Medium spiny neurons
nAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartic acid

NMDAR	NMDA receptor
P2X4R	Purinergic P2X subtype 4 receptor
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
PPTg	Pedunculopontine tegmental nucleus
qPCR	Quantitative polymerase chain reaction
TauT	Taurine transporter
TTX	Tetrodotoxin
VRAC	Volume regulated anion channels
VTA	Ventral tegmental area

INTRODUCTION

Alcohol use disorder (AUD)

Alcohol, a psychoactive substance with an addictive potential, has been used worldwide in many different cultures for thousands of years. Today, 5.1% of the global burden of disease and injury is attributed to alcohol intake. Excessive alcohol use is a leading risk factor for premature death, and worldwide 3 million people die from alcohol-related causes every year. The harmful use of alcohol is not only restricted to the particular individual, it also causes harm to family, friends as well as society. In addition to causing a significant health and social loss, alcohol has a great impact on the economic burden of the society (WHO, 2019). The annual cost in Sweden is estimated to 66 billion SEK (Svenska Folkhälsomyndigheten, 2019). When the consumption becomes severe, a physician can explore where Alcohol use disorder (AUD) is present. There are no quantitative or physiological assessments to use in order to confirm AUD. Instead, the diagnosis is based on fulfilling a certain number of criteria congregated in diagnostic manuals, The Diagnostic and Statistical Manual of Mental Disorders of the American Psychiatry Association, 5th edition (DSM-5) (table 1) or ICD-11 by WHO, used by physicians. Long-term alcohol consumption is a casual factor for more than 200 medical conditions (WHO, 2019), including various types of malign neoplasms, such as cancer in both the upper and lower digestive tract and breast cancer, but also cardiovascular and circulatory diseases (hypertension, dysrhythmias, stroke) as well as digestive diseases (hepatic steatosis, alcoholic hepatitis, cirrhosis) (Shield *et al.*, 2013). During prolonged drinking also brain atrophy and neurodegeneration occur leading to impairment of neurological function e.g. deficits in visuo-spatial and verbal learning, problem solving, memory function and perceptual motor skills and motor function (Harper & Matsumoto, 2005).

Table 1. *The presence of at least two of these criteria during the last 12 months indicates an Alcohol Use Disorder (AUD). The number of criteria fulfilled defines the severity of the AUD. Mild: 2 to 3 criteria, moderate: 4 to 5 criteria, and severe: 6 or more criteria (American Psychiatry Association, 2013).*

DSM-5 criteria for Alcohol Use Disorder

1. Had times when you ended up drinking more, or longer then you intended.

2. More than once wanted to cut down or stop drinking, or tried to, but couldn't.

3. Spent a lot of time drinking. Or being sick or getting over other aftereffects.

4. Wanted a drink so badly you couldn't think of anything else.

5. Found that drinking - or being sick from drinking - often interfered with taking care of your home or family. Or caused job troubles. Or school problems.

6. Continued to drink even though it was causing trouble with your family or friends.

7. Given up or cut back on activities that were important or interesting to you, or gave you pleasure, in order to drink.

8. More than once gotten into situations while or after drinking that increased your chances of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex).

9. Continued to drink even though it was making you feel depressed or anxious or adding to another health problem. Or after having had a memory blackout.

10. Had to drink much more than you once did to get the effect you want. Or found that your usual numbers of drinks had much less effect than before.

11. Found that when the effects of alcohol were wearing off, you hade withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure. Or sensed things that were not there.

Pharmacotherapy

For treating AUD both pharmacotherapy and psychosocial interventions are used. The treatment goal is achievement of abstinence, reduction in frequency and severity of relapse, and improvement in health and psychosocial functioning (European Medical Agency, 2010). To date, there are three drugs approved for the treatment of AUD by both the European Medical Agency (EMA) and the US Food and Drug Administration (FDA);

disulfiram, acamprosate and naltrexone. EMA have approved a fourth drug, nalmefene. Disulfiram inhibits the enzyme aldehyde dehydrogenase from degrading alcohol's primary metabolite acetaldehyde. The accumulation of acetaldehyde results in unpleasant feelings, such as headache, facial flushing, vomiting and chest pain among others (Barth & Malcolm, 2010). Disulfiram is the oldest of these drugs and acts as an aversive therapy, while the other three drugs belong to the new generation of pharmacological treatments. Acamprosate is a homotaurine analogue which mechanism of action has been suggested to involve inhibitory (GABAergic; (Boismare *et al.*, 1984; Daoust *et al.*, 1992) or glycinergic; (Chau *et al.*, 2010b; Chau *et al.*, 2018)) or excitatory neurotransmission, including modulation of N-methyl-D-aspartic acid (NMDA) or metabotropic glutamate receptors (mGluR), with restored balance between excitatory and inhibitory neurotransmission as a result (Rammes *et al.*, 2001; Harris *et al.*, 2002; Harris *et al.*, 2003). However, Spanagel and colleagues have suggested that it is the calcium moiety of the acamprosate molecule that is responsible for the pharmacological effect (Spanagel *et al.*, 2014). Naltrexone prevents the reinforcing effect of alcohol by acting as a competitive opioid receptor antagonist (O'Brien *et al.*, 1996). Nalmefene is also an opioid receptor antagonist with similar effects as naltrexone. However, unlike naltrexone, it has a partial agonistic effect at kappa opioid receptors, whereas naltrexone acts as a full antagonist at these receptors (Swift, 2013). Although these pharmacological treatments are available for patients with AUD, the effect sizes are poor (Kranzler & Van Kirk, 2001), and there is a great need for improved pharmacotherapy. To this end, studying mechanisms involved in the action of alcohol in the brain reward system is of high importance.

Addiction

Addiction is a chronic and relapsing brain disorder (McLellan *et al.*, 2000), characterized by loss of control over drug intake, impulsive drug seeking and intake despite adverse effects (DSM-5, table1). Discontinuation of drug intake may lead to physical as well as psychological withdrawal symptoms (Weiss & Koob, 2001), fulfilling one of the diagnostic criteria for AUD according to DSM-5 (table 1). Addiction is a heterogenic disorder and the lead period from recreational drug use to addiction is highly individual. The mechanisms underlying addiction probably involve several different neurocircuits and structures (Koob & Volkow, 2010), and social, environmental and developmental factors, as well as sex, personality traits and genetics, all have been shown to contribute to addiction development (Cloninger *et al.*, 1981; Cloninger, 1987; Chartier *et al.*, 2010; Bobzean *et*

al., 2014). The development of addiction also depends on the drug and exposure, as drugs differ in their addictive profile with alcohol representing one of the six most addictive drugs (Nutt *et al.*, 2007).

The neurobiological mechanisms behind the shift from impulsive to compulsive drug intake and the development of "loss of control" involve disruption of brain circuits involved in reward, learning and control (Koob & Volkow, 2010). In the early stages of drug intake the nucleus accumbens (nAc) plays an important role and mediates the rewarding sensation of the drug. However, it has been proposed that during the progression from impulsive and reward-driven behavior to compulsive and habit-driven drug-seeking behavior there appears to be neuroanatomical progression from the nAc to the dorsal striatum (DS), a key brain region for habit formation (Gerdeman *et al.*, 2003; Koob & Volkow, 2010). The development of addiction has in several review articles (Koob & Le Moal, 1997; Koob & Volkow, 2010; 2016) been described as a cycle composed of three stages: binge/intoxication, withdrawal/negative affect and preoccupation/anticipation. With repeated drug exposure the cycle is intensified and is believed to eventually result in the pathological state of addiction. During the binge/intoxication stage the drug is consumed due to its positive reinforcing effects and engages dopamine transmission in the nAc and then engages stimulus-response habits in the DS. Overstimulation of the reward system leads to loss of control and bingeing. At the withdrawal/negative affect stage the drug intake is rather driven by removal of aversive symptoms associated with withdrawal such as irritability, stress and anxiety, which have been tied to changes in the extended amygdala. The third stage, preoccupation/anticipation, involves processing of conditioned reinforcement by basolateral amygdala and contextual cues by the hippocampus and their interaction with the prefrontal cortex, which helps to execute desires despite negative consequences. Thus, this third stage of the addiction cycle is hypothesized to be a key component of relapse, a characteristic feature of the disorder

The brain reward system

The brain reward system regulates reward and motivation. The pleasurable feeling of natural rewards is essential for survival of the species; motivating the individual to engage in eating, mating and social interactions, and are therefore highly conserved among species. In the 1950s Olds and Milner initiated the discovery of what today is referred to as the brain reward system. They demonstrated that rats implanted with an electrode in the brain would

press a lever to receive electrical stimulation in certain brain areas. These areas were termed "reinforcing structures" (Olds & Milner, 1954). These pioneering researchers suggested that the stimulated brain areas were of importance for the pursuit of natural rewards such as food and sex. It was later shown that numerous drugs of abuse exert their habit-forming effect in these areas. Consequently, a common neuroanatomical circuitry of electrical self-stimulation, natural rewards and drugs of abuse was proposed (Wise, 1996). These "reinforcing structures" were later mapped and defined as the brain reward system. The reward system consists, among other areas, of the medial forebrain bundle, hippocampus, ventral tegmental area, amygdala, frontal cortex, septal and striatal regions (German & Bowden, 1974; Milner, 1991). Both drugs of abuse and natural rewards activate the reward system. However, drugs of abuse activate the system in a way that leads to stronger effects. Effects, that may in turn lead to neuronal changes and eventually addiction (Wise & Rompre, 1989).

Dopamine and the mesolimbic dopamine system

Dopamine is one of the three catecholamines (noradrenalin and adrenalin are the other two) and is synthesized both in neuronal terminals and in cell bodies in several different brain regions, but predominantly in the ventral tegmental area (VTA) and substantia nigra (Carlsson *et al.*, 1964; Dahlstrom & Fuxe, 1964). Dopamine is synthesized from the amino acid tyrosine, which is converted from phenylalanine in the dopamine neuron or transported over the blood-brain barrier into the brain. In the neuron tyrosine is converted to dihydroxyphenylalanine (L-DOPA), which then is decarboxylated to dopamine, where the hydroxylation of tyrosine to L-DOPA is the rate-limiting step of the synthesis. Dopamine is packed into vesicles located in the nerve terminal, released by exocytosis upon arrival of an action potential and binds to dopamine receptors. The synaptic cleft is cleared from dopamine by reuptake via the dopamine transporter and repacked to vesicles or degraded, mainly by two enzymes, catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO), resulting in the end-metabolite homovanillic acid (HVA) (Elsworth & Roth, 1997).

An important pathway of the brain reward system is the mesolimbic dopamine system (Engel & Carlsson, 1977; Wise & Bozarth, 1987), which is one of four dopamine pathways in the brain. In 1958, Arvid Carlsson and colleagues discovered dopamine as a neurotransmitter (Carlsson *et al.*, 1958). Subsequently, it was shown that dopaminergic cell bodies were predominantly located within the VTA and substantia nigra (Carlsson *et al.*, 1964; Dahlstrom & Fuxe, 1964). Dopaminergic neurons that project from the

VTA to the prefrontal cortex constitute the mesocortical dopamine system, which is involved in cognitive control, emotional response and motivation (Volkow *et al.*, 2004; Cools, 2008; Russo & Nestler, 2013). Dopaminergic neurons from the VTA also project to the nAc, amygdala and hippocampus and is therefore entitled the mesolimbic dopamine system. Much attention has been focused on the projection from the VTA to the nAc as it has been shown to be highly involved in mediating reward and reinforcing effects, including those of drugs of abuse (Le Moal & Simon, 1991; Kelley & Berridge, 2002). The nigrostriatal dopamine system where dopamine neurons project from the substantia nigra to the dorsal striatum has been shown to be involved in motor activation (Obeso *et al.*, 2008; Trudeau *et al.*, 2014) and habit-formation (Gerdeman *et al.*, 2003; Faure *et al.*, 2005).

Dopamine neurons have the ability to fire in two distinct manners: a phasic burst firing and tonic firing (Grace & Bunney, 1984a; b). Positive reinforcement is mainly associated with burst firing of the dopamine neurons, while tonic firing is responsible for maintaining basal levels of dopamine and to engage in cognitive, motivational and motor functions (Marinelli & McCutcheon, 2014). Dopaminergic cells in the VTA are under inhibitory control of GABAergic (γ-aminobutyric acid) interneurons within the VTA and GABAergic afferents from the nAc and ventral pallidum (Conrad & Pfaff, 1976). The excitatory afferents to the VTA are mainly glutamatergic and originates from the PFC, laterodorsal tegmental nucleus (LDTg), bed nucleus of the stria terminalis and lateral hypothalamus (Omelchenko & Sesack, 2007). Furthermore, cholinergic neurons projecting from the LDTg and pedunculopontine tegmental nucleus (PPTg) to the VTA enable dopaminergic phasic firing, via activation of nicotinic acetylcholine receptors (nAChR) (Blaha *et al.*, 1996).

The predominant class of neurons within the nAc is GABAergic medium spiny neurons (MSNs). There are five different types of dopamine receptors, which are classified as D1-like (D1 and D5) and D2-like (D2, D3 and D4) based on structure, biochemistry and pharmacology (Beaulieu & Gainetdinov, 2011). Both dopamine receptor 1 (D1R) and dopamine receptor 2 (D2R) are located on nAc MSNs (Gerfen, 1992). nAc D1R MSNs directly inhibit the dopaminergic ventral mesencephalon (the direct pathway), leading to disinhibition of the thalamus and promote motivated behavior. The nAc D2R MSNs inhibits the ventral pallidum, which inhibits the ventral mesencephalon (the indirect pathway), resulting in inhibition of the thalamus and motivated behavior (Kupchik & Kalivas, 2017). Furthermore, it has been demonstrated that activation of nAc D2R MSNs suppresses cocaine reward, whereas the opposite was shown for D1R MSNs (Lobo *et al.*, 2010). The

opposite effects of these two subtypes of nAc MSNs is consistent with the role of D1R and D2R MSNs in the dorsal striatum, where the direct (D1R containing) and indirect (D2R containing) pathways produce balanced behavioral output by acting in opposition (Albin *et al.*, 1989; Graybiel, 2000). However, emerging results suggests that the direct and indirect pathways of the nAc are not coded by MSN cell types. Kupchik and co-workers demonstrated that D1R-MSNs includes a portion of the classical indirect pathway by synapsing on ventral pallidum neurons that project to the ventral mesencephalon. Conversely, they demonstrated that nAc D2R-MSNs target ventral pallidum neurons that innervate the thalamus directly. Thus, these D2R-MSNs constitutes a direct pathway through the ventral pallidum that disinhibits the thalamus (Kupchik *et al.*, 2015). The nAc can be anatomically subdivided in two distinct regions; the core and shell (Zahm & Brog, 1992). The two subregions differ in their innervations and are functionally distinct. Dopaminergic innervation of the core is associated with the nigrostriatal system and dopaminergic innervation of the shell with the mesolimbic dopamine system (Deutch & Cameron, 1992). Besides dopaminergic innervation, the nAc also receives glutamatergic input, where the core receives glutamatergic control mainly from the prelimbic cortex and basolateral amygdala. The shell receives input from a larger number of areas such as the infralimbic cortex, ventral hippocampus, thalamus and the VTA (Gipson *et al.*, 2014). It has been suggested that dopamine has opposing roles within the core and shell regarding limbic information processing, as dopamine in the core is important for amygdala-dependent appetitive learning, whilst dopamine in the shell is important for hippocampal-dependent spatial control (Ito & Hayen, 2011). Furthermore, the shell appears to be of importance for reward induced by drugs of abuse. The core on the other hand is of importance for goal-directed behaviors (Ito *et al.*, 2004).

Dopamine and drugs of abuse

The increase of accumbal dopamine mediating the pleasurable feeling of natural rewards is the primary function of the brain reward system. However, most drugs of abuse, including alcohol, also increase dopamine in the nAc and give rise to an even greater dopamine response than natural rewards. Since both the amount and rate of dopamine increase is associated with the subjective sensation of the so-called "high", where a fast dopamine release and high amounts is positively correlated with the feeling of high (Volkow *et al.*, 1999; 2003; Koob & Volkow, 2016), drugs of abuse give rise to a greater feeling of "high" than natural rewards. Dopamine is also essential for motivation, memory and executive function (Volkow *et al.*, 2012), important

components in associative reward-related learning, which in turn is facilitated by dopamine increase in the nAc (Di Chiara, 1999).

The role of dopamine in addiction and reward is complicated. As mentioned above, not all drugs of abuse increase dopamine. Barbiturates, benzodiazepines and inhalants do not increase dopamine although they are rewarding and often abused (Wise, 1987; Koob, 1992; Balster, 1998). Administration of dopamine antagonists or lesioning of dopamine neurons decreases self-administration of several drugs (Roberts *et al.*, 1980; Corrigall *et al.*, 1992; Di Chiara *et al.*, 2004) but not of opiates and ethanol (Pettit *et al.*, 1984; Dworkin *et al.*, 1988; Rassnick *et al.*, 1993). On the other hand, low doses of dopamine antagonists have been shown to increase drug self-administration, (Yokel & Wise, 1975; Corrigall & Coen, 1991), which is commonly explained as a compensatory intake due to a blunted drug response. Dopaminergic neurons may also fire in response to aversive stimuli. However this response appears to be heterogeneous and segregated based on the location of dopamine neurons in the brain. For example in the rat, excitation induced by aversive stimuli is more likely to occur of neurons located in the lateral and ventral VTA than neurons located in the medial and dorsal VTA (Brischoux *et al.*, 2009; Valenti *et al.*, 2011; Marinelli & McCutcheon, 2014). Although, most researchers believe that the primary function of the mesolimbic dopamine pathway is to mediate reward, the variety of effects mediated by dopamine needs to be further elucidated in detail.

The pharmacology of ethanol

There are alcohols with different number of carbon atoms, where the alcohol used for human consumption contains two carbons and is entitled ethanol. Ethanol is the alcohol used in the studies that comprises this thesis, and the two will be used as synonyms. Due to the hydrophilic and lipophilic properties of the molecule ethanol has the ability to easily cross biological membranes such as the blood-brain barrier by passive diffusion into the central nervous system (CNS). Alcohol is metabolized in the liver by the enzymes alcohol dehydrogenase and cytochrome P450 to acetaldehyde, which is then converted to acetic acid by aldehyde dehydrogenase and finally broken down to carbon dioxide and water (Pohorecky & Brick, 1988).

Ethanol produces a number of different effects in humans that are both stimulative and sedative. At low doses ethanol produces disinhibition, euphoria and anxiolysis. At higher doses ethanol acts as a depressant resulting in impaired motor function and cognition and will eventually, with

increasing dose, lead to vomiting, unconsciousness and possible death (Hendler *et al.*, 2013).

Ethanol and ligand-gated ion channels

Ethanol has a rich and complex pharmacology and interacts with several different receptor systems, especially ligand-gated ion channels, where the interaction can take place both in a direct and indirect manner. Factors such as time of exposure and concentration of ethanol, as well as the receptor subunit composition appear to exert a high impact (Vengeliene *et al.*, 2008). In general, ethanol enhances the function of $GABA_A$ receptors (γ-aminobutyric acid receptor subtype A), glycine receptors (GlyR), 5-hydroxytryptamine (serotonin) subtype 3 receptors ($5\text{-}HT_3R$) and nAChR (Lovinger, 1999; Mihic, 1999; Narahashi *et al.*, 1999), and inhibits NMDA receptor (NMDAR) and purinergic P2X subtype 4 receptor (P2X4R) function (Lovinger *et al.*, 1989; Franklin *et al.*, 2014), Besides interacting with ligand-gated ion channels ethanol is also known to inhibit L-type Ca^{2+} channels (Wang *et al.*, 1994), and open G-protein activated inwardly rectifying K^+ channels (Kobayashi *et al.*, 1999; Lewohl *et al.*, 1999). Below follows a very brief description of ethanol's interaction with these receptor systems.

Ethanol potentiates $GABA_A$ receptor activity both direct and indirectly (Vengeliene *et al.*, 2008). Administration of GABA antagonists to rodents blocks the sedative effects of ethanol (Cott *et al.*, 1976; Liljequist & Engel, 1982), and facilitation of GABAergic neurotransmission appears to be involved in ethanol's anxiolytic effect and ethanol-induced impaired cognitive function as well as motor function (Ticku, 1990; Davies, 2003). The impact of ethanol on GABAergic transmission appears to be complex, depending on the concentration of ethanol, receptor localization as well as receptor subtype (Aguayo *et al.*, 2002; Korpi *et al.*, 2007; Kumar *et al.*, 2009). The time of ethanol exposure is also of importance as short-term consumption increases receptor function, whilst long-term consumption decreases it (Morrow *et al.*, 1990; Mihic, 1999; Davies, 2003). It has been shown that several of the candidate genes for AUD codes for $GABA_A$Rs and GlyRs (Korpi *et al.*, 2007). Ethanol enhances GlyR function (Perkins *et al.*, 2010), which may be a consequence of a direct interaction of ethanol with a group of amino acids that form a hydrophobic binding site for ethanol on the receptor (Harris *et al.*, 2008; Howard *et al.*, 2014; Burgos *et al.*, 2015). The binding site appears to be located at the transmembrane domain 2 and 3 (TM2, TM3), as mutations in these domains of the alpha subunit of the glycine receptor reduced or eliminated the ability of ethanol to potentiate glycine currents (Borghese *et al.*, 2012). Also residues of the loop 2 in the

extracellular region of the receptor constitute the binding site (Crawford *et al.*, 2007). It has also been proposed that ethanol enhances GlyR function, by increasing levels of taurine, a ligand for the GlyR, in the nAc (De Witte *et al.*, 1994; Adermark *et al.*, 2011b; Ericson *et al.*, 2011; Ericson *et al.*, 2019), which in turn has been shown to increase dopamine in the same region and in a strychnine dependent manner (Ericson *et al.*, 2006). The role of taurine in ethanol-induced dopamine increase will be discussed further in the subchapter "Interactions between taurine and ethanol in the CNS". Further it has been shown that glycine reuptake inhibitors decrease ethanol preference- and consumption in rats (Molander *et al.*, 2007; Lido *et al.*, 2012). Ethanol acts as a co-agonist at the nAChR and enhances the effect of acetylcholine (ACh) and nicotine if present (Marszalec *et al.*, 1999). nAChR in the VTA are indirectly involved in the ethanol-induced accumbal dopamine elevation (Blomqvist *et al.*, 1997; Ericson *et al.*, 2003; Ericson *et al.*, 2008). Ethanol potentiates the effects of 5-HT by direct interaction with the 5-HT$_3$R (Lovinger, 1991; Lovinger & White, 1991). Pharmacological manipulations of the serotonergic system and transgenic mice model indicate an involvement of this neurotransmitter in voluntary ethanol consumption (Engel *et al.*, 1998; Zhou *et al.*, 1998; Vengeliene *et al.*, 2008; Kasper *et al.*, 2013), and 5-HT$_3$R antagonism inhibited ethanol-induced dopamine release in the nAc (Carboni *et al.*, 1989). The effects of ethanol on the NMDAR appear so be exposure-dependent. Acute ethanol exposure results in inhibited receptor function, whilst receptor expression and function are increased during long-term exposure (Iorio *et al.*, 1992; Dodd *et al.*, 2000). Neuronal degeneration and cognitive impairment caused by ethanol are thought to be mediated by NMDA receptors (Vengeliene *et al.*, 2008). Furthermore, studies using NMDAR antagonists suggest that glutamate neurotransmission in the nAc modulates ethanol-self administration and reinforcement (Rassnick *et al.*, 1992; Biala & Kotlinska, 1999). It has been suggested that ethanol inhibits P2X4R function by acting as a negative allosteric modulator, shifting the adenosine triphosphate (ATP) concentration response curve to the right (Davies *et al.*, 2002; Davies *et al.*, 2005), and that also this receptor is involved in alcohol drinking behavior (Franklin *et al.*, 2014). Expression levels of the receptor messenger RNA (mRNA) is negatively correlated with alcohol intake (Tabakoff *et al.*, 2009), and mice lacking the P2X4R gene consume more ethanol than wild type controls (Wyatt *et al.*, 2014). Ivermectin, a positive modulator of P2X4R antagonizes ethanol-mediated inhibition of P2X4R (Asatryan *et al.*, 2010), and attenuates ethanol consumption and preference in mice (Yardley *et al.*, 2012; Asatryan *et al.*, 2014).

Ethanol and dopamine

Some of the first studies pointing towards a dopamine elevating property of ethanol were locomotor activity studies performed in mice. They showed that ethanol increased locomotion, a behavior related to ethanol-induced activation of the mesolimbic dopamine system (Carlsson *et al.*, 1974; Liljequist *et al.*, 1981; Wise & Bozarth, 1987; Engel *et al.*, 1988). Studies using *in vivo* microdialysis later confirmed the ability of ethanol to increase dopamine in the nAc. This is a robust phenomenon occurring regardless of whether alcohol is orally- (Weiss *et al.*, 1993) or systemically administrated (Di Chiara & Imperato, 1988; Jerlhag *et al.*, 2014; Ericson *et al.*, 2019), or perfused locally in the nAc (Yoshimoto *et al.*, 1992; Ericson *et al.*, 2003). Even the anticipation of alcohol has been shown to increase dopamine, and this increase could be part of the mechanisms underlying cue-induced relapse to alcohol intake (Weiss *et al.*, 1993; Lof *et al.*, 2007; Soderpalm *et al.*, 2009).

All of the different types of dopamine receptors appear to be involved in the actions of ethanol in the brain reward system. For example, the administration of agonists or antagonists of D1, D2 and D3 receptors influence ethanol intake (Pfeffer & Samson, 1988; Russell *et al.*, 1996; Cohen *et al.*, 1998), and antagonism of these three receptors reduce cue-induced reinstatement of alcohol-seeking behavior (Liu & Weiss, 2002; Vengeliene *et al.*, 2006). Furthermore, in particular the D2 receptor appears to be highly influenced by chronic alcohol intake both in humans and animals. In AUD patients, a reduction in both D2 receptor function and availability has been reported (Balldin *et al.*, 1993; Volkow *et al.*, 1996), and voluntary long-term alcohol consumption in rats reduced the mRNA expression of the D2 receptor within the nAc (Jonsson *et al.*, 2014; Feltmann *et al.*, 2018). Inversely, high levels of the D2 receptor may be protective against excessive alcohol intake, as overexpression of the receptor in the nAc has been shown to decrease ethanol preference as well as consumption in rats (Thanos *et al.*, 2001). Endogenous dopamine levels in the nAc are decreased in rats after long-term ethanol consumption (Diana *et al.*, 1993; Feltmann *et al.*, 2016). Reduced dopaminergic activity in the mesolimbic dopamine system has also been reported in AUD patients (Volkow *et al.*, 2007). If decreased dopaminergic activity is a cause or consequence of long-term alcohol intake is still a matter of debate.

The nAc-VTA-nAc circuit controlling ethanol-induced dopamine

During the last two decades, the present research group has formed a hypothesis about a neuronal circuitry mediating the dopamine elevating effect

of ethanol, involving GlyR in the nAc and nAChR in the VTA, where the endogenous amino acid taurine plays a key role.

Both systemic and local (VTA) administration of the nAChR antagonist mecamylamine inhibit accumbal dopamine increase after systemic ethanol administration (Blomqvist *et al.*, 1993; Blomqvist *et al.*, 1997). Thus, a direct interaction of ethanol with nAChR in the VTA was hypothesized. However, when ethanol was perfused in the nAc a dopamine increase in the same region was detected, but not when perfused in the VTA (Ericson *et al.*, 2003; Lof *et al.*, 2007). This dopamine increase was prevented following administration of mecamylamine in the VTA, which also reduced voluntary ethanol-intake and preference (Ericson *et al.*, 1998; Ericson *et al.*, 2003; Ericson *et al.*, 2008). Furthermore, voluntary ethanol-intake was demonstrated to increase both ACh in the VTA and dopamine in the nAc (Larsson *et al.*, 2005). These results were interpreted as activation of nAChR in the VTA, by increased levels of ACh in the same region, is preceded by an action of ethanol in the nAc. Thus, the first hypothesis of a direct interaction of ethanol with nAChR was revised to an indirect interaction.

It was demonstrated that tonically activated strychnine-sensitive GlyRs are expressed in the nAc and that they are of importance both for regulating dopamine levels per se, as well as ethanol-induced dopamine levels (Molander & Soderpalm, 2005b; a). In addition, this receptor population was also found to modulate voluntary ethanol intake as demonstrated by local (nAc) administration of glycine or the glycine receptor antagonist strychnine (Molander *et al.*, 2005). However, as there are few glycine immunoreactive fibers or cell bodies in the nAc (Rampon *et al.*, 1996), other endogenous ligands could participate in the activation of the GlyR. Indeed, local (nAc) administration of the endogenous amino acid taurine was shown to increase dopamine levels in the same region. This taurine-induced dopamine increase was, like shown for ethanol, inhibited by perfusion of strychnine in the nAc and mecamylamine in the VTA (Ericson *et al.*, 2006). Later, it was also demonstrated that an endogenous increase in accumbal taurine is required for ethanol to increase dopamine in the same region (Ericson *et al.*, 2011).

Taken together, ethanol is proposed to increase extracellular dopamine levels in the following manner: ethanol administration results in increased taurine levels, which activates GlyR in the nAc, presumably located on GABAergic neurons projecting to the VTA, leading to disinhibition of cholinergic neurons from the LDTg/PPTg and increased ACh output in the VTA. Subsequently, activation of nAChR in the VTA, presumably located on dopaminergic neurons projecting to the nAc, leads to increased accumbal

dopamine output (Soderpalm *et al.*, 2009) (fig. 1). By which mechanism ethanol increases endogenous taurine levels is not fully understood, and is one of the aims to explore in this thesis.
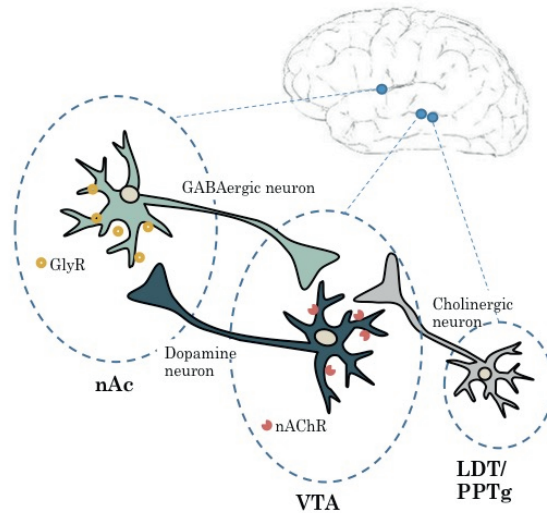
Figure 1. Schematic illustration of the hypothesized nAc-VTA-nAc circuitry. Taurine may interact with GlyR located on GABAergic neurons in the nAc. This results in increased acetylcholine levels in the VTA, leading to activation of nAChR on dopaminergic neurons projecting from the VTA to the nAc, and finally increased extracellular dopamine levels in the nAc. Image adopted with permission from Pei Pei Chau. GlyR=glycine receptor, nAChR=nicotinic acetylcholine receptor, nAc=nucleus accumbens, VTA=ventral tegmental area, LDT/PPTg= laterodorsal/pedunculopontine tegmental nucleus

Taurine

Taurine (*β*-amino ethane sulphonic acid), often referred to as an amino acid, is in the strict sense not an amino acid as it contains a sulfo group instead of the characteristic carboxyl group. The molecule acts as a zwitterion, showing low lipophilicity and is hence impermeable of biological membranes (Huxtable, 1992). Taurine is not believed to be incorporated into proteins as no aminoacyl tRNA synthesase has been found (Lambert *et al.*, 2015). Taurine is highly abundant in excitable and secretory tissues (Huxtable, 1989).

As early as 1915 taurine was proposed to act as an osmoregulator, which was later confirmed by several investigators (Huxtable, 1992). It is now known that one of the physiological functions of taurine is to act as an

osmoregulatory agent, both within and outside of the CNS, as the flow of taurine over cell membranes mostly are driven by changes in the external osmolarity (Huxtable, 1992; Oja & Saransaari, 1996). A reduction in external osmolarity increases taurine efflux from both astrocytes and neurons, as part of re-equilibration of osmotic pressure (Solis *et al.*, 1988; Oja & Saransaari, 1992; Pasantes-Morales *et al.*, 1993; Moran *et al.*, 1994b; Vitarella *et al.*, 1994; Oja & Saransaari, 1996; Deleuze *et al.*, 1998). Furthermore, microdialysis performed in the rat hippocampus (Lehmann, 1989) or nAc (Quertemont *et al.*, 2003) showed that perfusion of a hypoosmotic medium increased extracellular taurine levels, and taurine-deficient cultured astrocytes showed impaired cell volume regulation (Moran *et al.*, 1994b). In 1973, Huxtable and Bressler proposed taurine to act as a membrane stabilizer as they demonstrated that addition of the amino acid to the cell medium slowed the rate of loss of Ca^{2+} transport and ATPase activities of sarcoplasmatic reticulum from rat skeletal muscles caused by phospholipase C (Huxtable & Bressler, 1973). Since then, taurine has been shown to have antioxidative effects, for example by preventing cellular damage caused by reactive oxygen species such as nitrogen oxide (Gordon *et al.*, 1986; Gurujeyalakshmi *et al.*, 2000), and ozone (Banks *et al.*, 1992), as well as by maintaining efficient mitochondrial protein translation (Schaffer *et al.*, 2009). Taurine also modulates Ca^{2+}-dependent processes. This is most evident in the heart where taurine is positively inotropic in hearts exposed to subphysiological concentrations of Ca^{2+} and negatively inotropic when exposed to supraphysiological concentrations of Ca^{2+}. These positive and negative inotropic effects parallel the effects of taurine on Ca^{2+} binding to cardiac cell membrane and Ca^{2+} entry through the calcium channel (Huxtable, 1992). Taurine is also involved in physiological processes such as lung function and development (Lambert *et al.*, 2015). For example in the lung, taurine potentiates relaxation of precontracted airway smooth muscle cells through $GABA_A$Rs (Gallos *et al.*, 2012), and the offspring of cats fed taurine-free diet, display abnormal hind leg development, smaller brain- and body-weight, degeneration or abnormal development of the retina and visual cortex (Sturman, 1991).

In the adult human, taurine is synthesized predominantly in the liver but also to some extent in the brain (Stipanuk *et al.*, 2002; Stipanuk, 2004). However, for newborns dietary taurine is essential as children under the age of one years old are not able to synthesize sufficient amounts (Lambert *et al.*, 2015). Taurine is synthesized from cysteine via cysteine dioxygenase to cysteinsulfonate, which is converted to hypotaurine by cysteinsulfinate decarboxylase. Hypotaurine is then oxidized to taurine (Stipanuk *et al.*, 2002). A minor synthesis pathway of taurine occurs via degradation of

coenzyme A to cysteamine, which is then oxidized to hypotaurine by cysteamine dioxygenase (Dominy *et al.*, 2007). The daily human intake of taurine varies depending on which food that is consumed, as the amount of taurine varies greatly among different kinds of food. Meat and fish contain high amounts of taurine whilst a vegan diet is almost taurine deficient (Laidlaw *et al.*, 1990), resulting in individuals consuming a strict vegan diet to have decreased plasma taurine levels (14-22% lower) and 2-3 times less amount excreted in urine compared to omnivores (Rana & Sanders, 1986; Laidlaw *et al.*, 1988). As taurine is not metabolized in the mammal it is excreted via the urine or conjugated to bile acids, which are secreted via faeces (Huxtable, 1992).

As mentioned above, taurine is impermeable of biological membranes. Consequently, uptake of taurine into the cell is performed via the high-affinity, low capacity, Na^+ and Cl^--dependent taurine transporter (TauT) and the high capacity, proton-coupled but Na^+-independent β-amino acid transporter PAT1. Contrary to uptake into cells, taurine is released via volume-insensitive or volume-sensitive pathways. During isotonic conditions taurine, in low amounts, leaks from the cell to the extracellular environment through a volume insensitive pathway (Lambert *et al.*, 2015). By which mechanisms this occurs is not fully understood although it has been proposed that the TauT is responsible for this by working in reverse, releasing taurine from the cell (Lambert & Hoffmann, 1993). This is not likely however, due to the low cellular Na^+ concentration (Poulsen *et al.*, 2010). The release of taurine via the volume-sensitive pathway occurs within minutes following hypotonic exposure and involves the volume regulated anion channel (VRAC) (Lambert *et al.*, 2015; Jentsch, 2016).

VRACs are anion channels expressed in vertebrate cells. They are activated by cell swelling and regulate cell volume by the efflux of Cl^- and organic solutes such as taurine (Hoffmann *et al.*, 2009; Jentsch, 2016; Strange *et al.*, 2019). When osmotic cell swelling was introduced by removal of sucrose from the external bath solution, the VRAC current was activated following an increased cell-volume of 10%. Inactivation of the VRAC current can be obtained by increasing the external bath osmolality (Bond *et al.*, 1999; Strange *et al.*, 2019). Besides cell swelling, VRACs can also be activated by other factors, such as reactive oxygen species (Shimizu *et al.*, 2004; Liu *et al.*, 2009; Deng *et al.*, 2010), and by a reduction in intracellular ionic strength (Cannon *et al.*, 1998; Nilius *et al.*, 1998). In the brain, VRACs not only constitute a pathway for taurine release, but have also been found to release aspartate and glutamate (Akita & Okada, 2014; Mongin, 2016).

Within the CNS, taurine acts as an agonist at the GlyR (Curtis *et al.*, 1968; Haas & Hosli, 1973; Okamoto & Sakai, 1980; Taber *et al.*, 1986). In addition to taurine, glycine, beta-alanine and GABA are also capable of activating the receptor, with the following order of potency: glycine, beta-alanine, taurine and GABA (Lewis *et al.*, 2003; Pless *et al.*, 2007). Differences in response of these agonists depend on the expression system used, as glycine, beta-alanine and taurine act as full agonists in HEK293 cells and as partial agonists in Xenopus laevis oocytes (De Saint Jan *et al.*, 2001; Lewis *et al.*, 2003). Taurine also acts as an agonist at the $GABA_A R$ (Haas & Hosli, 1973; Okamoto & Sakai, 1980; Taber *et al.*, 1986; Bureau & Olsen, 1991), and as an antagonist at the NMDAR (Kurachi *et al.*, 1983; Lehmann *et al.*, 1984). Binding of taurine to the $GABA_B R$ has also been shown but with undefined functional effects (Kontro *et al.*, 1990; Kontro & Oja, 1990). A taurine receptor has been proposed to exist, however this protein has not yet been defined (Girard *et al.*, 1982; Okamoto *et al.*, 1983a; Wu *et al.*, 1992; Frosini *et al.*, 2003). Whether taurine fulfills the criteria to acts as a neurotransmitter or not within the CNS is a matter of debate.

Interactions between taurine and ethanol in the CNS

In one of the first studies examining the effect of taurine on ethanol-induced behavior, Arvid Carlsson and colleagues showed that taurine influenced motor behavior during ethanol treatment (Garcia de Yebenes Prous *et al.*, 1978). Since then, several studies have shown taurine to have the ability to affect ethanol-induced behavior. Taurine has been shown to both reduce and enhance ethanol-induced locomotion (Aragon et al., 1992) and sedation (McBroom *et al.*, 1986; Ferko & Bobyock, 1988). Administration of taurine enhanced conditioned place aversion produced by a low dose of ethanol, blocked conditioned placed aversion produced by an intermediate dose, and had no effect on a higher dose of ethanol (Aragon & Amit, 1993). Quertemont and co-workers have shown that oral taurine supplementation causes CPP at a low dose of ethanol, as compared to non-taurine supplemented animals. They suggested this finding to involve the interaction of taurine and dopamine in the mesolimbic dopamine system. As both taurine and low doses of ethanol increase dopamine levels, a simultaneous administration of these two substances may induce preference. They also demonstrated reduction of ethanol-induced aversion at a high ethanol dose by taurine supplementation, and proposed that this may be explained by the ability of taurine to maintain intracellular calcium homeostasis. Thus, the administration of taurine together with ethanol may restore any ethanol-induced changes in intracellular calcium, and will reduce any aversive behavioral effects (Quertemont *et al.*, 1998b). That taurine would reduce the

aversive effects of ethanol is further supported by a taurine-induced reduction of blood and liver acetaldehyde-concentrations caused by ethanol (Watanabe *et al.*, 1985). These studies indicate that the ability of taurine to modify the rewarding, aversive as well as behavioral effects of ethanol to be dependent on dose and treatment time of the drugs. Furthermore, taurine has also been shown to reduce ethanol consumption in rats. Acute systemic administration of taurine (50, 100 or 200 mg/kg) 15 minutes prior ethanol or water intake reduced ethanol consumption by approximately 25-40%, but did not influence ethanol intake at a dose of 10 mg/kg taurine. The water consumption was also reduced at the dose of 200 mg/kg, indicating a general influence on fluid intake at this dose (Olive, 2002).

In the first *in vivo* microdialysis study demonstrating an ethanol-induced increase of taurine in the nAc the aim was to investigate the effect of alcohol on the excitatory amino acid glutamate and two inhibitory amino acids, GABA and taurine. It was found that extracellular taurine levels increased 40 minutes after a systemic injection of 2 or 3 g/kg ethanol. The investigators speculated that the increase in taurine was due to compensatory mechanism, such as cell membrane stabilization and osmoregulation, to counteract the effects of acute ethanol on cell membrane, and on cell volume (Dahchour *et al.*, 1994). Subsequently, several microdialysis studies demonstrated increased extracellular accumbal levels of taurine in a dose-dependent manner after systemic ethanol administration (De Witte *et al.*, 1994; Dahchour *et al.*, 1996; Olive *et al.*, 2000; Quertemont *et al.*, 2003; Smith *et al.*, 2004; Ericson *et al.*, 2011), and during local administration of ethanol in the nAc (Adermark *et al.*, 2011a; Ericson *et al.*, 2017). Increased extracellular taurine levels in the nAc were also shown during operant ethanol self-administration, where the increase in taurine was positively correlated with ethanol dose (Li *et al.*, 2008), and greater in FIE (forced intermittent ethanol) rats compared to control rats (Li *et al.*, 2010). The magnitude of the taurine response after acute ethanol treatment also appears to be influenced by the level of ethanol-sensitization (as measured by locomotor activity), as low sensitized mice showed higher extracellular accumbal levels of taurine after an acute ethanol challenge than high sensitized mice (Nashed *et al.*, 2019).

We previously demonstrated that taurine is required for ethanol-induced dopamine increase (Ericson *et al.*, 2011). This study suggested a functional link between taurine and dopamine in response to ethanol. An interaction between taurine and dopamine in the brain is further supported by a behavioral study showing that endogenous brain levels of dopamine are increased in rats injected intracerebroventriculary with taurine, and that

dopamine depletion induced by alpha methyltyrosine is retarded by taurine (Garcia de Yebenes Prous *et al.*, 1978).

There are studies indicating a genetic influence on ethanol-induced levels of taurine in the nAc. Rats genetically bred for low alcohol sensitivity showed a delayed increase in taurine after systemic ethanol administration as compared to high alcohol sensitive rats, which response was similar to those of regular Wistar rats (Dahchour *et al.*, 2000). Differences have also been shown between Sardinian ethanol-preferring and Sardinian ethanol-non-preferring rats, where ethanol-induced taurine release in the alcohol-preferring rats was lowered by comparison to the ethanol-non-preferring rats (Quertemont *et al.*, 2000). Mice, lacking the epsilon isoform of protein kinase C, are behaviorally and biochemically "supersensitive" to ethanol and other allosteric modulators of the $GABA_AR$, and display increased endogenous extracellular levels of taurine in the nAc. In these mice, ethanol fails to increase accumbal taurine as well as dopamine levels (Olive *et al.*, 2000). Furthermore AUD patients may have reduced endogenous levels of taurine (Majumdar *et al.*, 1983), if this is a cause or consequence of excessive alcohol intake, or a consequence of malnutrition is still to be determined.

It is clear that taurine and ethanol interact in the CNS both on a behavioral and a biochemical level. As taurine has osmoregulatory effects, and as ethanol induces cell swelling, we have proposed that taurine is released from the cell into the extracellular environment due to osmoregulation (Adermark *et al.*, 2011b; Ericson *et al.*, 2011). However, more studies are needed to clarify the mechanisms involved.

Energy drinks and their interaction with ethanol

Energy drinks first appeared around 1960 in Asia and Europe, although it was the introduction of "Red Bull" in 1987 in Austria and in 1997 in the United States that really started the trend of an aggressive marketing of these drinks. Since then, the market has grown fast with hundreds of brands worldwide (Reissig *et al.*, 2009). Energy drinks usually contain high amounts of caffeine, taurine, glucoronolactone, glucose, vitamins and herbal supplements (McLellan & Lieberman, 2012), and are often marked to increase physical and mental attention, endurance and performance as well as weight loss. Children, adolescents and young adults consume the majority of energy drinks and adverse health effects have been reported when using these products (Seifert *et al.*, 2011). The immature and developing brain is vulnerable and may be influenced by both caffeine and taurine (Curran & Marczinski, 2017). Much focus has been put on the effects of energy drinks

on cardiovascular function as these drinks contain high amounts of caffeine. It is known that acute caffeine moderately can increase blood pressure and heart rate (Mesas *et al.*, 2011; Higgins & Babu, 2013), which may not be harmful in healthy adults. However, in the small body of a child or adolescent, with no developed tolerance to simulative substances, these effects can be significant (Curran & Marczinski, 2017). Therefore, several countries have at times banned the sales of energy drinks to minors (Ragsdale *et al.*, 2010; Seifert *et al.*, 2011). In the USA, as energy drinks are sold as food supplements and not as food (FDA), the product does not undergo the same regulatory processes and the amount of ingredients in energy drinks are not always known. This is alarming, considering the high intake of energy drinks among the young population. In Sweden, the total amount of caffeine in each energy drink has to be disclosed. However, there is no limit for the amount of taurine (Svenska Livsmedelsverket).

Besides intake of energy drinks alone, it is popular to consume them together with alcohol (Oteri *et al.*, 2007; Marczinski *et al.*, 2011; Seifert *et al.*, 2011). There are several reports indicating that this consumption is associated with risks. In 2008, O'Brien and colleagues reported that college students consuming the combination of alcohol mixed with energy drinks (AmED) compared to students drinking alcohol alone, consumed more alcohol on each drinking session and had twice as many episodes of weekly drunkenness. The increased alcohol consumption alone would increase the risk of violence/accidents and the development of AUD even without any direct pharmacological contribution of the ingredients of the energy drinks. It was further reported that the students consuming AmED had an increased risk of emergency room visits, driving intoxicated or riding a car with an intoxicated driver, as well as being engaged in undesired sexual activity (O'Brien *et al.*, 2008). Consuming AmED also attenuates some of the self-reported symptoms of alcohol intoxication, such as headache, weakness, and perception of motor coordination. However, objective measures like blood alcohol concentration, visual reaction time and motor coordination was not reported to be changed (Ferreira *et al.*, 2006). People's consumption of AmED would suggest an impaired ability to assess both their own and others levels of intoxication which would put both themselves and others at risk. Which ingredients that are responsible for these risks and the mechanisms of action underlying the effects are not clear. Subsequently, increased frequency in alcohol intake and consumption of larger amounts at each drinking session among AmED users, compared to those drinking alcohol alone, was shown to increase the risk for alcohol-related harm and the development of AUD (Price *et al.*, 2010; Arria *et al.*, 2011). It has also been shown that weekly or daily

energy drink consumption (note that this is not in combination with alcohol) is strongly associated with AUD (Arria *et al.*, 2011).

Taurine and caffeine as pharmacological active substances in energy drinks when combined with alcohol

Caffeine and taurine are the two major ingredients in energy drinks, and in general one can (approximately 250 ml) contains 80 mg caffeine and 1000 mg taurine (McLellan & Lieberman, 2012). A regular cup of coffee (150 ml) contains approximately 100 mg caffeine, and the daily intake of taurine for omnivores is about 120-130 mg (Svenska Livsmedelsverket 2019).

When reviewing the literature much focus is on caffeine as the pharmacological active ingredient in energy drinks *per se*, as well as when combined with alcohol. Preclinical studies support an interaction of caffeine and ethanol as it has been shown that a systemic injection with caffeine increases voluntary ethanol intake (Kunin *et al.*, 2000), promotes ethanol-induced conditioned taste aversion (Kunin *et al.*, 2001) and ethanol-induced locomotion (Hilbert *et al.*, 2013) in rodents. The combination of caffeine and ethanol induced conditioned place preference in mice, although the combination produced no greater effect than ethanol alone (Hilbert *et al.*, 2013). A common target for ethanol and caffeine is the adenosinergic system. It has been proposed that ethanol increases extracellular levels of adenosine in vitro (Nagy *et al.*, 1990; Krauss *et al.*, 1993), and antagonism of adenosine A_2 receptors (A_2R) enhances or attenuates ethanol self-administration depending on the concentration of antagonists used (Arolfo *et al.*, 2004). Furthermore, it has been suggested that A_2R are involved in the development of goal-directed ethanol drinking in mice (Nam *et al.*, 2013). Taken together, it has been hypothesized that ethanol exerts some of its behavioral effects by increasing extracellular levels of adenosine in the brain, with subsequent activation of adenosine receptors. As caffeine acts as a nonselective antagonist at adenosine receptors (Fredholm *et al.*, 1999) this could be part of a pharmacological mechanistic explanation for the interaction of caffeine and ethanol. Interestingly, dopaminergic mechanisms have been suggested to mediate some of the behavioral effects of caffeine (Garrett & Griffiths, 1997). A_2 receptors and dopamine D2-receptors are co-expressed in neurons of the nAc where they functionally interact with each other (Ferre *et al.*, 1991; Morelli *et al.*, 1995). Caffeine enhances dopaminergic activity, presumably by competitive antagonism at these A_2 receptors and thereby removing the negative modulatory effects of adenosine on dopamine D2-receptors (Garrett & Griffiths, 1997). This is interesting since ethanol is known to increase extracellular levels of dopamine in the nucleus accumbens,

an effect associated with positive reinforcement (Di Chiara & Imperato, 1988; Wise & Rompre, 1989; Spanagel, 2009).

As we have proposed that taurine and ethanol are interconnected in the brain reward system (see the above section "Interactions between taurine and ethanol in the CNS") we suggest that taurine could act as a pharmacological active ingredient in energy drinks when consumed together with alcohol. That both caffeine and taurine could have an impact on ethanol-induced behavior is further supported by the fact that administration of energy drink (containing caffeine and taurine) to mice reduces the depressant effects of ethanol and enhances ethanol-induced behavioral sensitization (Ferreira *et al.*, 2004; Ferreira *et al.*, 2013).

OBJECTIVES

Furthering the knowledge about ethanol's interaction with the brain reward system and especially it's interplay with dopamine and taurine may provide knowledge important for the development of new pharmacotherapies against alcohol use disorder (AUD). The overall aim of this thesis was to investigate correlations between taurine and dopamine during ethanol exposure, with special emphasis on neurochemical effects in the nAc.

The specific aims of the present thesis are the following:

- To define the impact of voluntary ethanol intake on both endogenous levels, as well as ethanol-induced elevation of taurine and dopamine (**Paper I**)

- To determine if repeated exposure to taurine affects neurochemical and/or behavioral responses to ethanol (**Paper II**)

- To characterize the origin of ethanol-induced taurine release (**Paper III**)

- To outline the modulatory role of energy drink constituents caffeine and taurine on ethanol-induced locomotor activity (**Paper IV**)

METHODOLOGY

More detailed descriptions of the materials and methods employed in this thesis are to be found in the individual papers. Below follows a brief description, including reflections, of the different experimental methodologies used in the present thesis.

Animals and animal models

Animal models have been used in preclinical studies for decades as a way to shed light on and increase the understanding of normal, as well as pathological, conditions in humans. The use of rodents to elucidate behavioral, environmental and neuronal mechanisms underlying neuropsychiatric disorders, such as AUD, is especially suitable, as the translatability to humans appears good. This is due to the fact that several key brain regions are highly conserved between species, and the genome of rodents is approximately 90% similar to the human genome (Mullins & Mullins, 2004). In all of the studies that constitute this thesis, outbred rat (Wistar) and mouse (NMRI) strains were used, in order to somewhat mimic the genetic variation in the human population and thereby enhance the translatability to the human situation. All experiments presented in this thesis were approved by the Ethics Committee for Animal Experiments, Gothenburg, Sweden.

In the microdialysis studies (paper I, II and III), for the intermittent ethanol consumption study (paper I), for the designer receptor exclusively activated by designer drugs (DREADDs)-experiments, for gene expression analysis (paper II) and for blood and cerebrospinal fluid (CSF) analysis (paper I and II) adult, male Wistar rats (Taconic, Denmark; Janvier Labs, France; Envigo Germany) were used. These rats were selected as our research group has an extensive experience with regard to neurochemistry and behavior displayed by this strain.

The Wistar rat is also commonly used in animal studies within the field of addiction, and is a foundation stock for several rat lines bred for high and low voluntary ethanol intake (Palm *et al.*, 2011). Moreover, it has been shown that the Wistar rat strain displays a high ethanol intake with pharmacological relevant blood ethanol concentrations when the intermittent ethanol consumption model used in this thesis is applied (Simms *et al.*, 2008). Nevertheless, studies report different results in ethanol intake in Wistar rats, emphasizing the vendor as an important parameter (Palm *et al.*, 2011). For

locomotor activity measurements (paper IV) adult, male NMRI (Charles River, Germany) mice were used instead of rats, since rats rarely display an increase of locomotion after acute ethanol administration (Linakis & Cunningham, 1979; Masur *et al*., 1986; Criswell *et al*., 1994; Chuck *et al*., 2006; Brabant *et al*., 2014). NMRI mice were selected as it has been shown that this strain shows robust ethanol-induced locomotion and accumbal dopamine increase (Engel *et al*., 1974; Engel *et al*., 1988; Jerlhag *et al*., 2009). All of the animals were allowed at least one week of acclimatization to the animal facility before any experiments were initiated. For the entire duration of the experiments, animals had access to standard rat feed and tap water ad libitum

Notably, Wistar rats were sometimes purchased from different vendors, which was due to the fact that Taconic, Denmark, stopped their marketing of Wistar rats. Therefore, we had to screen a number of Wistar rats from different suppliers in the microdialysis set-up. The rats were administrated ethanol and nAc taurine as well as dopamine were monitored. Unfortunately, we experienced that Wistar rats from the different vendors differed with respect to endogenous levels of taurine in the nAc. Even their body weight and anatomical features were different. The differences observed might be due to genetic variation, but also by the fact that different laboratory diets were used by the vendors. Furthermore, supplier-dependent differences have been shown in intermittent ethanol intake as well as in response to naltrexone treatment in Wistar rats (Momeni *et al.*, 2015). Nevertheless, their accumbal dopamine response after ethanol or ethanol in combination with taurine was the same. In this thesis, rats from different vendors were applied in the studies included in paper II and III. The following actions were taken to minimize the impact on the results: rats from the different vendors in each experiment were compared with respect to parameters important for the outcome, such as endogenous taurine and dopamine levels, the ethanol-induced taurine and dopamine increase, and the behavior of the animal was observed. Data presented in this thesis should not be significantly affected by the use of different vendors. However, it is not possible to completely exclude a potential impact and therefore, in general, it is preferable to as far as possible use rats from the same vendor. This also puts focus on the inherent heterogeneity among Wistar rats and the possibility to utilize their different attributes in study design.

As mentioned above, animal models constitute an important tool in the study of pathological processes underpinning AUD. However, addiction is a multifactorial disease and there is unfortunately not one single animal model that fully reflects the complex clinical picture of AUD. Therefore, several

different models are used to reflect the divers aspects, including relapse, cue-induced craving, sensation of reward and withdrawal symptoms (Spanagel, 2000). How well an experimental animal model reflects a certain human condition is termed validity. There are different forms of validity, such as face validity, construct validity and predictive validity. Face validity refers to how well the symptoms/behaviors observed in the animal model reflect those in the human medical condition. Construct validity refers to how similar the underlying pathophysiology in the animal model is to that in humans. Finally, predictive validity refers to how well the animal model predicts the effects of manipulations or treatments on the human condition. Since one experimental animal model seldom reflects the whole human medical condition, it is of great importance to cautiously select the model that is best suited for challenging the hypothesis in question, and often several different models are needed. The validity of the different models used in this thesis will be discussed further in the following subchapters.

In pre-clinical studies, including alcohol research, the use of male animals largely exceeds females. Importantly, sex differences in alcohol intake and ethanol-induced increase in nAc dopamine levels have been demonstrated in rodents (Lancaster & Spiegel, 1992; Blanchard *et al.*, 1993). Sex differences in alcohol use are also present in the human population (Erol & Karpyak, 2015). Thus, the same result cannot be expected in the opposite sex and studies including both sexes are needed. In pre-clinical studies employing female animals it is of importance to take into consideration the potential influence of the estrous cycle, as it has been demonstrated that alcohol intake varies in females depending on their estrous cycle (Forger & Morin, 1982). Traditionally in our laboratory male rodents have been used, and have therefore also been used in the studies of the present thesis. However, with regards to the above mentioned reasons we have started to include female animals in our studies. These studies are only at a preliminary stage and hence not included in this thesis.

Drugs and chemicals

Ethanol

For the intermittent drinking paradigm, 95% ethanol was diluted in tap water to a 20% ethanol solution. For all i.p. injections, 95% ethanol was diluted in 0.9% NaCl to a concentration of 15%. For blood and CSF analysis, ethanol was administrated 2.5 g/kg, i.p. For the locomotion study in paper II, rats were administrated a lower dose of ethanol (0.25g/kg, i.p.) to avoid a sedative effect. During the different microdialysis studies, rats were administrated 2.5

g/kg or 2.75 g/kg i.p. In microdialysis experiments where ethanol was administered by local perfusion via the microdialysis probe, 95% ethanol was diluted in Ringer's solution, which contains (in mmol/l): 140 NaCl, 1.2 $CaCl_2$, 3.0 KCl and 1.0 $MgCl_2$, to a concentration of 300mM. During locomotor activity measurements, mice were administered 1.75, 2.5 or 3.25 g/kg ethanol, i.p. In paper I, when ethanol was consumed orally by the rats, 20% ethanol solution was used as this concentration has been proven to establish pharmacologically relevant ethanol blood concentrations in the intermittent voluntary ethanol consumption model (Simms et al., 2008). The dose of 2.5 g/kg ethanol for i.p. administration was chosen as this is a high dose, yet not making the rat unconscious but still inducing measurable dopamine levels. The concentration of ethanol used for perfusion, via the microdialysis probe, in the nAc was based on a dose-response study performed in our laboratory (Ericson et al., 2003), this high, yet pharmacologically relevant concentration, was chosen as it increases dopamine levels in the nAc to approximately the same extent as 2.5 g/kg ethanol i.p. In paper IV, for systemic administration of ethanol to mice, two moderate doses (1.75 and 2.5 g/kg) were used has these have been shown to increase locomotor activity in mice (Carlsson et al., 1974; Liljequist, 1991; Blomqvist et al., 1994; Hilbert et al., 2013; Vallof et al., 2017). A higher dose (3.25 g/kg), with sedative effects (Hilbert et al., 2013) was used to account for the possibility that caffeine and/or taurine may diminish the sedative effects of ethanol.

Taurine

For all of the experiments, taurine was dissolved in 0.9% NaCl. The rats were given taurine 0.25 g/kg, i.p. for 10 days or intermittently for 15 days, or as an acute treatment during microdialysis experiments, or for blood and CSF analysis. For locomotor activity studies, mice were administered taurine 30, 60, 300 or 600 mg/kg, i.p. The dose of 0.25 g/kg for systemic administration was used as it has been demonstrated to increases taurine levels in plasma as well as in the extracellular fluid of the hippocampus (Lallemand & De Witte, 2004). In paper IV, four different doses of taurine was used, as it has been demonstrated that either low or high doses of taurine alone or in combination with ethanol may affect locomotor activity (Aragon et al., 1992; Ginsburg & Lamb, 2008; Whirley & Einat, 2008).

Caffeine

Caffeine was dissolved in 0.9% NaCl. Mice were given caffeine 15 mg/kg, i.p. intermittently for 18 days. During locomotor activity measurements, mice were administered 1, 5, 15 or 30 mg/kg caffeine i.p. These doses were used

because the threshold dose for caffeine-induced locomotor activity has been demonstrated to be approximately 3 mg/kg, and a maximum effect to be present at 20-30 mg/kg (Buckholtz & Middaugh, 1987; Hsu et al., 2009; Hilbert et al., 2013). The lowest dose of caffeine was chosen to account for possible additive effects when administrated together with ethanol and/or taurine.

Clozapine-N-oxide

The DREADD ligand Clozapine-N-oxide dihydrochloride (CNO) was dissolved in 0.9% NaCl and administrated 3 mg/kg, i.p, a dose within the recommended dose interval (Roth, 2016).

Tetrodotoxin

The Na^+ channel blocker tetrodotoxin (TTX) was dissolved in Ringer's solution to a concentration of 1 μM, and administrated by reversed microdialysis. This concentration was used as it has been shown to decrease taurine levels in the hypothalamus (Singewald et al., 1993).

Fluorocitrate

The glial metabolic inactivator dl-fluorocitrate was dissolved in Ringer´s solution to a concentration of 25 μM, and administrated by reversed microdialysis. A dose-response study performed in our laboratory showed that a dose of 25 μM was the highest dose without impact on the dopamine levels *per se*, and without negative behavioral effects (data not shown). The chosen dose decreased extracellular levels of glutamine by approximately 60% indicating intended effects on glial cells (data not shown).

DCPIB

The VRAC inhibitor DCPIB (4-(2-Butyl-6-7-dicholro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid) was dissolved in dimethyl sulfoxide (DMSO) (0.4% of the final solution) and diluted in Ringer's solution to a concentration of 100 μM, and administrated by reversed microdialysis. This dose of DCPIB was used as it has previously been shown to decrease the ischemia-induced release of aspartate and glutamate in the microdialysis setup (Zhang et al., 2008). In an attempt to increase the concentration of DCPIB, to enhance the pharmacological outcome of the experiment, the concentration of DMSO had to be increased to a point where DMSO alone had a major impact on both taurine and dopamine. This approach was concluded to be futile.

The different routes of administration were chosen to accommodate the specific aim. For mechanistic studies, where a local effect of the drug is desirable, local administration of the drug via the microdialysis probe was applied. Taurine, caffeine and ethanol were systemically administrated by i.p. injections to mimic the human situation where the drug is ingested orally, while at the same time controlling the amount of drug delivered. When a drug, aiming for specific targets is applied, its degree of specificity is important to take into consideration. Clearly, a high specificity is desirable in order to abate side effects, which can interfere with the results. Furthermore, the specificity of the drug generally decreases with increased dose.

Behavior

In this thesis two different behavioral animal models are used, which both utilize the ethological behavior of the animal: locomotor activity and voluntary drug intake in an intermittent drinking paradigm.

Locomotor measurements and behavioral sensitization

Behavioral sensitization and locomotor measurements were assessed in paper II in order to study the impact of repeated taurine administration on the acute effects of ethanol, and in paper IV to study the effects of the combination of caffeine and taurine on ethanol-induced locomotor activity in naïve and caffeine experienced animals.

Behavioral sensitization is a phenomenon where the same dose of a drug produces an increased response after repeated exposure. The increased drug-induced behavioral response is suggested to reflect neuroadaptations induced by repeated drug administration (Robinson & Berridge, 1993). Consequently, the model of behavioral sensitization is used in paper II and IV to study potential adaptations linked to dopamine related behavior after repeated taurine (0.25 g/kg, i.p.) administration for ten days (paper II) or an intermittent caffeine treatment regimen during fifteen days with a total of eleven injections (paper IV). The stimulatory effect of the drug was measured on the first and last day of the treatment periods.

The measurement of rodents' spontaneous activity is frequently used for studying the development of behavioral sensitization and drug-induced behavioral effects related to the brain reward system. This is based on the ability of ethanol, like most drugs of abuse, to increase locomotion in rodents, which is suggested to be related to ethanol-induced activation of the mesolimbic dopamine system (Carlsson *et al.*, 1974; Liljequist *et al.*, 1981;

Wise & Bozarth, 1987; Engel *et al.*, 1988). Therefore, locomotor stimulatory effects induced by acute drug treatment in drug naïve as well as in sensitized animals were monitored in paper II and IV. The open field arena used for these measurements consists of a two-layer grid of infrared beams, which makes it possible to measure both horizontal and vertical activity. Activity is registered when the animal's movement causes beam breaks. All animals were habituated to the test box before acute drug challenge, in order to abolish impact of the rodents' innate exploratory behavior to novel environment with the drug-induced effects. In both paper II and IV were the stimulatory effect analyzed with regards to locomotor activity (horizontal beam breaks) and in paper II it was also analyzed with regards to rearing activity (vertical beam breaks).

It is considered that behavioral sensitization is a valid model for studying neuroadaptations induced by repeated drug intake (Steketee & Kalivas, 2011). Although taurine and caffeine are not usually referred to as classic drugs of abuse (e.g. ethanol, nicotine, amphetamine), in our studies behavioral sensitization was found to be induced, which indicates some kind of neuronal adaptations. One important aspect of the behavioral sensitization model and the measurement of locomotion is that, normally and in this thesis, drug administration occurs in a non-voluntary manner. This is in contrast to the human situation where initial drug intake is largely a voluntary behavior. It is important to keep in mind that locomotor activity should be regarded as an approximation of dopaminergic neurotransmission and not as a direct measurement. To further elucidate the mechanism of action it is necessary to add other methods such as microdialysis, electrophysiology and/or immunohistochemistry. However, the method is useful as a first step in exploring a hypothesis (as was done in paper IV), as it is relatively simple.

Intermittent ethanol consumption

In paper I the intermittent access 20% alcohol two-bottle-choice drinking paradigm was used in order to study tentative divergences between high and low ethanol consuming rats with regards to baseline, as well as ethanol-induced, taurine and dopamine levels in the nAc.

Voluntary ethanol consumption in rats is frequently used to model human alcohol intake and AUD, providing a valuable tool for studying neurochemical and behavioral changes induced by high ethanol intake over time. The intermittent access 20% alcohol two-bottle-choice drinking paradigm used in paper I has been shown to induce high and stable ethanol intake and pharmacologically relevant blood ethanol concentrations in Wistar

rats. Furthermore, good predictive validity has been demonstrated as treatment with acamprosate as well as naltrexone reduce alcohol intake in this model (Simms *et al.*, 2008). The paradigm is based on voluntary drug intake as rats are allowed to drink in their home cage and choose when and how much to drink. In addition to one water bottle, the animals are given one bottle of alcohol for three 24-hour sessions per week. As rats are nocturnal animals and drink more during the dark phase, the alcohol is presented to the rats in the beginning of the dark cycle. This paradigm, as opposed to a continuous access paradigm, consists of repeated cycles of drinking and abstinence, which has been shown to increase voluntary alcohol intake (Wise, 1973). Besides inducing a high consumption, this model reflects the human drinking pattern, in terms of a voluntary and intermittent consumption. During the entire experiment, rats are provided regular rat feed ad libitum and their consumption is monitored in order to exclude alcohol intake due to calorie loss. Furthermore, the concentration of alcohol used is of importance for the results. With low concentrations the taste is mild and sweet and becomes aversive with very high concentrations. However, with low concentrations there is a risk of pharmacological irrelevance as the amount of fluid ingested by the animal is limited by physical constrains (Sanchis-Segura & Spanagel, 2006). Generally, rats prefer lower concentrations of ethanol (Sanchis-Segura & Spanagel, 2006; Palm *et al.*, 2011). However there is an individual variability as some rats will chose higher concentrations (Palm *et al.*, 2011). A concentration of 20% alcohol has been shown to be preferable (Simms *et al.*, 2008). A disadvantage with this method is that in order to monitor individual intake, the animals are normally single-housed. As rodents are gregarious animals this has a negative impact for their wellbeing and might thereby influence the results.

Biochemical measurements

In this thesis, samples from blood, CSF and accumbal extracellular fluid were sampled and analyzed by high performance liquid chromatography (HPLC) in order to measure levels of dopamine and amino acids, mainly taurine. Samples of the extracellular fluid were obtained by performance of *in vivo* microdialysis in the rat nAc.
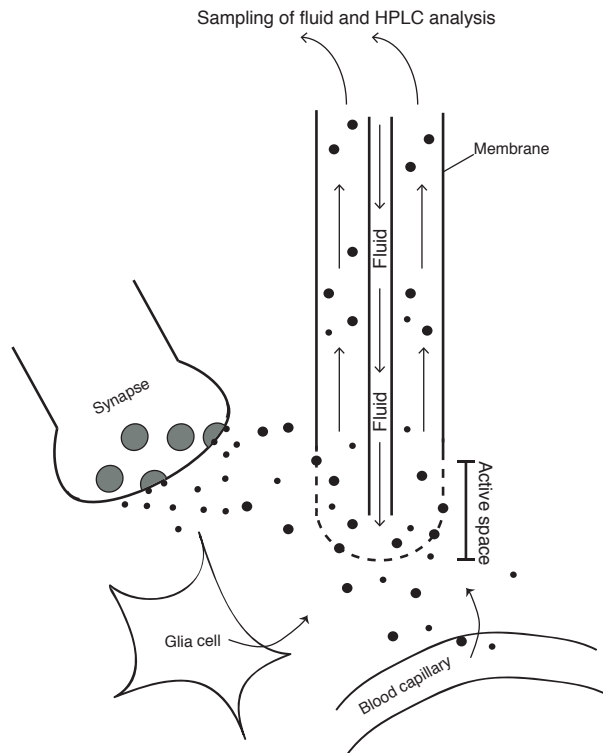
In vivo microdialysis

In 1974, Ungerstedt and co-workers first described the method of *in vivo* microdialysis, which allows sampling of extracellular substances such as neurotransmitters and amino acids, in awake freely moving animals (Ungerstedt & Pycock, 1974). Via the implanted microdialysis probe, the

technique also enables delivery of drugs to specific brain regions, so called reversed microdialysis. Both types of microdialysis were applied in this thesis. In paper I, II and III this technique was used to quantify extracellular levels of taurine and dopamine in the nAc. Also other amino acids were detected when desirable. Wistar rats were used for all of the microdialysis experiments.

In brief, two days before the microdialysis experiment, a probe with a semi-permeable membrane is implanted into the brain region of interest. The active space of the membrane enables passive diffusion of solutes along its concentration gradient from the extracellular space into the probe or vice-versa. On the day of microdialysis experiment, the probe is perfused with an artificial cerebrospinal fluid (Ringer's solution), which may contain drug for appropriate pharmacological manipulations, and is then sampled from the outlet of the probe and analyzed by HPLC (fig. 2).

Figure 2. *Descriptive image of the in vivo microdialysis set-up. A probe with a semi-permeable membrane enables molecules to diffuse (along its concentration gradient) from the extracellular space into the probe or vice versa, from which the fluid is sampled and analyzed by high performance liquid chromatography (HPLC). Image by Julia Morud Lekholm; Nicotine sensitization and the effects of extended withdrawal, Doctoral thesis.*

Microdialysis does not change the net fluid balance in the tissue surrounding the probe, which enables sampling for a longer time period. Another

advantage with the technique is that each animal can serve as its own control, thereby reducing the number of subjects (Li *et al.*, 2006). The are several different modifications available in order to optimize the outcome, such as sampling rate, the membranes' molecular cut-off, flow rate of the perfusate and length of the active space. In the set-up used in this thesis, the sampling rate is set to every 20th minute. This rather slow sampling rate is commonly applied, as it is necessary for obtaining sufficient volume of fluid and concentration of solute needed for HPLC analysis. Consequently, the time-resolution is low and fast events like burst firing of dopamine neurons are not possible to detect with this technique. For this electrophysiological recordings or *in vivo* voltammetry may be used. However, *in vivo* microdialysis is suitable for elucidating effects after chronic systemic treatment, (e.g. the drinking paradigm used in paper I, repeated taurine treatment used in paper II), or pharmacological manipulations (paper III) on neurotransmission, as well as showing good predictive validity. Furthermore, the possibility to implant more than one probe to the same animal enables manipulations and measurements of different brain areas simultaneously making the technique a powerful tool for studying neural circuits. Another aspect to take into consideration is the excovery (the concentration of drug in target tissue) and recovery (the amount of solute that is diffusing from the tissue to the probe) of the probe. Both vary due to the cut-off of the membrane, perfusion rate and intrinsic properties of the solute, e.g. pH, molecular weight, shape and polarity. Insertion of the probe will also have a pronounced impact as gliosis will occur in the surrounding tissue (Norton *et al.*, 1992) and the surgery may cause mechanical damage and blood clots. To give the animal sufficient amount of time to recover after the surgery and to avoid a substantial amount of gliosis, the microdialysis experiment was initiated 48 hours after insertion of the probe. After completed experiment, brains were dissected and visually examined. Brains with incorrect probe placement and visible signs of error, e.g. bleeding, were excluded from data analysis. As discussed above, there are several factors that affect the excovery, which makes it difficult to select an appropriate concentration of drug for perfusion via the probe. Additionally, there is a concentration gradient in the tissue surrounding the probe with the highest excovery next to the probe. Results from our research group (unpublished data) have concluded that perfusion with 300 mM ethanol results in 60-70 mM in the surrounding tissue and the excovery rate is estimated to be approximately 24 %. In order to avoid speculations and include uncertainties the concentration of the perfused drug is stated and not the assumed concentration in the tissue.

In all of the studies that comprise this thesis, the coordinates for probe placement is aimed for the nAc core-shell borderline region, suggesting that

we are sampling in both the core and shell region. It has been shown that this area possesses a high density of GlyR (unpublished data), which we suggest are of importance for ethanol-induced dopamine release in the nAc. Furthermore, dopamine was increased in the nAc core-shell borderline but not in the core or shell during ethanol consumption (Howard *et al.*, 2009). In our studies, coordinates for the core-shell borderline region have been proven successful when studying the dopamine elevating properties of ethanol after local (paper III), systemic (paper I, II and III) and oral administration (paper III).

Blood and CSF sampling

In paper I and II, blood and CSF were collected from naïve rats, rats with a history of ethanol consumption or rats systemically treated with ethanol or taurine to allow quantification of taurine in these fluids.

Briefly, the animals were anesthetized, the heart exposed and blood collected from the left ventricle. Blood was drawn from the left and not right ventricle, as the outer wall of the left ventricle is thicker, this to reduce the risk of collapse of the ventricle. After the blood was withdrawn, the heart was punctured allowing the animal to bleed out. The cisterna magna of the animal was then immediately exposed and CSF sampled. The animal was drained from blood prior to the sampling of the CSF in order to avoid contamination of taurine from the blood into the CSF. CSF samples containing visual traces from blood were excluded from further analysis.

HPLC analysis

High performance liquid chromatography (HPLC) is a common technique used for separation, detection and quantification of molecules in a mixture. In the present thesis this technique is used for the analysis of accumbal extracellular samples obtained by microdialysis as well as samples from blood and CSF.

Briefly, pumps use high pressure to press the sample, diluted in a mobile phase, through a column (stationary phase) filled with adsorbent material. Due to the intrinsic properties of each analyte, the interaction with the adsorbent material differ, leading to different flow rates and separation as they are pumped through the column. A detector generates a signal equivalent to the amount of the specific molecule enabling quantification. In this thesis, dialysate, blood and CSF samples were analyzed with regards to amino acids, primarily taurine. However, other amino acids as serine, glycine, glutamate and glutamine were also monitored when desirable. To

this end, a reversed phase-HPLC system consisting of a non-polar stationary phase and fluorescence detection was used. For the detection and quantification of dopamine in dialysate two different systems were used in parallel, one system utilized an ion exchange column and the other system a reversed phase column. As the dopamine molecule can be increased or decreased in its oxidation state, electrochemical detection was used for both systems. In order to identify and quantify amino acids as well as dopamine, internal standards were used. Four baseline samples for each animal were obtained before any drug challenge and an internal difference more than ±10% was not accepted. Subsequent samples were calculated as percentage of baseline, which can be considered to reflect changes in volume transmission. However, absolute concentrations of extracellular analytes can also be obtained by using this technique, for instance when endogenous levels are of interest (paper I, II).

The columns are expensive and easily degraded by particles in the sample or solvent and by irreversible binding of impurities. Thus, it is important that samples may not contain big particles, such as proteins and cells. Due to the semipermeable membrane of the microdialysis probe this is automatically avoided in the dialysate. Serum is obtained from the blood samples allowing detection of taurine by HPLC analysis. Although serum is the fraction of the blood containing the minimum numbers of particles, the serum as well as the CSF samples are deprotonated with trichloric acid and filtrated prior to analysis. The particle size of the material in the column influences the analysis. Small particle size gives better resolution because it provides a more uniform flow through the column, and because the distance through which solute must diffuse in the column and mobile phase is on the order of the particle size. In other words, the smaller the particles, the less distance solute must travel. The drawback with small particle size is increased resistance to solvent flow. Thus, a smaller particle size requires a higher pressure, but provides a shorter optimum run time and lower detection limit (Harris, C.D., 2010). The temperature used is also of importance. Heating a column usually decreases the viscosity of the solvent, thereby reducing the required pressure or permitting faster flow. However, increased temperature can also cause degradation and decreased lifetime of the column (Harris, C.D., 2010). The polarity of the solvent influences the rate of elution of the solute. In reversed phase chromatography the column is nonpolar or weakly polar and the solvent is more polar. Thus, a less polar solvent has higher eluent strength and the solvent molecules will more easily displace the solute molecules on the column, resulting in increased elution rate of the solute (Harris, C.D., 2010).

Chemogenetic manipulations

In paper III, we utilized the ability of the DREADD technique to with a high degree of specificity, manipulate non-neuronal signal transduction in a cell-type-specific manner, and combine this chemogenetic tool with *in vivo* microdialysis.

Designer receptors exclusively activated by designer drugs (DREADDs)

DREADDs are a class of chemogenetically-engineered proteins that permit temporal as well as spatial control of G-protein signaling *in vivo*. The receptor is solely activated by synthetic ligands such as CNO, when bound creates a conformational change in the receptor and activation of the G-protein. Further effect depends on the type of G-protein (Armbruster *et al.*, 2007; Roth, 2016). In paper III the technique was used in order to modulate the activity of nAc astrocytes and *in vivo* microdialysis was used to quantify extracellular levels of taurine and dopamine in the nAc.

Three weeks prior to the microdialysis experiment, rats were transfected with astrocyte-specific excitatory DREADDs (GFAP-Gq-DREADD) or a control plasmid (sham) in the nAc. On the day of microdialysis, animals were systemically administrated ethanol and the specific DREADD-ligand CNO, and accumbal taurine- and dopamine levels were monitored.

The DREADDs technique was developed about ten years ago by Bryan Roth and colleagues at the University of North Carolina (Armbruster *et al.*, 2007). Thus, this is a relatively new technique, which entails a number of concerns. One aspect is a potential constitutive activity of the DREADD-receptor. So far, there are no reports indicating that. However, just because there are no reports showing constitutive activity it does not imply the absence of it. Therefore, it is of importance to apply the lowest expression level of the DREADDs needed for the current experimental design and to carefully select appropriate controls. Another concern is the aspect of receptor regulatory processes. Because hM3Dq is subjected to the same regulatory processes (phosphorylation, desensitization, internalization, downregulation) as other G-protein coupled receptors (Alvarez-Curto *et al.*, 2011) prolonged activation with CNO could lead to an attenuated response due to desensitization and/or downregulation of hM3Dq. However, no such attenuation of regulatory responses has been reported (Alexander *et al.*, 2009; Krashes *et al.*, 2011). That this phenomenon would be present in the study of paper III is not very likely as no repeated dosing of CNO occur. To date, in most of the studies where the technology is used, the DREADDs are expressed in neurons and only in a few studies are the receptors expressed in astrocytes. The current

opinion is that activation of Gq-DREADDs leads to depolarization of neurons and enhances excitability, and activation of inhibitory DREADDs (Gi-DREADDs) leads to attenuation of neuronal firing (Armbruster *et al.*, 2007; Urban & Roth, 2015). However, their functional consequences on astrocytes are less known. It has been shown that activation of Gq-DREADDs (which are used in paper III) increase $[Ca^{2+}]_i$ which is in turn likely to affect gliotransmission. A Gq-DREADD expressing astrocyte is then presumed, when activated by CNO, to be in an activated state (Agulhon *et al.*, 2013; Bull *et al.*, 2014; Scofield *et al.*, 2016; Durkee *et al.*, 2019).

CNO appears to be pharmacological and behavioral inert in rodents if administrated at the recommended dose (0.1-3 mg/kg) (Roth, 2016), which was done in paper III. Importantly, CNO can be back metabolized to clozapine (Roth, 2016), although to a very low extent. The risk of back-metabolization of CNO to clozapine should be minimized by administrating the lowest possible dose, and adequate controls, where CNO is administrated to animals expressing control plasmid (sham), should be used. This is also important with regards to reducing possible clozapine-like side effects, such as hypotension and sedation. However, the *in vivo* mechanism of action of CNO at DREAADs is debated. Gomez and colleagues (2017) claim that CNO is not blood brain barrier (BBB) permeable, and that back-metabolized clozapine, derived from systemic administrated CNO, is the ligand for DREADDs. Therefore it is suggested that subthreshold doses of clozapine should be used instead of high doses of CNO (Gomez *et al.*, 2017) The back-conversion to clozapine has also been shown to produce clozapine-like interoceptive stimulus effects in rodents (Manvich *et al.*, 2018). Additionally, clozapine is already approved for clinical use and very low doses of clozapine are required for DREADDs activation, reducing undesirable side effects. Thus, the use of clozapine, rather than CNO, will most likely increase the translational potential of DREADDs as clinical CNS therapy. However, the major concern for using DREADDs as CNS therapy would be the insertion of an exogenous receptor into the brain.

Immunohistochemistry

Immunohistochemistry is a common technique used for detection and visualization of proteins (antigens) in a tissue sample. It utilizes the principle of antibodies binding to specific antigens and visualization is microscopy-based. In paper III this method was used to detect and assess astrocyte-specific DREADD transfection.

Even though a fluorescent reporter is connected to the DREADD-construct, the signal may need to be amplified. In paper III, the DREADD was HA-tagged in a vector with IRES-mCitrine and immunohistochemistry with fluorescence detection was required for visualization. There are two different approaches available for the use of immunohistochemistry: the direct and indirect. In comparison with the direct method, the indirect method utilizes both a primary antibody and a secondary antibody with a fluorescence reporter molecule, which amplifies the signal by increasing the number of fluorophore molecules per antigen (Odell & Cook, 2013). In the study with astrocyte-specific DREADDs (paper III), a primary anti-HA, anti-GFAP or anti-NeuN antibody, and secondary Alexa Flour 488 or Alexa Flour 555 antibody were used to visualize HA-tagged DREADD and GFAP- or NeuN-positive cells.

Sample preparation is critical for successful visualization. This requires proper tissue collection, fixation and sectioning. Reduced non-specific staining can be achieved by transcardial perfusion of the rats with buffer and paraformaldehyde, to postfixation of the brains in paraformaldehyde and by applying blocking buffer to the brain slices. The specificity and concentration of the antibodies are also significant factors. Furthermore, anti-HA antibody also labels areas of damage, e.g. cannula tracks. However, this can be distinguished visually and spatially from the HA-antigen. A significant problem with immunofluorescence is photobleaching, which results in loss of activity of the fluorophore. This can be controlled by increasing the concentration of the fluorophores, reducing the timespan or intensity of light exposure or by using more robust flourophores that are less prone to bleaching, e.g. Alexa Flours as was done in paper III (Ramos-Vara, 2005; Im *et al.*, 2019).

Gene expression

Gene expression analysis is used to study the occurrence or activity of genes. Quantification of mRNA is one way to achieve this, and quantitative polymerase chain reaction (qPCR) is the most common approach used. In paper II, this method was used to monitor the mRNA levels of several different genes (the taurine transporter, several different GABA$_A$ receptor subunits and glycine receptor subunits, glutamate decarboxylase 1 and 2) suggested to be of importance for ethanol-induced taurine and dopamine release. The gene expression levels within the nAc between rats systemically treated with taurine or vehicle for fifteen days were compared.

qPCR is based on conventional polymerase chain reaction (PCR), meaning a repetitive series of cycles giving an exponential accumulation of a specific DNA (deoxyribonucleic acid) fragment where the number of target DNA copies doubles in every PCR cycle. In qPCR, fluorescence is measured in every cycle enabling continuous assessment of the amount of PCR product. The amount of fluorescence is proportional to the initial amount of template. By using complementary DNA (cDNA) transcribed from extracted mRNA as template, a relative quantification of mRNA expression in a tissue sample can be obtained and comparison between samples can be made (Livak & Schmittgen, 2001).

To decrease a possible impact on the mRNA levels, the animals were decapitated without any anesthesia and dissection of the tissue was performed in a cold and RNase free manner. Furthermore, the tissue was immediately frozen after dissection and stored in -80 °C until RNA extraction and cDNA synthesis, which were also performed under RNase free conditions. The sensitivity and accuracy of PCR is largely dependent on the specificity of the primers used (Thornton & Basu, 2015). In the study of paper II, primers from the company Qiagen were used and although the exact sequence is not revealed they state high specificity. Furthermore, it is important to note that this method does not provide information about the biological function of the protein that the target gene is encoding. For instance, a receptor can be inactive due to internalization or being in an inactive state even though the mRNA expression is high.

Statistical analysis

When performing statistical analysis the first step is to consider if to use a parametric- or a non-parametric test and is most important with small data sets, as the power of non-parametric tests is low. The decision of when to use a parametric test and when to use a non-parametric test requires perspective and knowledge about the data set and the sample population and is *i.a.* based on whether the data set is assumed to be normally distributed or not. In addition, data can be plotted in a histogram and if a bell-shape can be detected normal distribution can be assumed. However, this is difficult with small sample sizes, as used in this thesis. Another approach is to perform a normality test, which is used to determine if data are well modeled by a normal distribution and will show a p-value for this. With small sample sizes, non-normal distribution is hard to detect with this method due to limited power. A third alternative, which is used for statistical analysis in this thesis, is to use a quantile-quantile normality plot (Q-Q plot), where the actual

residuals are plotted against the predicted residual assuming sampling from a Gaussian distribution (Campell *et al.,* 2007).

Statistical analysis was performed by using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The statistical significance was determined by unpaired t-test, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, two-way ANOVA followed by Tukey's multiple comparisons test, two-way ANOVA with repeated measures (treatment group x time) followed by Tukey's multiple comparisons test or Pearson's correlation coefficient as described in the different papers.

RESULTS AND DISCUSSION

Ethanol high-consuming rats show a blunted taurine increase after acute ethanol (paper I)

Chronic ethanol consumption is associated with a decreased activity in the mesolimbic dopamine system in both humans and animals (Diana *et al.*, 1993; Schulteis *et al.*, 1995; Volkow *et al.*, 2007; Connor *et al.*, 2008; Feltmann *et al.*, 2016). As taurine appears to be of importance for ethanol to increase dopamine in the rat nAc (Ericson *et al.*, 2011), investigating if similar adaptations occur with respect to taurine is important. Thus, the aims of this study was to assess possible divergences in endogenous levels, as well as ethanol-induced increases in extracellular taurine and dopamine levels in the nAc of rats voluntary consuming high or low amounts of ethanol. These rats will be referred to as ethanol high- and low-consuming animals.

Blunted increase in taurine following acute ethanol administration in ethanol high-consuming rats

By means of *in vivo* microdialysis we show that neither taurine nor dopamine release following an acute systemic injection of ethanol was affected by seven weeks of intermittent ethanol consumption when comparing all ethanol-consuming rats with ethanol-naïve rats. However, interesting results were revealed when the rats were divided by their ethanol consumption into high- or low-consuming animals. Although there were no differences in endogenous levels of taurine in the nAc, CSF or blood when comparing ethanol high- and low-consuming rats (see fig. 3C-E in paper I), we found that high-consuming rats exhibited both a reduced and slower increase in taurine after acute ethanol treatment as compared to low-consuming rats that displayed a normal rapid increase (see fig. 2 in paper I). These findings are in line with previous studies performed in ethanol high-preferring and high-sensitive animals, who displayed reduced but prolonged ethanol-induced elevation of taurine, as compared to non-preferring and low-alcohol sensitive animals (Quertemont *et al.*, 2000; Quertemont *et al.*, 2002). Thus, there appears to be an inverse relationship between the intensity of ethanol-induced taurine increase and ethanol intake as well as preference. It has been suggested that taurine attenuates the aversive and toxic effects induced by ethanol (Watanabe *et al.*, 1985; Quertemont *et al.*, 1998b), which is in agreement with the hypothesis of taurine being released to counteract ethanol-induced cell swelling, membrane alterations and calcium homeostasis

disruption (Quertemont *et al.*, 2000; Olive, 2002). Quertemont and co-workers suggest that ethanol preferring and high-sensitive rats are not as sensitive to the aversive effects of ethanol as their counterparts, non-preferring and low-sensitive rats, and consequently not as much taurine needs to be released (Quertemont *et al.*, 2000; Quertemont *et al.*, 2002). Thus, the ethanol high-consuming rats in our study may not be as sensitive to the aversive effects of ethanol as the low-consuming rats, and thereby display a blunted ethanol-induced taurine release. A reduced sensitivity to the aversive effects of ethanol could also be a part of the explanation to why they consume more ethanol. Thus, this argues for a pre-existing low dopaminergic tone and attenuated reactivity to ethanol-induced taurine elevations. However, if the blunted ethanol-induced taurine release displayed in animals consuming high amounts of ethanol is a consequence of excessive ethanol intake or a pre-existing phenomenon is a question we are not able to answer with the results from this study.

Decreased dopamine tone in ethanol high-consuming animals

Interestingly, we found that ethanol intake and extracellular levels of dopamine, as measured by *in vivo* microdialysis in the nAc correlate, where animals with the lowest dopamine tone consumed the largest amount of ethanol (see fig. 1H, I, K, L in paper I). Other studies have also shown that repeated high ethanol exposure is associated with low nAc dopamine levels in experimental animals (Diana *et al.*, 1993; Kashem *et al.*, 2012; Feltmann *et al.*, 2016). The observed correlation between dopamine levels and alcohol intake may imply that a low dopamine tone is a risk factor for drinking whereas a high dopamine tone is protective. This is supported by the fact that high postsynaptic dopaminergic capacity appears to be protective against alcohol consumption and AUD (Thanos *et al.*, 2001; Thanos *et al.*, 2005; Volkow *et al.*, 2006). However, it could also be the other way around, that an excessive alcohol intake reduces dopamine levels, in line with, e.g. Diana and colleagues (Diana *et al.*, 1993). A third possibility is a combination of the two above, i.e. a low dopamine tone promotes alcohol intake that, if excessive, further reduces dopamine levels.

High-consuming animals display a drinking pattern associated with AUD

High and low ethanol-consuming animals displayed a different drinking pattern, where high-consumers started on a high level of alcohol intake and maintained this throughout the study, whereas low-consumers started high but rapidly reduced their alcohol intake (see fig. 1B, C in paper I). The drinking pattern observed in high-consumers, which intermittently consumed large amounts of ethanol, is believed to increase the risk for AUD

(Wolffgramm & Heyne, 1995). The different drinking patterns observed in high- and low-consumers have been suggested to be related to the anxiety level of the animals, as low- and high-consuming animals display high and low anxiety-like behavior, respectively. High-consuming rats also show a higher risk-taking behavior than low-consuming ones (Momeni & Roman, 2014).

We also found that both alcohol intake and endogenous dopamine levels varied significantly between subjects. An individual variance is obviously present in the human population as well, since some individuals appear more resistance and some more sensitive to the development of AUD. Several studies emphasize the need for individual screening of animals, which may lead to a deeper understanding of the relationship between e.g. the dopaminergic status and alcohol intake, as well as of the impact of the velocity and magnitude of the ethanol-induced taurine elevation. It is possible, or maybe even likely that the outcome in terms of AUD is a combination of an underlying spontaneous vulnerability for high/low ethanol consumption and ethanol-induced adaptations (Meinhardt et al., 2015).

In conclusion, the results from this paper suggest that rats consuming high amounts of ethanol respond with a blunted taurine elevation in response to acute ethanol treatment. Further, ethanol intake correlates to extracellular levels of dopamine, where rats with a high ethanol-consumption exhibit a lower accumbal dopamine tone than those consuming low levels of ethanol. Thus, these data support the previously proposed association between low basal dopamine levels and excessive ethanol intake. If and how a blunted ethanol-induced taurine response is associated with a decreased dopamine tone would be of interest to investigate in future studies.

The influence of sub-chronic taurine administration on ethanol-induced behavior

and neurotransmission (paper II)

As mentioned before, taurine is an important endogenous substance for the elevating properties of ethanol (Ericson *et al.*, 2011), and the intake of taurine has escalated over the last decade largely due to increased consumption of taurine-containing energy drinks (Seifert *et al.*, 2011). Whether long-term exposure to large amounts of taurine influences ethanol-induced dopamine neurotransmission is not clear. Thus, to explore if repeated exposure to taurine affects neurochemical and/or behavioral responses to ethanol, rats were daily exposed to large amounts of taurine systemically. The behavior

and accumbal transmission were assessed after ten or fifteen days of treatment.

Systemic administration of taurine increases taurine levels in the CNS

In vitro studies have demonstrated active transport of taurine across the BBB (Benrabh *et al.*, 1995; Tamai *et al.*, 1995), and the blood-CSF-barrier (BCSFB) into the brain (Chung *et al.*, 1994; Chung *et al.*, 1996). In the present study we found that an acute systemic injection of taurine produced elevated levels of taurine in accumbal extracellular fluid (see fig. 3 in paper II). There is a theoretical possibility that a leakage of taurine from the blood into the CNS occurs due to disruption of the BBB caused by the microdialysis probe. Therefore, rats without a microdialysis probe were systemically administrated taurine. This injection significantly increased taurine levels in the blood as well as in the CSF (see fig. 3 in paper II). The ability of taurine to pass from the periphery into the brain is further supported by the additive effect observed following co-administration of ethanol and taurine on nAc taurine levels (see fig. A, B in paper II).

In our study, taurine's ability to enter the brain appears not to be altered by sub-chronic taurine treatment as no changes in endogenous levels of nAc taurine (see fig. 5B) were detected. This is further supported by the fact that we did not find any changes in the mRNA expression of the TauT (see fig. 5A), which indicates that this system is not downregulated by sub-chronic taurine exposure. However, others have performed in vitro studies and shown that the mRNA expression of TauT is downregulated and the taurine uptake into cells reduced following long-term exposure to taurine (Han *et al.*, 1997; Bitoun & Tappaz, 2000; Kang *et al.*, 2002; Voss *et al.*, 2004).

Repeated taurine treatment induces behavioral sensitization but did not influence

ethanol-induced dopamine elevation

Repeated taurine treatment did not influence the ability of taurine to enter the brain and we found it to induce behavioral sensitization as measured by locomotor activity and rearing (see fig. 1 in paper II). Interestingly, daily taurine exposure did not have an impact on these behaviors following acute administration of taurine and ethanol together (see fig. 1 in paper II). This could be a dose-related phenomenon as there are studies demonstrating bi-phasic effects of taurine on ethanol-induced behavior (Olive, 2002; Fontana *et al.*, 2016). It has also been shown that local administration of taurine, which results in higher taurine levels in the brain compared with the systemic injection administrated in paper II, decreases locomotion (Garcia de Yebenes Prous *et al.*, 1978). We show that co-administration of ethanol and taurine

produce an additive increase in accumbal taurine (see fig. 2A, B in paper II). Thus a theoretical possibility is that the co-administration results in such high taurine levels that signaling systems that will counteract locomotion become activated. For example taurine is known to exert agonistic effects at the GABA receptor (Huidobro-Toro *et al.*, 1987; Olive, 2002), a receptor known to be involved in the sedating effects within the CNS (Brohan & Goudra, 2017).

In this paper we also demonstrated that sub-chronic taurine treatment did not have an impact on ethanol-induced dopamine elevation in the nAc (see fig. 6E, F). This could be explained by a tight regulation of brain taurine concentrations during fluctuations in taurine blood concentrations (Lefauconnier *et al.*, 1978), or, as previously suggested, that only a small amount of taurine is needed as a co-factor for ethanol-induced dopamine elevation (Ericson *et al.*, 2011; Soderpalm *et al.*, 2017), making the extra amount of taurine redundant. This latter suggestion is further supported by the finding that no additive effect on ethanol-induced dopamine levels was produced when co-administrated with taurine (see fig. 2C, D in paper II). It could also be that the dose of taurine and/or treatment time was not sufficient to induce adaptations. However, in a recent (unpublished) study performed in our laboratory we found that rats administrated taurine-enriched (2% taurine) drinking water for five weeks were exposed to a higher dose of taurine (1.66 g/kg/day) per day compared to the systemic injections in this paper (II). Nevertheless, no changes in taurine or dopamine levels following acute ethanol treatment were found, as compared to water-drinking control subjects. Administrating even higher concentrations of taurine may include the risk of using physiologically irrelevant concentrations.

The consumption of taurine-containing energy drinks together with ethanol has been shown to be associated with severe consequences, such as increased alcohol intake and harmful behavior (Marczinski, 2011; Seifert *et al.*, 2011; Caviness *et al.*, 2017). Although we did not find any impact of sub-chronic taurine administration on ethanol-induced dopamine increase, exposure to large amounts of taurine during a longer period of time could still have an impact on positive reinforcement since taurine induced behavioral sensitization. A possible mechanism may involve the postsynaptic side of the dopaminergic system, as behavioral sensitization has been linked to enhanced dopamine release and increased postsynaptic dopamine receptor sensitivity (Fung & Lau, 1988; Akimoto *et al.*, 1989; Kazahaya *et al.*, 1989; Molander & Soderpalm, 2003).

We conclude that systemically administrated taurine has the ability to enter the CNS in physiologically relevant concentrations, and that this is not influenced by sub-chronic taurine treatment. Although, sub-chronic taurine exposure does induce behavioral sensitization to the drug, it does not influence the dopamine elevating properties of ethanol. However, further studies are needed to evaluate if frequency, length or dose of taurine exposure will have an impact of ethanol-induced taurine and dopamine elevation in the nAc.

The role of astrocytes in regulating taurine and dopamine interactions during ethanol exposure (paper III)

One of taurine's fundamental actions is to act as an osmoregulator (Huxtable, 1992), where taurine is released from the cell into the extracellular space to counteract cell swelling induced by a decreased osmolarity in the extracellular environment (Solis *et al.*, 1988; Pasantes-Morales *et al.*, 1993; Moran *et al.*, 1994a; Vitarella *et al.*, 1994; Deleuze *et al.*, 1998). As ethanol changes the extracellular milieu and induces cell-swelling (Kimelberg *et al.*, 1993; Allansson *et al.*, 2001; Adermark *et al.*, 2011b), we hypothesized that taurine is released for osmoregulatory purposes following acute ethanol exposure (Ericson *et al.*, 2011). However, the origin of ethanol-induced taurine release is not known. Thus, in order to determine if the rise in accumbal taurine after ethanol exposure mainly derives from neurons or astrocytes and if VRACs are involved, both pharmacological and chemogenetic manipulations of the rat nAc were performed.

Ethanol-induced taurine increase in not dependent on action potentials

As a result of perfusion with TTX in the nAc, we conclude that the rise of accumbal taurine levels following ethanol are independent of action potential mediated neurotransmission (see fig. 2A, C in paper III). This suggests that taurine release in the nAc after ethanol exposure derives from astrocytes, which also has been suggested based on findings in cell cultures (Kimelberg *et al.*, 1993; Aschner *et al.*, 1998). Arguing against this suggestion is the fact that metabolic inhibition of astrocytes by fluorocitrate failed to block the ethanol-induced increase (see fig. 5B, D). However, osmoregulation is an essential feature for maintaining cell volume homeostasis (Huxtable, 1992). Thus, if taurine is released for osmoregulatory purposes to counteract cell swelling induced by ethanol, fluorocitrate administration may not be sufficient to antagonize this basal mechanism, which is essential for cell survival. To further assess the role of nAc astrocytes in ethanol-induced taurine elevation we used astrocyte specific excitatory Gq-DREADDs.

Interestingly, chemogenetic activation of accumbal astrocytes not only potentiated the ethanol-induced taurine increase (see fig. 4B, C), it also potentiated the dopamine release during ethanol exposure (see fig. 4E, F). Additionally, when we used the opposite approach and decreased astrocyte activity by metabolic inhibition we found a reduction in ethanol-induced dopamine levels. Thus, the degree of activation or inhibition of the astrocyte appears to have an impact on dopamine levels following ethanol exposure, which further supports a significant role of this cell-type in ethanol-induced dopamine transmission (Adermark & Lovinger, 2006; Adermark *et al.*, 2011b; Bull *et al.*, 2014).

Ethanol-induced taurine release involves VRACs

VRAC is a swelling-activated channel that regulates cell volume. Upon activation it mediates the efflux of Cl⁻ and organic molecules such as taurine (Hoffmann *et al.*, 2009; Jentsch, 2016; Strange *et al.*, 2019). Increased efflux of taurine via the VRAC occurs within minutes following hypotonic exposure (Lambert *et al.*, 2015). As ethanol changes the extracellular environment and induces swelling of astrocytes (Kimelberg *et al.*, 1993; Allansson *et al.*, 2001; Adermark *et al.*, 2011b), in paper III we aimed to investigate the possibility that taurine may be released via VRACs to counteract ethanol-induced cell swelling. We found that VRAC antagonism reduced nAc taurine during ethanol exposure (see fig. 6B, D), further supporting the hypothesis that taurine is released due to osmoregulation. Although these channels are found both on astrocytes and neurons (Akita & Okada, 2014), the following circumstances argue for VRACs on astrocytes to be involved in ethanol-induced taurine release: astrocytes are the cell type predominantly involved in osmoregulation (Kimelberg *et al.*, 1992), where taurine acts as one of the primary osmolytes, and as mentioned above, ethanol induces cell swelling of astrocytes (Kimelberg *et al.*, 1993; Allansson *et al.*, 2001; Adermark *et al.*, 2011b). However, there is a possibility that other mechanisms than osmoregulation could be involved since increased taurine levels in the amygdala were detected after saline administration together with a conditioned ethanol-associated olfactory stimulus (Quertemont *et al.*, 1998b). However, this remains to be determined in future studies.

Based on the present study we suggest that ethanol-induced accumbal taurine release derives mainly from astrocytes and involves VRACs. We also suggest that increased or decreased activation of astrocytes and VRACs contribute to the regulation of dopamine levels following ethanol administration.

Energy drink constituents (caffeine and taurine) selectively potentiate ethanol-induced locomotion in mice (paper IV)

Not only is the consumption of the combination of energy drinks and alcohol associated with increased risk for binge drinking and development of AUD (Price *et al.*, 2010; Arria *et al.*, 2011), people consuming this mixture also display an impaired judgement and consequently expose themselves and others for risks (Ferreira *et al.*, 2006; O'Brien *et al.*, 2008; Marczinski *et al.*, 2012). If this is a result of a pharmacological interaction is not yet clear. Therefore it is of importance to study the interplay between two of the common energy drink ingredients, caffeine and taurine, on ethanol-induced behavior. Thus in this study three different doses of ethanol, four different doses of caffeine and four different doses of taurine were administered to mice and locomotor activity was assessed. Locomotor activity was used, as this is a straightforward model suitable for screening several doses of a drug. It is also known that moderate doses of ethanol increase locomotor activity in mice, an effect linked to the ability of ethanol to activate the mesolimbic dopamine system (Carlsson *et al.*, 1974; Liljequist *et al.*, 1981; Wise & Bozarth, 1987; Engel *et al.*, 1988).

Caffeine has an additive effect on ethanol-induced locomotion

In this study three different doses of ethanol were tested in the locomotor activity boxes. This was done in order to study the influence of caffeine and taurine on the locomotor stimulatory effect of ethanol, as well as on non-stimulatory ethanol doses. We found the middle dose (2.5 g/kg) of ethanol to have a stimulatory effect on locomotion, which is in line with previous studies (Liljequist, 1991; Blomqvist *et al.*, 1994). We also found caffeine to increase locomotion (see fig. 1C, D in paper IV), which could be linked to its ergogenic and hypothermic effects, as this has been discussed to be associated to changes in dopamine transmission (Quarta *et al.*, 2004; De Luca *et al.*, 2007; Zheng *et al.*, 2014). Interestingly, we showed that caffeine in specific doses potentiated the ethanol-induced locomotor activity (see fig. 2B-D in paper IV). The mechanisms underlying this interaction are not clear but it has been suggested that caffeine enhances locomotion by increasing dopaminergic neurotransmission in the nAc by antagonism of the adenosine receptors (El Yacoubi *et al.*, 2000; Karcz-Kubicha *et al.*, 2003; Quarta *et al.*, 2004; Kuzmin *et al.*, 2006), and that ethanol, besides increasing dopamine signaling in the nAc may enhance locomotion through interactions in the adenosinergic system (Nagy *et al.*, 1990; Krauss *et al.*, 1993; Arolfo *et al.*, 2004; Lopez-Cruz *et al.*, 2013; Nam *et al.*, 2013). Thus, this interaction may

be related to the drugs' combined effects on the mesolimbic dopamine system by modulating adenosine-signaling.

Taurine when administrated together with caffeine has a pronounced impact on ethanol-induced locomotion in naïve and caffeine experienced mice

Even though no influence of taurine could be seen when administered alone (see fig. 1E, F in paper IV), or in combination with ethanol (see fig. 3 in paper IV), we demonstrated that the combination of caffeine and taurine, at a specific dose combination, potentiated ethanol-induced locomotor activity compared to either drug alone (see fig. 4C in paper IV). As a large proportion of the human population has a continuous intake of caffeine, due to coffee consumption on a regular basis, it is of interest to explore the influence of caffeine and taurine on ethanol-induced locomotion in caffeine experienced animals. We found caffeine to induce behavioral sensitization to the drug (see fig. 5B in paper IV). Interestingly, the additive effect of the combination of caffeine and taurine on ethanol-induced locomotion was still present in these caffeine-sensitized animals (see fig. 5D in paper IV). Thus, even though not having an influence alone, taurine together with caffeine has a pronounced impact on ethanol-induced behavior. An interaction of caffeine and taurine on ethanol-induced behavior is further supported by a study where oral "energy drink" (containing caffeine and taurine) administration decreased the depressant effects of ethanol on locomotor activity and by that a modest ethanol-induced behavioral sensitization was increased by oral administration of this "energy drink" (Ferreira *et al.*, 2004; Ferreira *et al.*, 2013). Future studies are needed to elucidate the mechanisms underlying this observed potentiation, where joint targets may consist of $GABA_A$, glycine and NMDA receptors as well as calcium signaling, as all these are affected by both caffeine and taurine (Nehlig *et al.*, 1992; Schaffer *et al.*, 2000; Olive, 2002; Quarta *et al.*, 2004; Chan *et al.*, 2014).

Taken together we suggest that the combination of caffeine and taurine, at a specific combination of doses, increases ethanol-induced locomotion, a phenomenon previously linked to the reinforcing properties of the drug.

GENERAL DISCUSSION

Only a few pharmacotherapies are available for AUD with limited efficacy and with varying effects in patients, likely due to the complexity and heterogeneity of the disease (Heilig & Egli, 2006). Considering the severe consequences associated with excessive alcohol use and the enormous costs for the society there is a significant need for improved therapies. To enable development of this, a deeper understanding and knowledge about the processes involved in excessive alcohol intake and the development of addiction are needed. Changes in the mesolimbic dopamine system are thought to be a predominant underlying factor in excessive alcohol intake and AUD (Volkow *et al.*, 1996; Volkow *et al.*, 2007), and resent research has suggested taurine to play a central role in the dopamine elevating properties of ethanol. Thus the purpose of this thesis was to investigate correlations between taurine and dopamine during ethanol exposure, with special emphasis on neurochemical effects in the nAc.

In this thesis we show that ethanol increases extracellular levels of both taurine and dopamine in the nAc as measured by *in vivo* microdialysis. This occurred both in naïve (paper I, II, III) as well as in ethanol (paper I) or taurine (paper II) experienced animals, and when ethanol was systemically administrated by an i.p. injection (paper I, II, III) or by local perfusion via the microdialysis probe into the nAc (paper III) (table 2). In our studies ethanol, when systemically or locally administrated, increases accumbal taurine levels by approximately 40-60% (calculated as % of pre-drug baseline) in naïve animals, with some individual variance as well as variance between batches of animals. This is in line with the observed increase of taurine reported in some studies (Dahchour *et al.*, 1996; Smith *et al.*, 2004), but lower than what was reported in other studies (Dahchour *et al.*, 1994; Quertemont *et al.*, 2003). In our hands, systemic administration of ethanol increases dopamine levels by approximately 40-60%, whereas local perfusion of ethanol in the nAc increases dopamine levels by approximately 20-40%. Both the ethanol-induced taurine and dopamine elevation peaks at about the same time point after administration; at twenty minutes after an i.p. injection of ethanol, whereas the peaks tend to appear slightly later (about 20-40 minutes after the perfusion starts) during local perfusion of the drug. Thus the presented data in this thesis indicate that ethanol-induced taurine and dopamine elevations in the nAc are robust phenomena that occur simultaneously, despite different routes of administration and previous ethanol or taurine experience (table 2).

Table 2. Summary of the effects of different treatments on ethanol-induced taurine and dopamine levels as compared to baseline, and how the treatments relate to the effects produced by ethanol in naïve (untreated) rats. All measurements were performed on samples obtained from the nucleus accumbens by means of in vivo microdialysis. More detailed results can be found in papers I, II and III. TTX=tetrodotoxin, Gq-DREADDs=excitatory designer receptor exclusively activated by designer drugs, VRAC=volume regulation anion channel, n.s.=non-significant.

Treatment	TAURINE		DOPAMINE	
	Treatment +ethanol vs. baseline	Treatment +ethanol vs. Untreated +ethanol	Treatment +ethanol vs. baseline	Treatment +ethanol vs. Untreated +ethanol
Acute taurine treatment	↑	↑	↑	n.s.
Repeated taurine treatment	↑	n.s.	↑	n.s.
Repeated ethanol exposure	↑	n.s.	↑	n.s.
Action potential inhibition (TTX)	↑	↑	↓	↓
Astrocyte activation (Gq-DREADDs)	↑	↑	↑	↑
Astrocyte inhibition (Fluorocitrate)	↑	↑	↑	↓
VRAC inhibition (DCPIB)	↑	↓	n.s.	↓

We and others have suggested that taurine and dopamine are closely related with regards to ethanol's actions within the mesolimbic dopamine system (Olive, 2002; Ericson *et al.*, 2011; Soderpalm *et al.*, 2017). This is further supported by data presented in this thesis, since following pharmacological or chemogenetic manipulations, the direction of changes (increase or decrease) in ethanol-induced dopamine increase appears to follow the direction of changes in ethanol-induced taurine increase. As in paper III, the ethanol-induced dopamine and taurine levels were either both increased or decreased following treatment with CNO and ethanol in Gq-DREADDs transfected animals or with DCPIB and ethanol in naïve animals, respectively. Also, both the taurine and dopamine increase after acute ethanol remains unaffected by intermittent ethanol consumption (paper I) or sub-chronic taurine treatment (paper II) as compared to naïve animals. However in paper III, treatment with

fluorocitrate increased the taurine levels but decreased dopamine levels after ethanol treatment, which would argue against the importance of endogenous taurine as an essential component for ethanol-induced dopamine levels in the nAc (table 2).

That taurine is involved in the rewarding sensation of ethanol is however further supported by a study from Li et al, showing that accumbal taurine levels increased during the 15 first minutes during operant ethanol self-administration, and that the degree of taurine increase was positively correlated with the amount of ethanol consumed. Interestingly, this study also suggested that the two glycine receptor ligands, taurine and glycine, play different roles in the rewarding actions of ethanol. The greatest increase of glycine levels in the nAc was observed with the longest time-out period during operant ethanol self-administration, suggesting that glycine is involved in the anticipation of ethanol reward and taurine appears to be more involved in the reward itself (Li *et al.*, 2008).

The mechanism underlying the increase of taurine release following ethanol administration is not fully understood. In paper III, we hypothesized that the ethanol-induced taurine increase in the accumbal extracellular space is primarily due to osmoregulation and that taurine may be released via VRACs located on astrocytes. We found that antagonism of VRACs reduced ethanol-induced taurine levels and that taurine release following ethanol is independent of action potential firing, further supporting this hypothesis. Others have suggested that ethanol-induced taurine release may be due to neuronal depolarization (Smith *et al.*, 2004), which is further supported by the finding that taurine can be found in synaptic vesicles (Huxtable, 1992), and that depolarization of neurons result in taurine release (Kaczmarek & Davison, 1972; Collins & Topiwala, 1974; Shain & Martin, 1990). Furthermore, there appears to be regional selectivity with regards to ethanol's taurine enhancing effects as the ethanol-induced taurine increase was lower in the dorsal striatum compared to the nAc (Smith *et al.*, 2004). This is further supported by the finding that ethanol diluted in a hypertonic saline solution does not prevent ethanol from increasing taurine in the dorsal striatum (Ulenius *et al.*, 2017) in contrast to what was shown in the nAc (Ericson *et al.*, 2011). This would also argue against osmoregulation as the sole mechanism of action underlying ethanol's ability to increase taurine. Nevertheless, there is also a possibility that the ethanol-induced taurine elevation in the nAc derives from an increased biosynthesis. This is however not likely since the taurine turnover in the brain is slow, about 5 days (Collins, 1974; Spaeth & Schneider, 1974; Wright *et al.*, 1986). Another possibility would be that ethanol negatively interferes with the taurine

transporter, thereby preventing taurine uptake into e.g. astrocytes. However, this appears unlikely as ethanol did not interfere with the transport of taurine into the brain after systemic administration of taurine (paper II).

Although, based on the studies presented in this thesis (paper II and paper III), we suggest that ethanol-induced taurine release in the nAc mainly derives from central sources, more specifically astrocytes, we cannot totally discard a contribution of taurine from the periphery. Taurine has the ability to cross both the BBB (Benrabh *et al.*, 1995; Tamai *et al.*, 1995) and BCSFB (Chung *et al.*, 1994; Chung *et al.*, 1996) and in vitro studies have shown that short-term administration of ethanol impairs the BCSFB (Nixon *et al.*, 2008) and affect cytoskeletal and tight junctions proteins, which leads to an impairment of the BBB (Haorah *et al.*, 2005). On the other hand, Stewart and colleagues show that acute ethanol administration does not affect the permeability of horseradish peroxidase from the BBB (Stewart *et al.*, 1988). Thus, circulating taurine from the periphery could constitute a part of the observed taurine increase in the nAc following ethanol treatment, but further studies are needed to determine this. Despite origin and mechanism of action, it is likely that taurine has a neuronal impact. Taurine increases chloride conductance to hyperpolarize membranes, probably via activation of GlyR and GABAR (Okamoto *et al.*, 1983b; Ye *et al.*, 1997). As GlyRs are expressed in the nAc (Martin & Siggins, 2002; Molander & Soderpalm, 2005b; a; Forstera *et al.*, 2017), it is likely that taurine, via GlyR in the nAc, influence ethanol-induced dopamine release via the proposed nAc-VTA-nAc circuitry (Soderpalm *et al.*, 2009). And given the modulatory role of astrocytes in neuronal function (Walz, 2000; Akita & Okada, 2014; Adermark & Bowers, 2016) it is reasonable to think, as proposed in paper III, that these cells indirectly (via taurine) influence ethanol-induced dopamine levels.

Increased extracellular levels of taurine following ethanol exposure have been found in other regions of the brain than in the nAc, such as amygdala (Quertemont *et al.*, 1998a; Quertemont *et al.*, 1999), hippocampus (Dahchour & De Witte, 1999) and dorsal striatum (Smith *et al.*, 2004; Ulenius *et al.*, 2017). However, the mechanism by which this occurs appears to differ as administration of ethanol in a hypertonic saline solution did not prevent ethanol from increasing taurine or dopamine levels in the dorsal striatum (Ulenius *et al.*, 2017), whereas it did in the nAc (Ericson *et al.*, 2011). In the amygdala, taurine has been proposed to derive mainly from neurons (Quertemont *et al.*, 1999), since taurine has been detected in most of the neurons but only in a small portion of the astrocytes of the amygdala (Ottersen *et al.*, 1986), whilst we in paper III suggest that ethanol-induced

taurine release in the nAc mainly derives from astrcoytes. Thus there may be regional differences regarding both the origin of ethanol-induced taurine release as well as the underlying mechanisms of action.

The regulation of total body content of taurine seems to be rather rigorous as the rate of biosynthesized taurine is constant, and when dietary taurine is increased the half-life of elimination of biosynthetically derived taurine is decreased (Huxtable & Lippincott, 1982). This was demonstrated in cats fed a taurine-rich diet, which within days upregulated their secretion of taurine via the urine (Glass *et al.*, 1992). In line with these findings we did not find sub-chronic taurine treatment to affect the endogenous levels of extracellular taurine in the nAc (paper II). However, only a prolonged increase of taurine intake or depletion appears to cause changes in plasma taurine concentration, and a correlation between whole blood taurine and plasma taurine levels have been suggested to be found only following extremes of taurine depletion or taurine supplementation (Trautwein & Hayes, 1995). In line with this, it has been shown that vegans display lower plasma taurine concentration as well as decreased excretion of taurine via the urine (Rana & Sanders, 1986; Laidlaw *et al.*, 1988). In paper II, we found that sub-chronic taurine treatment did not influence extracellular levels of taurine in the nAc. In this study we did not measure taurine plasma concentrations after sub-chronic taurine treatment, raising the possibility of changed plasma concentrations. However, even if the taurine plasma concentration was changed it is likely that this would not effect accumbal extracellular taurine levels, since it has been shown that brain taurine concentrations are kept constant during changes in plasma taurine concentration (Lefauconnier *et al.*, 1978). Although patients with AUD display decreased levels of taurine in the blood, as compared to nonalcoholic individuals (Majumdar *et al.*, 1983), we found that intermittent voluntary ethanol consumption did not influence the endogenous levels of taurine in serum, or in the CSF or in the extracellular fluid of the nAc (paper I). This could mean that the time-period for ethanol consumption in paper I is too short in order to induce changes in endogenous taurine levels. Another alternative is that the decreased taurine levels found in AUD-patients are due to an overall malnutrition.

Finally, the question arises whether acute or repeated intake of large amounts of taurine is harmful with regards to excessive alcohol consumption and the development of AUD. Based on the studies presented in this thesis taurine could, probably together with caffeine, play a role in the reported increased alcohol consumption in humans drinking alcohol mixed with energy drinks as we found that the combination of caffeine and taurine had an additive influence on ethanol-induced locomotion (paper IV). We also found that sub-

chronic taurine treatment did not influence the dopamine elevating properties of ethanol, but did induce behavioral sensitization to taurine (paper II). The former speaking against and the latter arguing for the ability of taurine to influence positive reinforcement. Putting these findings together with previous studies (see "Interactions between taurine and ethanol in the CNS" in the introduction of this thesis) it appears that the effects of taurine on ethanol-induced dopamine and dopamine-related behavioral effects are largely dependent on the dose of the drugs as well as time of treatment period. Thus we cannot conclude that acute or repeated intake of large amounts of taurine is innoxious with regards to excessive alcohol consumption, and further studies are needed to determine this. With respect to taurine's actions in the periphery (Huxtable, 1992), excessive intake of large amounts of taurine may produce adverse effects on peripheral organs, such as the heart. For example, the intake of energy drinks is associated with adverse cardiovascular effects, particularly on blood pressure and heart rate. These effects are thought to be mediated by caffeine and a possible combination of caffeine and taurine (Grasser *et al.*, 2016). The detailed peripheral actions of taurine are however a discussion beyond this thesis.

The anti-craving substance and homotaurine analogue acamprosate has been shown to reduce ethanol-intake in laboratory animals (Boismare *et al.*, 1984; Czachowski *et al.*, 2001; Olive *et al.*, 2002; Cowen *et al.*, 2005; Gupta *et al.*, 2008; Chau *et al.*, 2010a; Lido *et al.*, 2012), an effect suggested to be related to the drug's dopamine elevating properties (Olive *et al.*, 2002; Cowen *et al.*, 2005; Chau *et al.*, 2018). Similar to both taurine (Ericson *et al.*, 2006; Ericson *et al.*, 2011) and ethanol (Blomqvist *et al.*, 1993; Blomqvist *et al.*, 1997; Ericson *et al.*, 2003; Molander & Soderpalm, 2005a), the dopamine action of acamprosate has been shown to involve GlyR in the nAc and nAChR in the VTA (Chau *et al.*, 2010b). Interestingly, taurine modestly reduces ethanol consumption in rats (Olive, 2002). Thus, theoretically there is a possibility that taurine could be used as treatment for patients suffering from AUD.

In conclusion, based on the results presented in this thesis we suggest that increased accumbal taurine levels following ethanol exposure is a robust phenomenon, mainly derives from astrocytes and involves VRACs. Furthermore, we suggest that sub-chronic, systemic administration of taurine may have long-lasting effects on the brain, in the present thesis displayed as behavioral sensitization, but that ethanol-induced dopamine release is not affected. We also conclude that a low dopamine tone in nAc is associated with excessive ethanol intake and that the active state of the astrocytes appears to influences dopamine levels following acute ethanol exposure.

Finally, we suggest that taurine could, probably together with caffeine, have a role in the reported increased alcohol consumption in humans drinking alcohol mixed with energy drinks.

FUTURE PERSPECTIVES

Based on studies presented in this thesis we can conclude that ethanol-induced taurine release in the nAc is a robust phenomenon. Studying the underlying mechanisms behind this phenomenon is important as it may contribute to furthering the knowledge about how ethanol increases accumbal dopamine levels. Although we suggest that taurine release following ethanol exposure mainly derives from astrocytes, involves VRACs and occurs due to osmoregulation, it needs to be further evaluated. Except for the VRAC inhibitor DCPIB, used in this thesis, there are a number of anion channel inhibitors, such as DIDS, SITS, NPPB and carbenoxolone, that can be used for inhibiting VRACs (Friard *et al.*, 2017). Additionally, to further evaluate the role of VRACs whole cell patch clamp electrophysiology recordings can be used for measuring VRAC-mediated chloride currents (Strange *et al.*, 2019) following ethanol exposure. To outline the involvement of astrocytes in ethanol-induced taurine release it would be beneficial to chemogenetically inhibit astrocytes by astrocyte-specific inhibitory DREADDs and, as was done in paper III, monitor taurine and dopamine levels by *in vivo* microdialysis.

There are studies showing increased taurine levels after i.p. injections of ethanol in doses that animals will self-administer (Smith *et al.*, 2004), and during operant ethanol self-administration (Li *et al.*, 2008), but to my knowledge there are no studies investigating accumbal taurine and dopamine levels during oral voluntary ethanol consumption. This is an important aspect as alcohol consumption in humans occurs both voluntary and orally and should be investigated in future studies.

As high ethanol-consuming animals display a lower tone of dopamine in the nAc as well as a blunted ethanol-induced taurine release (paper I), it would be beneficial to determine if this hypo-dopaminergic state is a preexisting phenomenon or if it develops over time as a consequence of excessive alcohol consumption. Theoretically this may be investigated by using guide cannulas for insertion of a microdialysis probe, enabling measurement of dopamine before and after excessive ethanol intake. Regardless of whether it is a preexisting phenomenon or not, a dopamine stabilizer, which has the ability to inhibit or stimulate dopamine-related behaviors depending on the prevailing dopaminergic tone (Sonesson *et al.*, 1994; Rung *et al.*, 2008), would constitute a beneficial pharmacotherapy (Feltmann *et al.*, 2016). Thus, regardless of a low or high dopaminergic tone and how it changes over time during prolonged ethanol intake, a dopamine stabilizer would normalize the

dopaminergic tone and consequently reduce ethanol intake. A dopamine stabilizer has been demonstrated to counteract downregulated accumbal dopamine output in long-term ethanol consuming rats (Feltmann *et al.*, 2016), and should be further evaluated for being used for attenuating alcohol intake in AUD-patients.

As increased locomotor activity induced by ethanol is linked to activation of the mesolimbic dopamine system (Carlsson *et al.*, 1974; Liljequist *et al.*, 1981; Wise & Bozarth, 1987; Engel *et al.*, 1988), it is commonly used for studying drug-induced behavioral effects related to the brain reward system. Since we found the combination of caffeine and taurine to have an additive impact on ethanol-induced locomotion, it may imply that this combination gives rise to a more intense activation of the reward system and consequently a lager positive reinforcing effects than ethanol alone. This could potentially be one of the reasons to why humans drinking alcohol mixed with energy drinks display an increased alcohol intake. Thus, the next step would be to determine if the drug-combination will have an additive impact on nAc dopamine levels following ethanol exposure. To this end, naïve rats as well as ethanol-experienced rats will be administered taurine, caffeine and ethanol, subsequently taurine and dopamine levels will be monitored by means of *in vivo* microdialysis. As the rate of dopamine elevation has been correlated with the subjective feeling of pleasure (Volkow *et al.*, 1999; 2003; Koob & Volkow, 2016), sampling every ten minutes instead of the regular twenty-minute sampling protocol would reveal not only the amount of drug-induced taurine and dopamine levels but also the rate of increase. Commonly, as well as in this thesis, *in vivo* microdialysis is coupled with HPLC to analyze the extracellular levels of taurine and other amino acids. However, one should consider using capillary electrophoresis coupled with laser-induced fluorescence detection (CE-LIF) instead of HPLC, which will provide a higher sensitivity and temporal resolution enabling detection of subtle changes of dopamine and amino acids such as taurine (Lada & Kennedy, 1996; Bowser & Kennedy, 2001; Bert *et al.*, 2002; Smith *et al.*, 2004). Finally, investigating whether caffeine and/or taurine influence the behavioral effects and subjective experience of ethanol in human laboratory studies is important considering the consumption by the young population.

Except taurine's interaction with ethanol and its potential involvement in AUD, a number of other interesting actions of the amino acid within the CNS have been demonstrated, such as anxiolytic and antidepressant effects, where it has been suggested to interact with dopamine and GlyR (Wu *et al.*, 2017; Jung & Kim, 2019). The potential anxiolytic effect of taurine has been demonstrated in animal models (Chen *et al.*, 2004; Kong *et al.*, 2006; Jung &

Kim, 2019), however the mechanism of action is not fully understood. Jung and co-workers (2019) have shown that taurine does not exert its anxiolytic effect through the GABA$_A$R or by influencing the GABA synthesis. They suggest that taurine reduces anxiety via strychnine-sensitive GlyR (Jung & Kim, 2019), which is further supported by another laboratory (Komatsu *et al.*, 2015). Interestingly, Jung et al also propose that taurine-induced anxiolytic behavior may be mediated by dopamine regulation as taurine has been shown to increase dopamine (Ericson *et al.*, 2003). One of the hypothesis of the pathogenesis of depression is the monoaminergic hypothesis, which state that depression is probably due to a deficiency in monoamine neurotransmitters including 5-HT, dopamine and noradrenaline (Lopez-Munoz & Alamo, 2009). Subjects in a rat model of depression display lower levels of these neurotransmitters, changes that were prevented by administration of taurine (Wu *et al.*, 2017). It was suggest that this occurs by a direct regulation of the neurotransmitters by taurine, or by an indirect action of taurine via regulation of the hypothalamic-pituitary-adrenal axis (Wu *et al.*, 2017). Thus, the interaction of taurine and dopamine within the CNS may not be solely interesting with regards to ethanol. Their influence on and interaction with other psychiatric disorders would be interesting to include in future studies.

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