Neurophysiological signatures of alcohol use disorder–like behavior in male rats

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Cover illustration: biocytin-filled prelimbic cortex neurons, recorded by Davide Cadeddu; histological processing and visualization by Lindsay Zentveld.

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“If the human brain were so simple that we could understand it,

we would be so simple that we couldn’t.”

— Emerson M. Pugh

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ABSTRACT

Alcohol use disorder (AUD) is a chronic and relapsing disorder characterized by loss of control over drinking despite negative consequences. Although alcohol use is widespread, only a subset of individuals develops maladaptive drinking patterns, transitioning from recreational to compulsive use and, in some cases, developing AUD. In this context, nicotine use is particularly common among individuals who drink alcohol and has been associated with heavier drinking and higher AUD risk in humans, while repeated nicotine exposure increases alcohol intake in animal models. However, the underlying neurophysiological mechanisms remain incompletely understood. The overall aim of this thesis was to investigate neurophysiological signatures associated with AUD-like vulnerability and to examine how repeated nicotine exposure modulates alcohol-related behaviors and synaptic transmission in male rats. To this end, models of voluntary alcohol intake and operant self-administration, followed by AUD-like behavioral assessments, were combined with patch-clamp, field potential recordings and *in vivo* microdialysis. The results showed that only a subset of rats developed an AUD-like vulnerable phenotype and that this was associated with increased neuronal excitability and altered mGluR2/3-mediated signaling in the prelimbic cortex (PL), as well as with a hypoactive profile in the basolateral amygdala (BLA). In addition, repeated nicotine exposure increased voluntary alcohol intake and reduced resilience to AUD-like behavior, which was associated with specific neurophysiological alterations in the PL. Prolonged nicotine exposure was also associated with altered dopamine (DA) signaling between the ventral tegmental area (VTA) and medial prefrontal cortex (mPFC), increased extracellular DA and glutamate levels in the mPFC and altered glutamate/glutamine regulation.

Acute alcohol and nicotine each reduced PL synaptic transmission through mechanisms involving nicotinic acetylcholine receptors (nAChRs) and D2/D3 receptors. In separate experiments, repeated nicotine exposure enhanced PL sensitivity to acute alcohol and nicotine. In conclusion, this thesis supports the presence of distinct neurophysiological signatures in the BLA and PL associated with AUD-like vulnerability and suggests that repeated nicotine exposure promotes alcohol intake and reduces resilience to AUD-like behavior through mechanisms that may involve neuroadaptations in the mPFC. These findings may therefore contribute to a better understanding of the neurobiological mechanisms underlying AUD and, ultimately, support the development of more targeted pharmacological treatment strategies.

Keywords: alcohol use disorder, alcohol, nicotine, electrophysiology, operant self-administration

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SAMMANFATTNING PÅ SVENSKA

Neurofysiologiska egenskaper associerade med alkoholbrukssyndromliknande beteende hos hanråttor

Alkoholbrukssyndrom (AUD) är ett kroniskt och återfallande tillstånd som kännetecknas av förlorad kontroll över drickande trots negativa konsekvenser. Även om alkoholanvändning är utbredd utvecklar endast ett fåtal individer maladaptiva dryckesmönster, där man går från rekreativ konsumtion till ett mer tvångsmässigt bruk och, i vissa fall, AUD. Nikotinanvändning är särskilt vanlig bland individer som dricker alkohol och har associerats med högre alkoholkonsumtion och högre risk för AUD, ett samband som även uppmärksammats i djurmodeller. De bakomliggande neurofysiologiska mekanismerna är dock fortfarande ofullständigt kartlagda. Det övergripande syftet med denna avhandling var att identifiera neurofysiologiska egenskaper kopplade till AUD-liknande sårbarhet i hanråttor, och utvärdera underliggande mekanismer som kan förklara sambandet mellan nikotinexponering och riskfyllt alkoholbruk. För detta ändamål kombinerades beteendemodeller, där alkoholintag och AUD-liknande beteendebedömningar studerades följt av neurofysiologiska analyser, med fokus på elektrofysiologiska mätningar i hjärnslicer och *in vivo*-mikrodialys i hjärnregioner som tidigare kopplats till sårbarhet för AUD. Resultaten visade att endast en del av råttorna utvecklade en AUD-liknande sårbar fenotyp och att detta var associerat med ökad neuronal excitabilitet och förändrad mGluR2/3-medierad signalering i prelimbiska cortex (PL), samt med en hypoaktiv profil i basolaterala amygdala (BLA). Upprepad nikotinexponering ökade frivilligt alkoholintag och minskade motståndskraften mot AUD-liknande beteende, vilket var associerat med specifika neurofysiologiska förändringar i PL. Långvarig nikotinexponering var även associerad med förändrad dopamin (DA)-signalering mellan ventrala tegmentområdet (VTA) och mediala prefrontala cortex (mPFC), ökade extracellulära nivåer av DA och glutamat i mPFC och förändrad glutamat/glutaminreglering. Vidare minskade akut alkohol och nikotin synaptisk transmission i PL, en effekt som förstärktes av upprepad nikotinexponering och förhindrades av blockad av nikotinerga acetylkolinreceptorer (nAChRs) eller D2/D3-receptorer. Sammanfattningsvis stödjer denna avhandling förekomsten av distinkta neurofysiologiska mönster i BLA och PL associerade med AUD-liknande sårbarhet och antyder att upprepad nikotinexponering främjar alkoholintag och minskar motståndskraften mot AUD-liknande beteende genom mekanismer som kan involvera neuroadaptationer i mPFC. Dessa fynd kan därför bidra till en bättre förståelse av de neurobiologiska mekanismer som ligger bakom AUD och, i förlängningen, stödja utvecklingen av mer riktade farmakologiska behandlingsstrategier.

LIST OF INCLUDED PAPERS

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

I. Cadeddu, D., Lucente, E., Ericson, M., Söderpalm, B., Adermark, L., & Domi, A. (2025). Impaired Synaptic Activity in the Basolateral Amygdala Is Associated With an Alcohol Use Disorder-Like Vulnerable Phenotype in Male Rats. *Addiction biology*, *30*(10), e70091. https://doi.org/10.1111/adb.70091

II. Domi, A., Cadeddu, D., Lucente, E., Gobbo, F., Edvardsson, C., Petrella, M., Jerlhag, E., Ericson, M., Söderpalm, B., & Adermark, L. (2024). Pre- and postsynaptic signatures in the prelimbic cortex associated with "alcohol use disorder" in the rat. *Neuropsychopharmacology*, *49*(12), 1851–1860. https://doi.org/10.1038/s41386-024-01887-2

III. Adermark, L., Lucente, E., Cadeddu, D., Stomberg, R., Ericson, M., & Söderpalm, B. Repeated nicotine exposure promotes alcohol intake and increases the sensitivity to ethanol's neuromodulatory effects in the prelimbic cortex of male rats. *Manuscript*.

IV. Cadeddu, D., Beretta, E., Söderpalm, B., Ericson, M., Lucente, E., & Adermark, L. Neurophysiological signatures underlying reduced resilience to alcohol use disorder-like behavior after nicotine exposure. *Manuscript*.

LIST OF ADDITIONAL PUBLICATIONS

The following list includes additional publications not included in the thesis.

I. Cadeddu, D., Loftén, A., Ademar, K., Söderpalm, B., Adermark, L., & Ericson, M. (2025). The involvement of GABA-rho receptors in regulating ethanol-induced elevation of dopamine, glycine and taurine within the nucleus accumbens of Wistar rats. *Frontiers in pharmacology*, *16*, 1668669. https://doi.org/10.3389/fphar.2025.1668669

II. Cadeddu, D., Lucente, E., Ericson, M., & Adermark, L. (2025). Amphetamine produces sex-and subregion-selective neuroplasticity in the amygdala of Wistar rats. *European Journal of Pharmacology*, 178302. https://doi.org/10.1016/j.ejphar.2025.178302

III. Loftén, A., Cadeddu, D., Danielsson, K., Stomberg, R., Adermark, L., Söderpalm, B., & Ericson, M. (2025). Reduced Alcohol Consumption Following Ablation of Cholinergic Interneurons in the Nucleus Accumbens of Wistar Rats. *Addiction biology*, *30*(2), e70022. https://doi.org/10.1111/adb.70022

IV. Domi, A., Lucente, E., Cadeddu, D., & Adermark, L. (2023). Nicotine but not saline self-administering or yoked control conditions produces sustained neuroadaptations in the accumbens shell. *Frontiers in molecular neuroscience*, *16*, 1105388. https://doi.org/10.3389/fnmol.2023.1105388

V. Adermark, L., Cadeddu, D., Lucente, E., Danielsson, K., Söderpalm, B., & Ericson, M. (2025). Morphine self-administration decreases intrinsic excitability of accumbal medium spiny neurons and suppresses the innate immune system in male Wistar rats. *Neurochemistry international*, *186*, 105965. https://doi.org/10.1016/j.neuint.2025.105965

VI. Domi, A., Lucente, E., Cadeddu, D., Bengtsson, N., Smedler, E., & Adermark, L. (2026). Psilocin mediates long-term synaptic depression in the prelimbic cortex through 5-HT_{2A} receptor-independent mechanisms. *Neuropharmacology*, *288*, 110854. https://doi.org/10.1016/j.neuropharm.2026.110854

VII. Edvardsson, C. E., Cadeddu, D., Ericson, M., Adermark, L., & Jerlhag, E. (2025). An inhibitory GLP-1 circuit in the lateral septum modulates reward processing and alcohol intake in rodents. *EBioMedicine*, *115*, 105684. https://doi.org/10.1016/j.ebiom.2025.105684

VIII. Olsson, Y., Lidö, H., Ademar, K., Cadeddu, D., Ericson, M., & Söderpalm, B. (2024). The GlyT1-inhibitor Org 24598 facilitates the alcohol deprivation abolishing and dopamine elevating effects of bupropion + varenicline in rats. *Journal of neural transmission (Vienna, Austria: 1996)*, *131*(1), 95–106. https://doi.org/10.1007/s00702-023-02701-x

IX. Lucente, E., Cadeddu, D., Edvardsson, C. E., Ericson, M., Jerlhag, E., & Adermark, L. (2024). Subregion specific monoaminergic signaling in the female rat striatum during nicotine abstinence. *Addiction Neuroscience*, *13*, 100184. https://doi.org/10.1016/j.addicn.2024.100184

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ABBREVIATIONS

aCSF — artificial cerebrospinal fluid

AMPA — α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA — analysis of variance

AP — action potential

APV — 2-amino-5-phosphonovaleric acid

AS — addiction score

AUD — alcohol use disorder

BLA — basolateral amygdala

CIN — cholinergic interneuron

CNQX — 6-cyano-7-nitroquinoxaline-2,3-dione

DA — dopamine

D2/D3 — dopamine D2 and D3 receptors

DSM-IV — Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition

DSM-5-TR — Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision

EPM — elevated plus maze

EtOH — ethanol

fEPSP — field excitatory postsynaptic potential

FR — fixed ratio

GABA — gamma-aminobutyric acid

$GABA_A$ — gamma-aminobutyric acid type A receptor

GlyR — glycine receptor

HCN — hyperpolarization-activated cyclic nucleotide-gated channel

HPLC — high-performance liquid chromatography

IC — current clamp

IL — infralimbic cortex

I/O — input–output

LMA — locomotor activity

mGluR2/3 — metabotropic glutamate receptor 2/3

mPFC — medial prefrontal cortex

MSN — medium spiny neuron

nAChR — nicotinic acetylcholine receptor

NAc — nucleus accumbens

NAcC — nucleus accumbens core

NAcSh — nucleus accumbens shell

NMDA — N-methyl-D-aspartate

PCA — principal component analysis

PFC — prefrontal cortex

PL — prelimbic cortex

PPR — paired-pulse ratio

PR — progressive ratio

SEM — standard error of the mean

sEPSC — spontaneous excitatory postsynaptic current

sIPSC — spontaneous inhibitory postsynaptic current

VC — voltage clamp

VTA — ventral tegmental area

1 INTRODUCTION

1.1 ALCOHOL

Alcohol, also referred to as ethanol (EtOH), is one of the most widely consumed psychoactive substances worldwide (World Health Organization, 2024). It has a long history of use across social, cultural and religious contexts (Sudhinaraset et al., 2016) and is found in alcoholic beverages such as wine, beer and spirits. Although most people consume alcohol for recreational purposes, some individuals develop patterns of use that become harmful, persistent and difficult to control (American Psychiatric Association, 2022). Indeed, alcohol use represents a major global public health, social and economic burden, affecting individuals, families, healthcare systems and society at large (World Health Organization, 2024). Chronic alcohol consumption contributes to the development of numerous conditions, including somatic conditions such as liver disease, pancreatitis, hypertension, cardiovascular disease, stroke and several forms of cancer (Shield et al., 2014) as well as psychiatric conditions such as depression, anxiety and other substance use disorders (Schuckit, 1996). In addition to its long-term health effects, alcohol increases the risk of both unintentional and intentional injuries by impairing judgment, coordination and behavioral control. As a result, alcohol contributes to road traffic injuries, self-harm, interpersonal violence, intimate partner violence and suicide (Chikritzhs & Livingston, 2021).

Globally, total alcohol consumption in 2019 was estimated at 5.5 liters of pure alcohol per person aged 15 years or older. In the same year, alcohol use was attributable to approximately 2.6 million deaths worldwide, including around 2 million deaths among men and 600,000 among women (World Health Organization, 2024). In Sweden, total alcohol consumption in 2024 was estimated at 8.4 liters of pure alcohol per inhabitant aged 15 years or older (Trolldal & Åkerblad, 2025). In the same year, 16% of the population aged 16 years or older reported risk consumption of alcohol (Folkhälsomyndigheten, 2026).

Although alcohol use remains a major public health concern globally and nationally, available data indicate modest declines in some indicators of alcohol consumption and hazardous use over time. Nevertheless, alcohol use continues to represent a substantial public health burden (Trolldal & Åkerblad, 2025; World Health Organization, 2024).

1.2 ALCOHOL USE DISORDER

In the *Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition, Text Revision (DSM-5-TR), alcohol use disorder (AUD) is defined as a problematic pattern of alcohol use leading to clinically significant impairment or distress (American Psychiatric Association, 2022). More broadly, AUD can be described as a chronic, relapsing medical condition involving loss of control over alcohol use despite adverse physical, psychological and social consequences (National Institute on Alcohol Abuse and Alcoholism, 2025b). Importantly, the previous DSM-IV diagnostic framework distinguished between alcohol dependence and alcohol abuse as separate diagnoses, whereas the DSM-5 integrated them into the broader diagnosis of AUD (American Psychiatric Association, 1994). In addition to the DSM-IV/DSM-5-TR, alcohol-related disorders are also classified in the *International Classification of Diseases* (ICD-11) (World Health Organization, 2022).

Worldwide, approximately 400 million people, corresponding to around 7% of the global population aged 15 years and older, are estimated to live with alcohol use disorders (World Health Organization, 2024). Earlier WHO estimates also indicate a marked sex difference in AUD prevalence, with substantially higher rates among men than among women (World Health Organization, 2018). In Sweden, approximately 4% of the adult population meets the clinical criteria for alcohol dependence (Andréasson et al., 2013). Despite the high prevalence and substantial disease burden, AUD commonly remains underdiagnosed and undertreated, with many individuals never receiving pharmacological treatment (Rehm et al., 2015; SAMHSA, 2023).

According to the DSM-5-TR, AUD is diagnosed based on the presence of at least two of 11 diagnostic criteria occurring within a 12-month period, reflecting a problematic pattern of alcohol use (American Psychiatric Association, 2022). These criteria include a range of behavioral, cognitive and physiological manifestations, such as strong cravings, unsuccessful attempts to cut down or control alcohol use and continued use despite negative physical or psychological consequences. AUD criteria also include tolerance and withdrawal. Tolerance is defined as a reduced sensitivity to alcohol following repeated exposure, such that larger amounts are needed to achieve intoxication or the desired effect. Importantly, tolerance can develop differently across alcohol effects. For instance, a person who uses alcohol can become less sensitive to some effects, such as sedation or intoxication, whereas alcohol's reinforcing effects may be less affected (Elvig et al., 2021). Withdrawal, by contrast, is characterized by physiological and psychological symptoms

following cessation or reduction of alcohol use, which may motivate continued drinking to relieve discomfort and negative affect (Koob & Volkow, 2016). A complete list of the criteria is presented in Table 1. Importantly, AUD severity is classified according to the number of criteria fulfilled during the 12-month period as mild (2–3 criteria), moderate (4–5 criteria) or severe (6 or more criteria) (American Psychiatric Association, 2022).

Table 1. DSM-5-TR diagnostic criteria for alcohol use disorder (AUD) and corresponding severity classification.

Diagnostic Criteria for Alcohol Use Disorder in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision (DSM-5-TR)
1. Alcohol is often taken in larger amounts or over a longer period than was intended.
2. There is a persistent desire or unsuccessful efforts to cut down or control alcohol use.
3. A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects.
4. Craving, or a strong desire or urge to use alcohol.
5. Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home.
6. Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.
7. Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
8. Recurrent alcohol use in situations in which it is physically hazardous.
9. Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
10. Tolerance, as defined by either of the following: a. A need for markedly increased amounts of alcohol to achieve intoxication or desired effect. b. A markedly diminished effect with continued use of the same amount of alcohol.
11. Withdrawal, as manifested by either of the following: a. The characteristic withdrawal syndrome for alcohol (refer to Criteria A and B of the criteria set for alcohol withdrawal). b. Alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms.
Note. Mild AUD = 2–3 symptoms; Moderate AUD = 4–5 symptoms; Severe AUD = ≥6 symptoms.

1.3 INTER-INDIVIDUAL VARIABILITY IN VULNERABILITY TO ALCOHOL USE DISORDER

Although alcohol use is widespread across the population, only a subset of individuals transition from recreational or controlled drinking to maladaptive patterns of alcohol use that may develop into AUD. This vulnerability reflects a complex interplay between genetic, environmental and individual-level factors (MacKillop et al., 2022). Twin and adoption studies have demonstrated a substantial genetic contribution to AUD, with heritability estimated at approximately 50% (Verhulst et al., 2015). AUD vulnerability has also been associated with several individual, clinical and socioeconomic factors, including male sex, lower sensitivity to some effects of alcohol, prior or concurrent substance use and comorbid psychiatric disorders such as depression, anxiety and schizophrenia (Hasin et al., 2007), alongside adverse socioeconomic conditions, low educational attainment and unemployment (Calling et al., 2019). In contrast, certain genetic polymorphisms in enzymes involved in alcohol metabolism, particularly alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), may exert protective effects against the development of AUD (Edenberg & McClintick, 2018). Beyond these genetic and environmental aspects, epigenetic modifications have also been implicated in the pathophysiology of AUD, further highlighting the interaction between genetic predisposition and environmental exposure (Pucci et al., 2019).

1.4 ALCOHOL AND NICOTINE CO-USE

Among co-occurring substance use patterns, nicotine use is particularly relevant in the context of alcohol use and AUD. Indeed, several epidemiological studies have demonstrated a strong association between alcohol and nicotine use, with these substances frequently co-used and mutually reinforcing each other (Bobo & Husten, 2000; DiFranza & Guerrera, 1990; Grant et al., 2004; Hurley et al., 2012; Weinberger et al., 2016). In a case-control study, DiFranza and Guerrera reported that 83% of individuals with alcoholism were smokers, compared with only 34% of non-alcoholic controls (DiFranza & Guerrera, 1990). This association has also been observed in other studies (Batel et al., 1995; Burling & Ziff, 1988; Falk et al., 2006) and more recent Swedish cohort data further show that nicotine use from both cigarettes and snus is associated with higher alcohol consumption during adolescence (Andersson et al., 2024). Longitudinal and epidemiological

evidence further indicates that adolescents who smoke show higher rates of hazardous drinking and AUD during late adolescence and adulthood compared with non-smokers (Bobo & Husten, 2000; Grucza & Bierut, 2006; Mathers et al., 2006). This comorbidity may be explained by shared genetic, environmental, social and neurobiological mechanisms. Experimental evidence further suggests that cross-reinforcement and cross-tolerance may contribute to alcohol–nicotine co-use. Cross-reinforcement is defined as the capacity of one substance to enhance the reinforcing effects of the other, whereas cross-tolerance refers to reduced sensitivity to one substance following repeated exposure to the other (Adams, 2017).

The close association between alcohol and nicotine use highlights the need for a deeper understanding of the mechanisms underlying their comorbidity, particularly how nicotine use may contribute to AUD vulnerability.

1.5 ALCOHOL EFFECTS ON NEUROTRANSMITTER SYSTEMS

Alcohol affects neurotransmission through the modulation of several neurotransmitter systems rather than acting on one specific receptor target. At the synaptic level, alcohol alters the excitatory/inhibitory balance, through effects on both glutamatergic and GABAergic signaling (Lovinger & Roberto, 2013). Indeed, it is associated with reduced glutamatergic transmission and enhanced GABAergic inhibition, although these effects may vary across brain regions (Abrahao et al., 2017). Acute alcohol exposure has also been repeatedly shown to increase extracellular dopamine (DA) levels within the mesolimbic system (Di Chiara & Imperato, 1986, 1988) while repeated alcohol exposure is primarily linked to several compensatory mechanisms in receptor expression and functioning, neurotransmitter release and neuronal excitability (Roberto & Varodayan, 2017).

Alcohol modulates glutamatergic neurotransmission through effects on ionotropic glutamate receptors, including NMDA and AMPA receptors (Joffe et al., 2018; Morisot & Ron, 2017; Sanchis-Segura et al., 2006). In particular, acute alcohol exposure is commonly associated with inhibition of NMDA receptor-mediated signaling (Lovinger et al., 1989). Chronic alcohol exposure and dependence have been associated with altered metabotropic glutamate receptor 2/3 (mGluR2/3)-mediated regulation of glutamate transmission (Meinhardt et al., 2013), which under physiological conditions regulates

glutamate release and contributes to the control of excitatory synaptic transmission (Cartmell & Schoepp, 2000). Moreover, alcohol may also influence astrocyte-dependent glutamate homeostasis (Adermark & Bowers, 2016), a process supported by the conversion of glutamate into glutamine by glutamine synthetase (Tani et al., 2014). In parallel, $GABA_A$ receptors represent a major target of alcohol (Olsen & Liang, 2017), which generally enhances $GABA_A$ receptor-mediated signaling, though effects can be region specific (Proctor et al., 1992; Roberto et al., 2003).

Alcohol also interacts with nicotinic acetylcholine receptors (nAChRs), the main molecular target of nicotine, providing an important mechanistic link between nicotine exposure and alcohol-related behaviors. Although alcohol does not act as a classical agonist at these receptors, experimental evidence indicates that alcohol can modulate nAChR function (Aistrup et al., 1999). In line with this, nAChRs have been implicated in alcohol-induced activation of mesolimbic DA transmission (Ericson et al., 2003; Söderpalm et al., 2000) and repeated nicotine exposure has been reported to increase voluntary alcohol intake in rats (Blomqvist et al., 1996; Olausson et al., 2001). In addition, pharmacological studies have shown that acute blockade of nAChRs with the nAChR antagonist mecamylamine reduces voluntary alcohol intake (Ericson et al., 1998) and prevents alcohol-induced DA increase in the NAc (Blomqvist et al., 1993; Blomqvist et al., 1997), supporting the involvement of nAChRs in the reinforcing properties of alcohol. Further support comes from studies using the α4β2 nAChR partial agonist varenicline, which has been shown to decrease alcohol intake (Söderpalm et al., 2020). Importantly, repeated nicotine exposure has been associated with increased nAChR expression (Flores et al., 1992), an effect that can be further modulated by alcohol exposure (Dohrman & Reiter, 2003). In addition, glycine receptors (GlyRs) in the NAc have been implicated in the regulation of basal and alcohol-induced DA release and voluntary alcohol intake in rats (Molander et al., 2005; Molander & Söderpalm, 2005a, 2005b).

A detailed understanding of how alcohol affects neurotransmitter and receptor systems is essential for a better understanding of AUD. Further research is therefore needed to clarify these mechanisms and to identify more precise therapeutic targets that could improve treatment outcomes.

1.6 PHARMACOLOGICAL TREATMENTS FOR ALCOHOL USE DISORDER

Treatment for AUD typically involves a combination of pharmacological and psychosocial interventions aimed at reducing alcohol consumption and craving, preventing relapse and improving overall quality of life and psychological well-being (Anton et al., 2006). Strategies are generally individualized based on patient characteristics, treatment goals, AUD severity and the presence of psychiatric or medical comorbidities (Bahji, 2024). Psychological interventions include cognitive behavioral therapy, motivational enhancement therapy and participation in mutual-help groups such as Alcoholics Anonymous (Antonelli et al., 2022). Currently, in the United States, three medications are approved by the Food and Drug Administration (FDA) for the treatment of AUD: disulfiram, acamprosate and naltrexone (National Institute on Alcohol Abuse and Alcoholism, 2025a; SAMHSA, 2023). In Europe, nalmefene has also been approved by the European Medicines Agency (EMA) (Paille & Martini, 2014). Additional medications are approved in specific countries: for example, sodium oxybate is approved for AUD treatment in Italy and Austria (Guiraud et al., 2022), whereas baclofen has been approved in France (Agabio et al., 2023). Furthermore, several medications are prescribed off-label for AUD, including topiramate, gabapentin, varenicline and ondansetron, due to emerging evidence supporting their efficacy in reducing alcohol consumption and relapse risk. Benzodiazepines remain the first-line treatment for acute alcohol withdrawal (Witkiewitz et al., 2019).

Disulfiram inhibits ALDH, the enzyme responsible for the breakdown of acetaldehyde, a toxic intermediate produced during alcohol metabolism. Alcohol consumption during disulfiram treatment leads to acetaldehyde accumulation, producing aversive effects such as skin flushing, tachycardia, hypotension, palpitations, nausea, vomiting, dizziness, dyspnea and blurred vision. This reaction discourages alcohol consumption and supports abstinence. However, adherence is often limited by poor tolerability and the requirement for complete abstinence from alcohol and other ethanol-containing products (Jørgensen et al., 2011; Skinner et al., 2014).

Acamprosate (calcium acetylhomotaurinate) is thought to restore the imbalance between excitatory and inhibitory neurotransmission associated with chronic alcohol exposure, although its precise mechanism of action remains incompletely understood (Ademar et al., 2023; Mann et al., 2008). Preclinical studies suggest that GlyRs in the NAc contribute to the mechanism

of action of acamprosate. Indeed, local blockade of these receptors with strychnine prevented the acamprosate-induced increase in extracellular DA in the NAc (Chau, Stomberg, et al., 2010) and abolished the alcohol intake-reducing effect of acamprosate (Chau, Höifödt-Lidö, et al., 2010). Moreover, acamprosate has been proposed to modulate glutamatergic signaling, including weak antagonism at NMDA receptors and effects on mGluR5, thereby reducing craving and alleviating protracted withdrawal symptoms. Due to minimal hepatic metabolism, acamprosate is particularly suitable for individuals with hepatic impairment (al Qatari et al., 1998; Mann et al., 2008).

Naltrexone is a competitive opioid receptor antagonist that has evidence for reducing heavy drinking and relapse risk in AUD. It attenuates the reinforcing effects of alcohol by blocking opioid receptors, primarily μ-opioid receptors, with lower affinity for κ- and δ-opioid receptors and is available in both oral and long-acting injectable formulations. However, its use may be limited in patients with significant hepatic impairment (Avery, 2022).

Nalmefene acts as an antagonist at μ- and δ-opioid receptors and a partial agonist at κ-opioid receptors. It is administered on an as-needed basis, typically 1–2 hours before anticipated alcohol consumption, in individuals with a high drinking risk level who do not require immediate detoxification or exhibit significant withdrawal symptoms (Mann et al., 2016).

In addition to standard treatments, the smoking cessation agent varenicline, a partial agonist at α4β2 nAChRs, has shown potential for reducing alcohol consumption and craving in individuals with AUD (de Bejczy et al., 2015; Litten et al., 2013). One proposed mechanism is that varenicline modulates nAChR-dependent mesolimbic dopaminergic signaling involved in the reinforcing effects of both nicotine and alcohol (Ericson et al., 2009). Preclinical studies have also shown that varenicline reduces alcohol consumption and seeking in rats (Steensland et al., 2007). Notably, a recent randomized clinical trial showed that varenicline, alone or in combination with bupropion, a DA and norepinephrine reuptake inhibitor, reduced alcohol consumption in individuals with moderate-to-severe AUD, with the largest effects observed for the combination treatment among participants with high medication compliance (Söderpalm et al., 2025).

Despite the availability of several evidence-based pharmacological treatments, AUD remains substantially undertreated. Current medications show modest to moderate efficacy and considerable interindividual variability in treatment response (Witkiewitz et al., 2019). These limitations highlight the need for further investigation into the mechanisms underlying AUD and for the development of more targeted treatment strategies.

1.7 DEFINING A CLINICALLY RELEVANT MODEL TO STUDY AUD-LIKE BEHAVIOR

To gain a deeper understanding of the behavioral, neurobiological and pharmacological mechanisms underlying AUD, animal models that capture selected features of AUD and other substance use disorders represent valuable complementary tools (Koob, 2000; Spanagel, 2017). These models allow controlled investigation of specific mechanisms that are difficult to isolate in human studies. However, capturing the complexity and heterogeneity of the human condition in laboratory animals remains a major challenge, as no single animal model can fully reflect all aspects of AUD. Different paradigms are therefore used to model distinct components of AUD, including voluntary alcohol consumption, motivation to obtain alcohol, compulsive-like intake and relapse-like behavior. Current animal models of addiction include voluntary drug consumption under non-operant and operant conditions, as well as reinstatement paradigms that model relapse-like behavior (Spanagel, 2017).

Among non-operant paradigms, the two-bottle choice procedure is one of the most widely used approaches to assess voluntary alcohol consumption and preference (Blomqvist et al., 1996; Richter & Campbell, 1940). In this model, animals are given free access to two bottles, one containing water and the other containing a drug solution, such as an alcohol solution. Depending on the experimental planning, access to alcohol can be scheduled as either continuous or intermittent. This paradigm is useful for assessing baseline alcohol consumption, preference, changes in consumption patterns following pharmacological, genetic or environmental manipulations. However, because alcohol is freely available, non-operant models provide limited information about motivation, effort-related responding or compulsive-like alcohol seeking (Spanagel, 2017).

In contrast, operant self-administration models are considered particularly suitable for investigating more complex aspects of addiction that resemble features observed in humans (Panlilio & Goldberg, 2007). Under this paradigm, animals are required to perform an operant response, such as lever pressing or nose poking, in order to obtain access to the drug (Staddon & Cerutti, 2003). Using operant self-administration, Deroche-Gamonet et al. developed an animal model to study human addiction-like behaviors in cocaine-exposed rats. This model is based on three signs of loss of control over drug use described in the DSM-IV: (i) inability to refrain from drug seeking, (ii) high motivation for the drug and (iii) maintained drug use despite negative consequences (American Psychiatric Association, 1994). These signs were used to develop a multisymptomatic 0/3 criteria model to capture addiction-like behaviors in rats (Deroche-Gamonet et al., 2004). This approach was later adapted to model AUD-like behaviors (Domi et al., 2019; Jadhav et al., 2017).

Despite the widespread use of both rats and mice as animal models to study alcohol addiction, rats are generally considered more suitable for learning complex behaviors, making them particularly valuable for investigating addiction-like phenotypes. In particular, rats generally show robust operant responding and are well suited for procedures assessing individual differences in alcohol seeking, motivation, and compulsive-like behavior (Ellenbroek & Youn, 2016; Spanagel, 2017). Therefore, all experiments in this thesis were conducted using rats.

1.8 OPERANT CONDITIONING

Operant conditioning is a form of associative learning in which the probability of a behavior is shaped by its consequences (Staddon & Cerutti, 2003). This principle has its origins in Edward Thorndike's law of effect, according to which positive consequences increase the likelihood that a behavior will be repeated, whereas negative consequences reduce it (Thorndike, 1898). This principle was later developed by Burrhus Frederic Skinner, who introduced the term operant conditioning and demonstrated, using the Skinner box, how behavior can be shaped by reinforcement or punishment (Skinner, 2019). In contrast to operant conditioning, which focuses on the relationship between an action and its outcome, classical conditioning, first described by Pavlov, refers to learning associations between stimuli (Pavlov, 2010).

In addiction research, operant self-administration paradigms, in which animals are required to perform an action such as pressing a lever to obtain a drug reward, are widely used to model voluntary drug seeking and drug taking (Staddon & Cerutti, 2003). This approach allows the experimenter to assess several aspects of addiction-like behaviors. For example, fixed ratio schedules can be used to measure stable alcohol reinforced responding, whereas progressive ratio schedules are used to assess the motivation to obtain the drug. In addition, punishment-coupled sessions, in which drug delivery is associated with an adverse consequence such as a mild footshock, allow researchers to model continued alcohol use despite negative outcomes (Panlilio & Goldberg, 2007). Understanding how these behaviors emerge requires knowledge of the neurobiological mechanisms underlying addiction.

1.9 NEUROBIOLOGY OF ADDICTION

Addiction can be defined as a chronic and relapsing disorder characterized by a loss of control over drug intake, compulsive drug-seeking and drug-taking behavior and adverse emotional states in the absence of the drug (American Psychiatric Association, 2022). The course of addiction can be described as a recurring three-stage cycle including the following stages: binge/intoxication, withdrawal/negative affect and preoccupation/anticipation (Koob & Le Moal, 1997; Koob & Volkow, 2010, 2016). Each stage is associated with partly overlapping but functionally distinct neurocircuitry, neurotransmitter systems and neuroadaptations.

The binge/intoxication stage is driven by the rewarding and reinforcing properties of a drug, which activate the brain's reward circuitry within the mesolimbic DA pathway. Acute drug intake increases DA release from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), producing reinforcing effects that strengthen drug-taking behavior (Di Chiara & Imperato, 1988). Over time, repeated drug exposure produces persistent neuroadaptations within these reward-related circuits, leading to increased incentive salience, defined as the attribution of motivational value to drug-related stimuli and cues (Robinson & Berridge, 1993, 2008). With repeated use, behavioral control gradually shifts from goal-directed actions to more habitual patterns of drug seeking, a transition associated with the progressive recruitment of the dorsal striatum (Zapata et al., 2010). Together, these adaptations promote the transition from voluntary drug use to compulsive drug seeking and taking (Belin et al., 2008; Everitt & Robbins, 2005; Koob & Volkow, 2016).

The withdrawal/negative affect stage emerges following the cessation or reduction of drug use and is characterized by impaired reward functionality and increased recruitment of the stress system (Koob & Le Moal, 2005). During this stage, decreased dopaminergic and serotonergic transmission within the NAc contributes to blunted sensitivity to natural rewards, which in humans may manifest as anhedonia and reduced motivation (Weiss et al., 1996). In parallel, increased corticotropin-releasing factor and dynorphin signaling, together with hyperactivity of the extended amygdala, promote negative emotional states such as irritability, anxiety and dysphoria (Koob, 2008). In animal models, the withdrawal stage is characterized by elevated reward thresholds, anxiety-like behavior and reduced motivation for natural rewards. Together, these neuroadaptations support the transition from drug use driven by positive reinforcement to continued use maintained by negative reinforcement, such that drug seeking becomes less motivated by rewarding effects and more by the avoidance of aversive withdrawal-related states (Koob et al., 2014; Koob & Le Moal, 2008; Koob & Volkow, 2016).

The preoccupation/anticipation stage, often referred to as the craving stage, is characterized by persistent thoughts about drug use, impaired executive function and enhanced responsiveness to drug-related cues that can persist even after extended periods of abstinence (Koob & Volkow, 2010). Additionally, drug-associated environmental cues, stressful experiences and exposure to contexts previously associated with drug use can trigger craving and relapse (Sinha, 2001). These processes are mediated by dysfunction within interconnected brain regions including the PFC, hippocampus and basolateral amygdala (BLA). Impairments in the PFC contribute to deficits in executive functions such as inhibitory control, decision-making, attention regulation and behavioral flexibility. At the same time, the hippocampus and the BLA support associations between drug-related memories, emotional states and environmental cues, thereby increasing the salience of drug-associated stimuli (Goldstein & Volkow, 2011; Koob & Volkow, 2010, 2016; Sinha, 2011).

Overall, this three-stage cycle provides a framework for understanding AUD as an addictive disorder. Among the brain regions implicated in AUD, the mPFC, BLA, VTA and NAc were selected for investigation in this thesis.

1.10 BRAIN REGIONS RELEVANT TO AUD-LIKE BEHAVIOR

AUD is associated with functional and structural alterations within several brain regions that regulate reward processing, motivation, emotional learning and executive control (Makris et al., 2008; Sullivan & Pfefferbaum, 2005; Wrase et al., 2007). Among these brain regions, the mPFC, BLA, VTA and NAc are the focus of this thesis. Notably, these regions do not operate as isolated structures but interact within the mesocorticolimbic circuitry, with each region contributing distinct functional roles (Sesack & Grace, 2010). The VTA and the NAc are involved in mediating the rewarding and reinforcing effects of alcohol (Di Chiara & Imperato, 1986, 1988), the PFC modulates decision-making, inhibitory control and the regulation of motivated behavior (Miller & Cohen, 2001) while the BLA regulates emotional and associative learning (Crofton et al., 2022; Keistler et al., 2017).

1.10.1 MEDIAL PREFRONTAL CORTEX

Anatomically, the rodent medial prefrontal cortex (mPFC) is suggested to be partly analogous to human medial frontal regions, including the dorsomedial prefrontal cortex (dmPFC), dorsal anterior cingulate cortex (dACC) and ventromedial prefrontal cortex (vmPFC) (Howland et al., 2022; Uylings et al., 2003). The mPFC represents a central hub involved in executive control, decision-making, behavioral flexibility and the regulation of motivated behaviors (Dalley et al., 2004) and it is commonly divided into the anterior cingulate cortex (ACC), prelimbic cortex (PL) and infralimbic cortex (IL) (Anastasiades & Carter, 2021; Hoover & Vertes, 2007). The PL and IL have often been associated with opposing roles, with the PL generally promoting and the IL suppressing drug-seeking behavior (Moorman et al., 2015). Notably, neurobiological alterations within the PL have been observed following chronic alcohol exposure, supporting the relevance of this region as an important site of alcohol-induced neuroadaptations (Barbier et al., 2021; Domi et al., 2024; Hughes et al., 2020).

The mPFC receives inputs from several brain regions, including other cortical areas, the VTA, the amygdala, the thalamus and the hippocampus (Hoover & Vertes, 2007). At the same time, it sends projections to regions such as the NAc, dorsal striatum, amygdala, VTA and other subcortical regions. Through these projections, the mPFC exerts top-down regulation over limbic, striatal and midbrain circuits (Ding et al., 2001; Gabbott et al., 2005).

At the neuronal level, glutamatergic pyramidal projection neurons constitute the major neuronal population of the mPFC, accounting for approximately 80–90% of cortical neurons, whereas GABAergic interneurons represent the remaining 10–20% and regulate local circuits (Howland et al., 2022; Riga et al., 2014).

1.10.2 BASOLATERAL AMYGDALA

The BLA is part of the amygdaloid complex subdivided into lateral (LA), basal (BA) and basomedial (BM) amygdala. The LA represents a major input region, receiving sensory information from thalamic and cortical afferents, which is then processed through intra-BLA connections and relayed to downstream targets. While reciprocally connected with the PFC and hippocampus, the BLA sends projections to the central nucleus of the amygdala (CeA), the NAc and the bed nucleus of the stria terminalis (BNST) (Janak & Tye, 2015; Sah et al., 2003). The BLA is a cortical-like structure composed mainly of glutamatergic principal projection neurons, which account for approximately 80% of the neuronal population, together with a smaller but highly diverse population of GABAergic interneurons that regulate local network activity (Hájos, 2021; Janak & Tye, 2015; McDonald, 2020).

Although the BLA is commonly associated with anxiety-like behavior, fear conditioning and extinction, increasing evidence indicates that it also plays a central role in reward learning and addiction-related behaviors (LeDoux, 2003; Sepahvand et al., 2023; Wassum & Izquierdo, 2015). In particular, BLA-to-NAc excitatory projections promote cue-evoked reward seeking and motivated responding in operant tasks, highlighting a circuit mechanism through which BLA activity may influence reward-directed behavior (Ambroggi et al., 2008; Stuber et al., 2011). Consistent with this, several studies have implicated the BLA in alcohol intake, cue-induced alcohol seeking, reinstatement-like behavior and vulnerability to AUD-like phenotypes (Cadeddu et al., 2025; Keistler et al., 2017; Morales et al., 2018; Price & McCool, 2022).

1.10.3 VENTRAL TEGMENTAL AREA

The VTA is a heterogeneous midbrain region critically involved in motivated behavior, reward processing and reinforcement learning (Berridge & Robinson, 1998; Morales & Margolis, 2017; Trutti et al., 2019). As part of the mesocorticolimbic circuit, the VTA contributes to stimulus-outcome and action-outcome learning, thereby shaping how organisms assign value to environmental stimuli and adapt behavior based on previous consequences. It

also influences decision-making and goal-directed behavior in response to reinforcement, aversion and salient stimuli (Lammel et al., 2014).

This broad behavioral repertoire reflects the marked neuronal and connectivity heterogeneity of the VTA. Although DA neurons represent the largest neuronal population, comprising approximately 55–65% of VTA neurons, the VTA also contains substantial GABAergic populations, accounting for around 30%, as well as smaller populations of glutamatergic neurons, estimated at approximately 2–3% (Hou et al., 2024). In addition, some VTA neurons are capable of co-releasing multiple neurotransmitters. The activity of VTA DA neurons is regulated by inputs originating from several brain regions as well as by local GABAergic and glutamatergic neurons, which also send projections to many of the same target regions innervated by VTA DA neurons (Morales & Margolis, 2017). Notably, evidence has shown that distinct VTA neuronal subpopulations are associated with specific neural networks and can differentially contribute to reward-, aversion-, motivation- and learning-related behaviors (Cohen et al., 2012).

Accordingly, the VTA is positioned as an integrative hub, receiving major cortical, limbic and brainstem inputs from regions such as the PFC, NAc, ventral pallidum, lateral hypothalamus, lateral habenula, dorsal raphe nucleus, laterodorsal tegmental nucleus/pedunculopontine tegmental nucleus and rostromedial tegmental nucleus, while sending outputs primarily to the NAc and PFC, with additional projections to the amygdala, hippocampus, ventral pallidum, bed nucleus of the stria terminalis and lateral habenula (Morales & Margolis, 2017).

The VTA is well established as a key region involved in the acute rewarding and reinforcing effects of alcohol. Indeed, alcohol can increase VTA DA neuron activity and elevate DA release in the NAc, a mechanism shared with several other drugs of abuse (Blomqvist et al., 1997; Di Chiara & Imperato, 1986, 1988; Ericson et al., 1998; Gessa et al., 1985).

1.10.4 NUCLEUS ACCUMBENS

The NAc is a major component of the ventral striatum and plays a central role in motivational, reward and emotional processes (Kelley, 2004; Salgado & Kaplitt, 2015). It is divided into two main subregions: the shell and the core (NAcSh and NAcC). NAcSh is involved in reward valuation, motivational salience and relapse-related behavior, and has been implicated in the reinforcing properties of novelty and drugs of abuse (Chaudhri et al., 2010; Di

Chiara, 2002; Reed et al., 2015; Zahm, 1999). NAcC is more closely associated with cue-guided reward seeking, instrumental responding and the translation of reward-predictive information into goal-directed actions (Ambroggi et al., 2011).

The NAc receives dopaminergic projections from the VTA as well as dense glutamatergic inputs from the PFC, BLA, hippocampus and thalamus. In turn, it sends predominantly GABAergic outputs to downstream targets, including the VTA and ventral pallidum, thereby regulating mesolimbic dopaminergic signaling (Brog et al., 1993; Ding et al., 2001; Tye, 2012; Xia et al., 2011).

The NAc is primarily composed of GABAergic MSNs, which account for approximately 90-95% of the neuronal population, with the remainder consisting of GABAergic and cholinergic interneurons (CINs) (Dautan et al., 2014; Kawaguchi et al., 1995; Warner-Schmidt et al., 2012). Additionally, the MSN population can be further divided based on DA receptor subtype expression into D1-MSNs and D2-MSNs, which are associated with partially distinct projection pathways and can differentially regulate reward-related behavior (Gerfen & Surmeier, 2011). Notably, although CINs represent only 1-3% of the neuronal population, they play an important modulatory role in regulating MSN activity and NAc output (Oldenburg & Ding, 2011; Witten et al., 2010), and have been shown to be necessary for alcohol-induced DA elevation (Loftén et al., 2021) and to be implicated in the regulation of alcohol intake (Loftén et al., 2025).

Acute alcohol exposure enhances mesolimbic DA transmission by increasing the activity of VTA DA neurons, elevating extracellular DA levels in the NAc (Di Chiara & Imperato, 1986, 1988; Ericson et al., 1998; Gessa et al., 1985). With repeated exposure, alcohol induces neuroadaptations within the NAc and broader reward-related circuits, including changes in receptor function, neurotransmitter release, synaptic plasticity, neuronal excitability and intracellular signaling pathways (Lovinger & Roberto, 2013; Roberto & Varodayan, 2017).

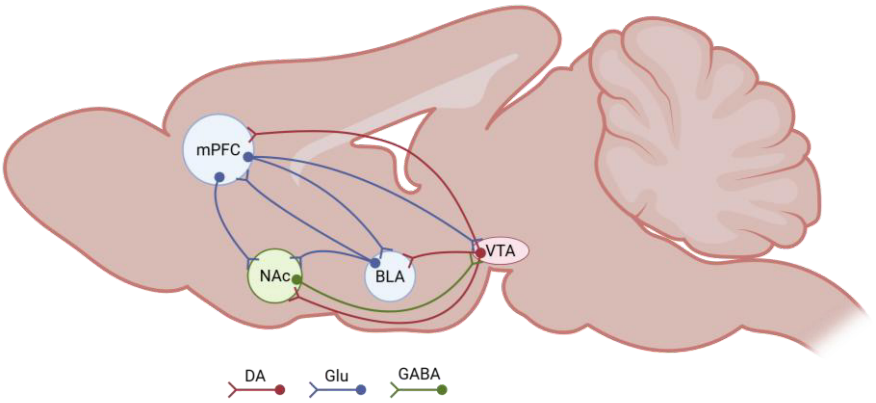

Figure 1. Simplified drawing of the rat brain areas investigated in this thesis, highlighting selected dopaminergic, glutamatergic and GABAergic connections. The figure includes the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), basolateral amygdala (BLA) and ventral tegmental area (VTA). Created with BioRender.com.

2 AIM

The overall aim of this thesis was to investigate AUD-like vulnerability, with special emphasis on its corresponding neurophysiological signatures, and to examine how nicotine exposure modulates alcohol-related behaviors and neurophysiological responses in male rats. More specifically, this thesis aimed to determine how individual differences in AUD-like behavior are reflected in synaptic transmission and neuronal excitability within addiction-related brain circuits, with particular focus on PL, BLA, VTA and NAcSh. The following aims are organized according to the papers and manuscripts included in the thesis.

I. To determine whether neurophysiological properties in BLA are associated with AUD-like vulnerability.
II. To identify pre- and postsynaptic signatures in PL associated with AUD-like behavior.
III. To investigate neurobiological mechanisms underlying the association between nicotine use and alcohol misuse.
IV. To outline neurobiological underpinnings in PL and NAcSh contributing to nicotine-induced escalation of alcohol intake and AUD-like vulnerability.

3 METHODS

This section provides an overview of the methods used in this thesis and discusses key methodological considerations. Detailed descriptions of the materials and procedures are provided in the individual papers. Throughout the thesis, “alcohol” is used when referring to drinking behavior, AUD-like behavior and clinical or translational contexts, whereas “EtOH” is used when referring to experimentally administered ethanol in pharmacological, electrophysiological or in vivo microdialysis experiments.

3.1 ANIMALS

All studies were conducted in outbred male Wistar rats. Animals were obtained from Charles River, Germany, for Papers I, II and IV, whereas in Paper III, experiments were performed in rats obtained from Inotiv, the Netherlands. Depending on the experiment, rats were either group-housed, pair-housed or single-housed. Across studies, rats were housed under controlled light/dark cycle conditions with ad libitum access to food and water. All procedures were conducted in accordance with the guidelines of the Swedish National Committee for Animal Research and approved by the relevant animal ethics committee in Gothenburg, Sweden. All efforts were made to minimize animal suffering and distress.

All studies were conducted in male rats. The exclusive use of males limits the generalizability of the findings across sexes and should be considered when interpreting the results.

3.2 EXPERIMENTAL DESIGN BY PAPER

The included studies used related experimental approaches but differed in their behavioral procedures, brain regions and neurobiological assessments. Table 2 provides a brief overview of the main experimental design of each paper and manuscript.

Table 2. Overview of the experimental designs used in the included papers and manuscripts. Abbreviations: AUD, alcohol use disorder; EPM, elevated plus maze; LMA, locomotor activity; PL, prelimbic cortex; mPFC, medial prefrontal cortex; BLA, basolateral amygdala; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; VTA, ventral tegmental area.

Study	Behavioral approach	Brain regions	Neurobiological assessment
Paper I	Two-bottle choice followed by operant alcohol self-administration and AUD-like behavioral assessment	BLA	Whole cell patch clamp and field potential recordings
Paper II	LMA in a novel environment and EPM, followed by two-bottle choice, operant alcohol self-administration and AUD-like behavioral assessment	PL, BLA and NAcC	Whole cell patch clamp and field potential recordings
Paper III	Two-bottle choice combined with repeated nicotine or saline exposure, followed by quinine adulteration and LMA assessment	PL/mPFC and VTA	Whole cell patch clamp, cell attached patch clamp and field potential recordings, together with *in vivo* microdialysis
Paper IV	Two-bottle choice combined with repeated nicotine or saline exposure, followed by operant alcohol self-administration and AUD-like behavioral assessment	PL and NAcSh	Whole cell patch clamp and field potential recordings

3.3 VOLUNTARY ALCOHOL INTAKE IN THE TWO-BOTTLE CHOICE PARADIGM

In all studies included in this thesis, voluntary alcohol intake was assessed using two-bottle choice paradigms, in which rats could freely choose between an alcohol solution and water. The main outcome measures included alcohol intake, water intake, total fluid intake and alcohol preference, calculated as the amount of alcohol solution consumed relative to total fluid intake.

In Papers I and II, the two-bottle choice procedure was used as a pre-exposure phase before operant alcohol self-administration, while avoiding sucrose fading or water deprivation. In Papers III and IV, voluntary intake measurements were used as primary outcome measures of nicotine-induced changes in alcohol consumption, and in Paper III, these measurements were also used in separate analyses based on baseline alcohol preference and alcohol consumption, depending on the experiment.

In Papers I and II, rats received intermittent 24 h access to 10% alcohol three times per week for four weeks. Similarly, in Paper III, voluntary alcohol intake was assessed using an intermittent two-bottle choice paradigm, with three 24 h sessions per week. In the main nicotine-treatment experiment, rats received 6% alcohol for one week followed by 12% alcohol during two baseline weeks, three weeks of nicotine or saline treatment and two post-treatment weeks. A subset then received quinine-adulterated alcohol for one week, whereas another subset was used for electrophysiological recordings. In a separate cohort used to compare low and high alcohol consumers, rats received 6% alcohol for one week followed by 12% alcohol for five weeks. In Paper IV, rats received access to alcohol and water during repeated limited-access two-bottle choice sessions on weekdays, for a total of 33 sessions across approximately seven weeks. Alcohol concentration was 6% during the first week and was then raised to 15% after the first week. In contrast to the 24 h access procedures used in Papers I–III, intake in Paper IV was measured at 30 min, 2 h and 6 h after the start of each session. This design allowed the assessment of both early-phase drinking, measured at 30 min, and longer session intake, measured at 2 h and 6 h, thereby providing a more detailed temporal profile of nicotine-induced changes in alcohol consumption. In Paper IV, alcohol and water were administered via graduated burettes to facilitate repeated intake measurements within each session, whereas standard drinking bottles were used in the intermittent access studies.

Housing conditions during the two-bottle choice procedure differed slightly across papers according to the specific experimental aims. Depending on the experiment, rats in Paper III were either group-housed or single-housed. Rats used in the two-bottle choice experiments were single-housed to allow individual measurements of alcohol intake. In Papers I and II, rats were pair-housed because the two-bottle choice procedure was primarily used to habituate rats to alcohol before operant self-administration rather than to obtain individual-level drinking measures. Rats in Paper IV were also pair-housed; however, cage mates were separated by a perforated Plexiglas divider during each drinking session to allow individual intake measurements while limiting social isolation. The divider was then removed at the end of each session. Notably, across paradigms, the position of bottles or burettes was alternated when relevant to minimize side bias. These experimental differences should be considered when interpreting and integrating findings across the included studies.

3.4 NICOTINE TREATMENT

Repeated nicotine exposure was used in both Papers III and IV to assess behavioral, electrophysiological and neurotransmitter-related aspects of nicotine-induced neuroadaptations, either alone or in relation to their effects on alcohol-related behaviors.

In both studies, rats received systemic subcutaneous injections of nicotine or saline (0.9% NaCl) over a three-week period, for a total of 15 injections. Nicotine hydrogen tartrate was dissolved in 0.9% NaCl, pH-adjusted with $NaHCO_3$, and administered at 1 mg/kg, corresponding to 0.36 mg/kg free-base nicotine. This paradigm has been shown to produce behavioral sensitization to the locomotor-stimulant effects of nicotine, which can persist for several weeks after the last drug injection (Morud et al., 2016).

In Paper III, a subset of rats was assigned to nicotine or saline treatment after a baseline drinking period and received injections during the two-bottle choice period to assess whether repeated nicotine exposure promoted voluntary alcohol intake, aversion-resistant drinking when alcohol was adulterated with quinine and long-lasting neurophysiological changes in the PL and VTA. Alcohol intake was also monitored after nicotine discontinuation, allowing assessment of whether nicotine-induced changes persisted beyond the injection period. In addition, a separate subset of rats received nicotine or saline injections and was tested for locomotor activity to evaluate nicotine-induced

behavioral sensitization. Approximately one week after the last injection, some rats were used for *ex vivo* electrophysiological recordings in PL, whereas others underwent *in vivo* microdialysis in mPFC.

In Paper IV, rats were assigned to receive nicotine or saline injections based on their baseline alcohol intake and preference in order to obtain two balanced groups. Treatment was given approximately 30 min prior to access to alcohol and water burettes. After nicotine or saline treatment, rats underwent a readjustment phase before operant alcohol self-administration training, as described in the following section. After the behavioral assessment, rats were used for electrophysiological recordings in the PL and NAcSh.

3.5 OPERANT ALCOHOL SELF-ADMINISTRATION

Operant alcohol self-administration was used in Papers I, II and IV. Following the two-bottle choice period, rats were trained in operant boxes and later assessed for AUD-like behaviors. Rats were placed in operant boxes containing an active lever, where pressing resulted in the delivery of 0.1 mL of alcohol into a receptacle, and an inactive lever, where pressing had no consequences. Each session lasted 30 min and consisted of two 10 min alcohol-available periods (drug periods) separated by a 10 min period during which alcohol was not available (no-drug period). The no-drug period was signaled by the activation of the house light. Alcohol delivery was paired with illumination of a cue light for 5 s and was followed by a 10 s timeout period, during which active lever pressing was not associated with additional alcohol delivery. Active and inactive lever presses, including presses during the timeout period, were recorded during both the drug and no-drug periods. In order to facilitate the acquisition of the operant response, self-administration training started under a fixed ratio 1 (FR1) schedule, where a single active lever press resulted in the delivery of a single alcohol reward. Subsequently, the fixed ratio was increased to fixed ratio 3 (FR3), where three active lever presses were required to obtain a single alcohol delivery, providing a more stable measure of responding. The number of lever presses during the no-drug period was used to measure persistence of responding when alcohol was not available (criterion 1). In Papers I and II, rats were trained to self-administer alcohol over approximately 60 sessions, starting with 10% alcohol under FR1, followed by FR3 and an increase in alcohol concentration to 15%. In Paper IV, rats were trained in operant boxes following a readjustment phase after nicotine/saline treatment. Operant training started with 15% alcohol under FR1 and was then shifted to FR3.

After the FR3 period, rats underwent a single progressive ratio (PR) session. Here, rats had to press the active lever an increasing number of times according to a predefined progressive ratio schedule of reinforcement to obtain a single alcohol reward. The maximum number of active lever presses reached within the predefined time limit was defined as the breakpoint, which was used as an index of motivation for alcohol (criterion 2).

Following the PR session, resistance to punishment (criterion 3) was assessed using a mild electrical footshock. In this test, the first active lever press resulted in activation of a new cue light, the second active lever press triggered a mild footshock (0.25 mA, 0.5 s) and the third active lever press resulted in delivery of 0.1 mL of alcohol. If the rat failed to complete the sequence within one minute, the sequence was restarted. In Papers I and II, resistance to punishment was assessed across three consecutive punishment sessions, whereas in Paper IV a single punishment session was used. Responding during punishment was compared with responding during preceding baseline sessions to calculate a resistance score.

3.6 EVALUATION OF ADDICTION-LIKE BEHAVIORS

The evaluation of AUD-like behaviors was performed in Papers I, II and IV using a multisymptomatic approach based on three addiction-like criteria: 1) persistence of responding when alcohol was not available, 2) motivation for alcohol under a progressive ratio schedule of reinforcement and 3) continued alcohol seeking despite adverse consequences (Deroche-Gamonet et al., 2004). Each measure was then normalized to a z-score for each rat and aggregated to obtain the addiction score (AS). This overall score provided a measure of AUD-like behavior, allowing individual differences in addiction-like vulnerability to be further assessed.

In Paper I, rats with an addiction score above the 75th percentile of the total population were classified as vulnerable, whereas rats below the 25th percentile were classified as resilient to developing AUD-like behaviors. In Paper II, a rat was considered positive for a criterion when the score for that specific behavior was within the top 34% of the total distribution, corresponding to values above the 66th percentile. Rats were therefore primarily classified based on the number of criteria met (0 crit, 1 crit, 2 crit or 3 crit), while addiction score was also calculated. In Paper IV, the addiction

score was used to classify rats, with animals below the 20th percentile defined as resilient and animals above the 80th percentile defined as vulnerable to developing AUD-like behaviors. Although all studies were based on the same three behavioral dimensions, differences in the evaluation of AUD-like behaviors should be considered when comparing vulnerable and resilient subgroups across studies.

3.7 LOCOMOTOR ACTIVITY

The locomotor activity (LMA) test was used to assess animal movement within an open-field arena, providing measures of motor behavior, exploratory activity and behavioral responses to pharmacological treatments. In a novel environment, locomotor activity reflects the animal's exploratory response and can be used as a baseline behavioral measure. In drug exposure studies, repeated assessment of locomotor activity can also be used to evaluate behavioral sensitization, defined as an enhanced locomotor response following repeated exposure to the same drug (Walsh & Cummins, 1976). LMA was recorded in an open-field arena consisting of a Plexiglas cube-shaped chamber (40 × 40 cm) equipped with photocell beam breaks used to monitor ambulatory counts and total distance traveled.

In Paper II, LMA was assessed before alcohol exposure as a measure of novelty-induced locomotor activity. Rats were placed in the open-field arena for 60 min, and LMA was quantified as the total distance travelled during the first 30 min of the session. This measure was included to evaluate whether baseline exploratory activity was related to later vulnerability to AUD-like behavior.

In Paper III, LMA was measured in a subset of rats in response to the first and last nicotine or saline injection to assess behavioral sensitization, which is commonly observed following repeated drug administration, including repeated exposure to cocaine, amphetamine or nicotine (Clarke & Kumar, 1983; Ericson et al., 2010; Steketee & Kalivas, 2011). Each LMA session started with a 60 min habituation period in the room, followed by a 30 min habituation period in the open-field arena. Rats then received a single injection of either saline or nicotine, and their locomotor activity was monitored for an additional 30 min to assess the acute locomotor response to the treatment.

3.8 ELEVATED PLUS MAZE

The elevated plus maze (EPM) consists of two open arms and two enclosed arms arranged in a plus-shaped configuration and elevated above the floor. The EPM was used to assess anxiety-like behavior based on the natural conflict between exploration of open spaces and avoidance of exposed elevated areas (Pellow et al., 1985; Walf & Frye, 2007).

In Paper II, EPM testing was performed before alcohol exposure to assess whether pre-existing behavioral traits were related to later vulnerability to AUD-like behavior. Rats were initially positioned on the center platform of the maze facing an open arm and were allowed to explore the apparatus for 5 min. Anxiety-like behavior was estimated using both the percentage of time spent in the open arms and the number of open-arm entries.

3.9 *IN VIVO* MICRODIALYSIS

In vivo microdialysis is a sampling technique that allows the measurement of extracellular analytes, such as neurotransmitters, amino acids and metabolites, in specific brain regions of awake and freely moving animals (Chaurasia, 1999). This technique consists of the implantation of a microdialysis probe equipped with a semi-permeable membrane connected to an inlet and an outlet tube. On the day of the experiment, the probe is perfused with Ringer's solution, a physiological salt solution that mimics the ionic composition of extracellular fluid. Molecules in the extracellular environment diffuse across the active space of the semipermeable membrane, while dialysate is collected for subsequent biochemical analysis using high-performance liquid chromatography (HPLC). This approach can be combined with pharmacological manipulations while neurochemical changes are monitored over time. In addition, reverse microdialysis can be used to locally deliver pharmacological compounds into the extracellular space of a specific brain region (Darvesh et al., 2011; Ericson et al., 2008; Tossman & Ungerstedt, 1986).

In vivo microdialysis was performed in Paper III to assess extracellular levels of dopamine, glutamate and glutamine in the mPFC following systemic EtOH administration in nicotine- and saline-treated rats. Five days after the last nicotine or saline injection, rats were deeply anesthetized and underwent surgery for implantation of a custom-made I-shaped microdialysis probe targeting the mPFC. Following recovery from surgery, microdialysis was

performed in awake and freely moving animals. Probe placement was verified at the end of the experiment, and only rats with correct positioning were included.

3.10 *EX VIVO* ELECTROPHYSIOLOGY

Electrophysiology refers to a group of techniques used to measure the electrical properties of excitable cells, such as neurons and muscle cells, as well as excitable tissues such as brain and heart tissue (Hodgkin & Huxley, 1952). In neuroscience, it is used to study neuronal excitability and synaptic transmission by recording changes in membrane potential and ionic currents. Electrophysiology can be classified as *in vitro* when performed on isolated cells, *ex vivo* when performed on tissue preparations such as brain slices and *in vivo* when performed in living animals. In this thesis, *ex vivo* electrophysiology was used to assess neurophysiological adaptations associated with alcohol intake, nicotine exposure and AUD-like behaviors in the selected brain regions. All procedures were conducted in continuously oxygenated aCSF under controlled temperature conditions.

3.10.1 BRAIN SLICE PREPARATION

Brain slices were prepared for *ex vivo* electrophysiological recordings as follows. Rats were deeply anesthetized with isoflurane, decapitated and the brain was rapidly removed and transferred to ice-cold modified artificial cerebrospinal fluid (aCSF) continuously oxygenated with 95% O_2 and 5% CO_2. The brain was sliced using a Leica VT1200S vibratome (Leica Microsystems AB, Bromma, Sweden) to obtain brain slices of 250 µm thickness containing the brain regions of interest for each study. These included: the BLA in Paper I, the PL, NAcC and BLA in Paper II, the mPFC and VTA in Paper III and the PL and NAcSh in Paper IV. Coronal slices were used for cortical and striatal regions, whereas horizontal slices were prepared for the VTA and, in one experiment in Paper III, for the mPFC. Slices were then transferred to standard oxygenated aCSF and allowed to recover at 30–33 °C for 30 min, after which they were kept at room temperature for an additional 30 min before recordings.

3.10.2 FIELD POTENTIAL RECORDING

Field potential recording is an extracellular electrophysiological technique used to measure the collective activity of a population of neurons in response to local stimulation (Buzsáki et al., 2012). During recordings, brain slices were continuously perfused with preheated (30 °C) and oxygenated aCSF (95 % O_2,

5 % CO_2), and a recording electrode, consisting of a glass micropipette, was placed in close proximity to a tungsten stimulating electrode. Electrical stimuli were delivered through the stimulating electrode at 20 s intervals (0.05 Hz) and the resulting evoked field responses were recorded. Stimulation intensity was set to approximately half of the maximum evoked response. After a stable 10-min baseline was established, neurobiological responses were assessed through bath application of specific pharmacological compounds. When applicable, a cocktail of CNQX and APV was used to validate the glutamatergic nature of the evoked field responses.

In some experiments, an input–output (I/O) protocol consisting of stepwise stimulation intensities of 12, 18, 24, 30, 36, 42 and 48 μA was applied to assess the stimulus-response relationship. To investigate changes in presynaptic release probability, a paired-pulse protocol was used, consisting of two identical stimuli delivered 50 ms apart. The paired-pulse ratio (PPR) was calculated as the ratio of the second response to the first response. A PPR value close to 1 indicates little or no paired-pulse modulation. Values greater than 1 indicate paired-pulse facilitation, which is generally associated with lower initial neurotransmitter release probability, whereas values lower than 1 indicate paired-pulse depression, which may reflect higher release probability.

In this thesis, field potential recordings were performed in brain slices containing the PL, BLA, NAcSh and NAcC. In Papers I, II and IV, the $GABA_A$ receptor antagonist bicuculline was used to assess $GABA_A$-receptor-mediated inhibitory control over the evoked field response. In Paper II, the mGluR2/3 agonist LY354740 was bath-applied to assess mGluR2/3-mediated modulation of synaptic transmission while in Paper III, brain slices were acutely exposed to alcohol or nicotine, as well as to pharmacological blockers targeting selected receptor systems.

3.10.3 PATCH CLAMP RECORDING

Patch clamp recording is an electrophysiological technique used to measure ionic currents and membrane voltage from individual neurons, originally developed by Neher and Sakmann (Neher & Sakmann, 1976). In this technique, a borosilicate glass recording pipette is used to record electrical activity from individual cells in different configurations. In this thesis, cell attached and whole cell configurations were used. Cell attached recordings were used to monitor spontaneous neuronal firing without rupturing the membrane, thereby preserving the intracellular environment. From the cell attached configuration, further negative pressure or a small electrical pulse can

be applied to rupture the membrane patch under the pipette tip, thereby moving to the whole cell configuration, where neuronal currents, membrane properties and action potential firing can be measured from individual neurons.

Whole cell recording can be performed using voltage clamp (VC) or current clamp (IC) mode. VC mode consists of holding the membrane voltage at a defined potential, typically -65 mV, while measuring spontaneous postsynaptic currents. Excitatory (sEPSCs) or inhibitory (sIPSCs) currents can be recorded depending on the internal solution used to fill the glass electrodes, which in this thesis included potassium gluconate or cesium chloride-based solutions, respectively. Measurements of spontaneous currents in VC included frequency, which was used as an indirect index of presynaptic input, and amplitude, rise time and decay time, which were used as indirect indices of postsynaptic properties. On the other hand, IC recording consists of measuring changes in membrane voltage in response to stepwise current injections. It was used to assess neuronal excitability, including resting membrane potential, rheobase, defined as the minimum current necessary to evoke the first action potential, action potential voltage threshold and frequency of action potentials. Additionally, whole cell recording allows the measurement of passive membrane properties such as membrane capacitance and membrane resistance, while access resistance was monitored as a quality control parameter.

During patch clamp recordings, brain slices were transferred to a recording chamber and continuously perfused with temperature controlled, oxygenated aCSF. Neurons were visualized using an upright microscope and, depending on the brain region, were selected based on their morphology and electrophysiological properties.

In the studies included in this thesis, whole cell recording was performed in the PL, BLA and NAcSh, while cell attached recording was employed in the VTA to monitor spontaneous firing activity of putative DA neurons. Notably, the complementary use of patch clamp and field potential recording allowed a more complete assessment of both synaptic and intrinsic neuronal mechanisms.

3.11 VISUALIZATION OF NEURONS

Visualization of biocytin-filled neurons was performed to confirm the correct localization and morphology of whole cell recorded neurons. Biocytin was included in the internal solution used to fill glass recording electrodes and diffused into the recorded neuron throughout the recording. Following whole cell recording, brain slices containing biocytin-filled neurons were fixed overnight in 4% paraformaldehyde, cryoprotected when required and washed in PBS. Slices were then incubated with fluorescently conjugated streptavidin Alexa Fluor 594, which binds with high affinity to biocytin, allowing visualization of the recorded cell. Following additional washes, slices were mounted and imaged using fluorescence or confocal microscopy. Visualization of biocytin-filled neurons was used in Paper II and Paper IV.

3.12 STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA), STATISTICA and, when applicable, Python-based analysis tools. Data are presented as individual values and/or mean ± SEM, with statistical significance set at $p < 0.05$. The numbers of animals and recordings are reported in the individual papers. When applicable, values from multiple recordings obtained from the same animal were averaged to obtain a single value per animal before statistical analysis. Statistical tests were selected according to experimental design and data type. Comparisons between two groups were performed using t-tests, whereas comparisons involving multiple groups were analyzed using one-way or two-way ANOVA. Fisher's exact test was used to assess the association between treatment group and resilient classification in Paper IV. Repeated-measures ANOVA was used when data were collected repeatedly over time, including voluntary alcohol intake, operant self-administration and some electrophysiological experiments. Mixed-effects models were used in cases of missing values in the dataset. Post hoc tests were performed to identify specific group differences when significant main effects or interactions were detected among multiple groups. Correlation analyses were conducted using Pearson's correlation to investigate associations within and between behavioral and neurophysiological data. To explore the interdependence between behavioral and electrophysiological measurements, PCA was performed in Papers II and IV. Electrophysiological data were extracted and analyzed using Clampex (Molecular Devices, Foster City, CA, USA), Mini Analysis 7 (Synaptosoft, Decatur, GA, USA) and compiled using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

4 RESULTS AND DISCUSSION

The main findings from the four papers included in this thesis are summarized and discussed below. The aim of this section is to highlight the behavioral, neurophysiological and neurochemical results most relevant to the overall thesis framework and to relate them to previous literature, while full experimental details and complete statistical analyses are presented in the individual papers.

4.1 AUD-LIKE VULNERABILITY IS ASSOCIATED WITH NEUROPHYSIOLOGICAL ALTERATIONS IN BLA AND PL

In humans, the transition from controlled alcohol use to AUD does not occur in all individuals but rather depends on several individual biological and environmental factors (MacKillop et al., 2022). Similarly, evidence based on AUD-like animal models shows that rats also display inter-individual variability in the vulnerability to developing an AUD-like phenotype (Domi et al., 2019; Jadhav et al., 2017). In this thesis, voluntary alcohol intake was assessed using a two-bottle choice paradigm prior to operant alcohol self-administration, followed by behavioral testing to evaluate the development of AUD-like behavior in rats. The AUD-like model used was based on the following DSM-IV-based traits: (1) inability to refrain from drug seeking, (2) high motivation for the drug and (3) maintenance of drug use despite negative consequences (American Psychiatric Association, 1994). Building on previous research (Deroche-Gamonet et al., 2004), we evaluated rats for three addiction criteria consisting of: persistence in alcohol seeking when alcohol was not available, motivation for alcohol under a progressive ratio schedule of reinforcement and resistance to punishment. Across Papers I, II and IV, these behavioral dimensions were used to identify animals showing resilient or vulnerable AUD-like phenotypes. Following behavioral characterization, electrophysiological assessments were conducted to examine associations between the behavioral phenotype and neurophysiological properties in the BLA, PL and NAc.

4.1.1 ONLY A SUBSET OF RATS DEVELOPS AN AUD-LIKE VULNERABLE PHENOTYPE

Although the classification strategies differed across Papers I, II and IV, results were in line with previous studies showing that only a subset of rats develops an AUD-like vulnerable phenotype (Domi et al., 2019; Jadhav et al., 2017). Additionally, rats considered vulnerable to AUD-like behavior consistently showed higher addiction scores and higher values in the individual behavioral measures represented by the three addiction criteria. These findings confirm that the vulnerable phenotype did not depend only on one isolated behavioral measure, but rather reflected a multidimensional behavioral profile. This interpretation was further supported in Papers I and II, where vulnerable rats obtained a higher number of alcohol rewards compared with resilient rats, suggesting that AUD-like vulnerability was accompanied by increased alcohol-reinforced responding. In Paper IV, a retrospective analysis of the two-bottle choice data at the 6 h time point was performed to compare resilient saline rats with vulnerable rats, including both saline- and nicotine-exposed animals. Results showed significantly higher alcohol intake and alcohol preference in vulnerable rats compared with resilient saline rats. Additionally, further analysis comparing resilient saline rats and combined vulnerable rats showed a tendency toward higher alcohol rewards in vulnerable rats during the FR3 period, although this effect did not reach statistical significance. Importantly, alcohol rewards alone do not define AUD-like vulnerability but rather provide an additional behavioral measure consistent with the vulnerable phenotype. Collectively, these findings further support an association between later AUD-like vulnerability and higher voluntary alcohol drinking during the two-bottle choice phase. Although this result should be interpreted cautiously, because vulnerable saline- and nicotine-exposed rats were combined in this analysis, the tendency was consistent with the pattern observed in Papers I and II and can be considered supportive rather than conclusive.

4.1.2 AUD-LIKE VULNERABILITY IS ASSOCIATED WITH A HYPOACTIVE BLA PROFILE

When looking at the electrophysiological results, Paper I showed that AUD-like vulnerability was associated with reduced excitatory synaptic activity in BLA. Specifically, whole cell recordings showed decreased sEPSC frequency in vulnerable rats compared with both resilient rats and water-drinking controls, suggesting reduced presynaptic excitatory activity onto BLA neurons, which may reflect altered excitatory drive from brain regions

projecting to BLA. Additionally, sEPSC amplitude was reduced in vulnerable rats compared with controls, whereas no differences were observed in sEPSC kinetics. The negative correlations between addiction score and both sEPSC frequency and amplitude further support an association between AUD-like vulnerability and reduced BLA excitatory synaptic activity. Field potential recordings showed no group difference in bicuculline-induced disinhibition. However, stimulus–response curves showed a significant group effect, with lower evoked fEPSP amplitudes in vulnerable rats than in water controls, further supporting reduced excitatory neurotransmission in the BLA of vulnerable rats. In addition, current clamp results showed reduced intrinsic excitability in BLA neurons from vulnerable rats, as reflected by increased rheobase, lower AP frequency and higher AP latency.

Overall, findings from Paper I suggested that AUD-like vulnerability was associated with a hypoactive BLA profile, characterized by reduced excitatory synaptic transmission and decreased neuronal excitability. This interpretation was further supported by correlation analyses showing negative associations between addiction score and sEPSC frequency, sEPSC amplitude and the total number of APs. Thus, this hypoactive BLA profile may reflect altered processing of reward-related and motivational information (Wassum & Izquierdo, 2015). Importantly, altered BLA function has previously been linked to chronic alcohol exposure and alcohol-related behaviors (Gass et al., 2011; Läck et al., 2007; Price & McCool, 2022). However, previous studies reported increased BLA glutamatergic activity or excitability, which did not align with the hypoactive profile observed in Paper I. Importantly, the different outcome may reflect differences in experimental design, including active versus passive alcohol exposure, withdrawal state, stress-related components and timing of recordings. Finally, the absence of operant training in water-drinking controls should be considered when interpreting results.

4.1.3 ALTERED PL SYNAPTIC REGULATION AND INTRINSIC EXCITABILITY IN VULNERABLE RATS

Paper II extended this investigation by assessing neurophysiological properties in PL associated with AUD-like behavior. Whole cell results demonstrated that rats that fulfilled all three AUD-like criteria showed reduced sEPSC frequency in PL pyramidal neurons compared with water-drinking controls, 0 crit rats and 1 crit rats. In contrast, sEPSC amplitude, rise time and decay time were not altered, suggesting reduced spontaneous glutamatergic input rather than postsynaptic changes. Similar to Paper I, sEPSC frequency negatively

correlated with addiction score, further supporting the association between AUD-like behavior and neurophysiological alterations even across different brain regions. Given that the PL receives projections from the BLA (Krettek & Price, 1977), the decreased excitability of BLA neurons observed in Paper I could hypothetically contribute to the reduced excitatory input observed in the PL in Paper II. However, this represents a circuit-level hypothesis based on findings from separate cohorts and was not tested, as the recorded sEPSCs did not selectively assess excitatory projections from the BLA to the PL.

Despite the reduction in spontaneous excitatory input, current clamp results demonstrated increased intrinsic excitability in PL neurons from 3 crit rats compared with the other groups, as suggested by greater voltage changes in response to current injections and a tendency toward increased AP frequency. Input resistance was significantly higher in neurons from 3 crit rats as compared to 0 crit, 1 crit, 2 crit and water-drinking controls, whereas membrane capacitance did not differ among groups. Thus, the increased input resistance may contribute to the greater voltage responses by reducing current leakage across the membrane. One possible explanation could be that reduced excitatory input is accompanied by a compensatory increase in intrinsic excitability, whereby PL neurons adjust their input-output function to maintain neuronal output despite lower excitatory synaptic input (Desai et al., 1999; Pratt & Aizenman, 2007). Therefore, 3 crit rats may display a state in which PL neurons receive less spontaneous excitatory input but respond more readily when depolarized. Ultimately, this altered balance between excitatory input and intrinsic responsiveness may contribute to disrupted PL-dependent control over alcohol-directed behavior in AUD-like vulnerable rats.

In addition, Paper II assessed the mGluR2/3-mediated regulation of PL glutamatergic transmission in association with the AUD-like phenotypes. Field potential recordings following bath application of the mGluR2/3 agonist LY354740 reduced the amplitude of evoked field responses in all groups, but this effect was blunted in 3 crit rats. Moreover, LY354740 increased PPR in controls and 0–2 crit rats, whereas no change was observed in 3 crit rats. Given the role of mGluR2/3 in regulating glutamate release (Cartmell & Schoepp, 2000), these findings suggest impaired presynaptic control over glutamatergic transmission in PL of AUD-like vulnerable rats. This was in line with a previous study showing loss of mGluR2 function in association with escalated alcohol seeking in alcohol-dependent rats (Meinhardt et al., 2013). Importantly, Paper II did not distinguish between mGluR2 and mGluR3 or determine whether the observed effects occurred at specific excitatory inputs.

Future studies using receptor-subtype-selective pharmacological tools and pathway-specific approaches are therefore needed.

In contrast, no difference in GABAergic inhibitory control was detected among the 0–3 crit groups following bicuculline bath application. However, bicuculline-induced disinhibition was enhanced in alcohol-exposed rats compared with water-drinking controls, suggesting an effect associated with repeated alcohol exposure rather than specific vulnerability to an AUD-like phenotype. Notably, when interpreting these results, it should be considered that the water-drinking controls were not exposed to operant training, although a supplementary experiment in Paper II did not detect a significant effect of footshock exposure on PL glutamatergic synaptic transmission or on mGluR2/3-mediated depression.

In Paper IV, additional analysis further supported an association between AUD-like vulnerability and altered PL neurophysiology. Electrophysiological assessment comparing resilient saline-treated rats with vulnerable rats, including both saline- and nicotine-injected animals, showed more pronounced bicuculline-induced disinhibition in vulnerable animals, suggesting heightened $GABA_A$-mediated inhibitory control over PL output. Given the central role of GABAergic microcircuits in regulating PFC output, alterations in this system may affect the regulation of alcohol-related behaviors (Fish & Joffe, 2022).

Further comparison of the field-potential findings regarding bicuculline-induced disinhibition in Papers II and IV may suggest that GABAergic inhibitory tone in the PL is differentially affected across stages of alcohol exposure, given the longer operant alcohol self-administration period in Paper II. Although differences in alcohol exposure, nicotine treatment and behavioral classification should be considered, no differences were detected among groups meeting 0–3 criteria in Paper II, whereas vulnerable rats showed greater disinhibition than resilient saline-treated rats in Paper IV. One possible interpretation is that alterations in GABAergic neurotransmission may emerge during earlier phases of alcohol exposure, potentially contributing to the progression of alcohol-drinking behavior, but become less apparent as animals acquire more extensive alcohol experience. However, this interpretation remains speculative and requires further investigation.

Additionally, current clamp results from Paper IV further revealed increased AP frequency in vulnerable rats compared with resilient saline rats, supporting an association between AUD-like vulnerability and increased intrinsic excitability of PL neurons. Notably, this finding is consistent with Paper II, where 3 crit rats also showed increased intrinsic excitability. Moreover, correlation analyses and PCA from Paper IV revealed an association between PL neurophysiological properties and addiction score, with AP firing and bicuculline-induced disinhibition contributing to the separation between resilient and vulnerable phenotypes. This further supports the idea that AUD-like behavioral vulnerability is associated with functional alterations in PL. However, because vulnerable saline- and nicotine-exposed rats were combined in this comparison, this result should be interpreted cautiously, as potential treatment-dependent effects cannot be excluded. Nevertheless, these findings may help outline neurophysiological correlates of AUD-like behavior.

4.1.4 LIMITATIONS

Across papers, the selected study designs did not allow determination of whether the observed neurophysiological alterations preceded the development of AUD-like behavior or emerged as a consequence of alcohol exposure. Therefore, these findings should be interpreted as associations between behavioral vulnerability and neurophysiological properties, rather than as evidence of causality. The PCA findings from Paper IV should also be interpreted cautiously because of the small sample size and the non-independent selection of groups and variables. Future longitudinal studies using *in vivo* recordings before, during and after alcohol exposure could help identify predictive markers of vulnerability and clarify whether these neurophysiological changes represent pre-existing traits or consequences of repeated alcohol exposure.

4.1.5 CONCLUDING REMARKS ON AUD-LIKE VULNERABILITY

Taken together, Papers I, II and IV indicate that AUD-like vulnerability is associated with region-specific neurophysiological alterations within BLA and PL. These findings support the idea that AUD-like behavior is not driven by a single neurophysiological alteration, but by distributed adaptations across addiction-related circuits.

4.2 NICOTINE EXPOSURE PROMOTES ALCOHOL DRINKING AND REDUCES RESILIENCE TO AUD-LIKE BEHAVIOR

Alcohol and nicotine are frequently co-used, and epidemiological studies have consistently shown a strong association between nicotine use, higher alcohol consumption and AUD (Bobo & Husten, 2000; DiFranza & Guerrera, 1990; Grant et al., 2004; Hurley et al., 2012; Weinberger et al., 2016). In line with this clinical association, preclinical studies have shown that repeated nicotine exposure can increase voluntary alcohol intake in rats (Blomqvist et al., 1996; Olausson et al., 2001) as well as operant alcohol behavior responding (Domi et al., 2020; Lê et al., 2000). In Papers III and IV, this thesis further investigated this association by integrating voluntary alcohol intake under a two-bottle choice paradigm and operant alcohol self-administration in combination with repeated nicotine exposure.

4.2.1 REPEATED NICOTINE EXPOSURE INCREASES ALCOHOL INTAKE AND PREFERENCE

Papers III and IV used different experimental designs to assess alcohol drinking behavior during the two-bottle choice paradigm. In Paper III, intermittent access (three days/week), which has long been established to allow escalation of alcohol intake in animal models (Simms et al., 2008; Wise, 1973), was used, and intake was monitored every 24 h. In contrast, Paper IV used 6 h limited access, with multiple measurements during the day, including 30 min, 2 h and 6 h from the start of the session. Moreover, alcohol concentrations differed slightly, with 6–12% alcohol solutions used in Paper III, whereas 6–15% alcohol solutions were used in Paper IV. Nevertheless, repeated nicotine exposure increased alcohol intake over the longer access periods in both studies, corresponding to 24 h in Paper III and 6 h in Paper IV. After nicotine treatment ended, increased intake persisted at 24 h in Paper III and was detected at 30 min, but not at 6 h, in Paper IV.

In this context, nAChRs may represent key mechanistic candidates underlying the association between escalated alcohol intake and nicotine use. Indeed, prolonged nicotine treatment has previously been associated with long-lasting neuroadaptations involving nAChRs (Baker et al., 2013; Govind et al., 2012; Vallejo et al., 2005). Chronic nicotine exposure has been shown to upregulate α4β2-containing nAChRs in rat brain (Flores et al., 1992) and increase nicotine

binding sites in human brain (Benwell et al., 1988). Although alcohol does not act as a classical agonist at nAChRs, studies in cultured rat cortical neurons and recombinant human nAChRs have shown that alcohol can modulate nAChR function and potentiate ACh-evoked responses at specific receptor subtypes (Aistrup et al., 1999; Cardoso et al., 1999). Thus, nicotine-induced upregulation of nAChRs could enhance the reinforcing effects of alcohol through increased nAChR target availability. Further supporting the role of nAChRs, evidence has shown that nAChR blockade with nAChR antagonist mecamylamine reduces EtOH-induced increases in extracellular DA levels in NAc and decreases voluntary alcohol intake (Blomqvist et al., 1997; Ericson et al., 1998). To better outline this role of nicotinic receptors, Paper III further investigated the neurophysiological basis of nicotine promoting effects on alcohol intake and examined the mechanisms underlying the acute effects of EtOH and nicotine, which will be described in the following section.

Notably, two-bottle choice results from Paper IV indicated a time-dependent effect on alcohol drinking behavior, with nicotine-exposed rats showing reduced alcohol intake during the treatment period at the 30 min time point. Conversely, following nicotine cessation, increased alcohol intake was observed at 30 min, together with enhanced alcohol preference at both 30 min and 6 h. Importantly, bidirectional effects of nicotine on alcohol intake have been reported in previous studies (Alén et al., 2009; Dyr et al., 1999), suggesting that the direction of nicotine's effect may depend on treatment phase, timing of measurement and experimental design. The temporary decrease in alcohol intake during nicotine treatment may reflect a transient substitution-like effect, whereby nicotine-induced activation of mesolimbic DA signaling temporarily reduces the drive to consume alcohol. In line with this interpretation, administration of the nAChR partial agonist varenicline has been shown to prevent alcohol-induced DA release (Ericson et al., 2009). Nicotine-induced changes in DA signaling were further investigated in Paper III using *in vivo* microdialysis, and these data are discussed in more detail in the following section. An alternative explanation is that nicotine may initially produce aversive effects, temporarily reducing alcohol intake, whereas tolerance to these effects may develop over repeated exposure (Clarke & Kumar, 1983).

Additional analysis of the two-bottle choice data from both Papers III and IV revealed a baseline preference-dependent effect of repeated nicotine exposure on alcohol drinking. In Paper III, nicotine increased alcohol preference in rats with low baseline alcohol preference, whereas no corresponding effect was

observed in rats with high baseline alcohol preference. Similarly, in Paper IV, nicotine-exposed rats with low baseline preference showed increased alcohol intake and preference after repeated nicotine exposure at both the 30 min and 6 h time points. In contrast, rats with high baseline preference did not show significant changes between baseline and the post-injection period. Thus, despite differences in experimental design, both studies indicate that the effect of repeated nicotine exposure was most evident in animals with initially lower alcohol preference.

One possible interpretation is that animals with high baseline preference already show elevated alcohol intake, leaving less room for further nicotine-induced escalation, whereas low-preference animals may be more sensitive to nicotine-induced adaptations that promote alcohol drinking. Thus, nicotine exposure may act as a factor that shifts a subset of animals from a lower-drinking phenotype toward higher alcohol consumption, thereby contributing to inter-individual variability in alcohol-related outcomes. This finding is consistent with previous evidence suggesting the relevance of baseline alcohol preference in nAChR-mediated effects on alcohol drinking behavior (Blomqvist et al., 1996), where mecamylamine reduced alcohol intake in high-preferring but not low-preferring rats. Another explanation might be that high-preference rats display a ceiling effect, whereby activation of the mesolimbic DA system by alcohol is already sufficient to support elevated intake and preference, making further nicotine-induced increases more difficult to detect. Importantly, the absence of nicotine-induced effects on alcohol drinking behavior of high-preference rats should not be interpreted as evidence that nicotine has no pharmacological effect on these animals.

Field potential data from a separate cohort in Paper III, discussed in the following section, examined nicotine-induced synaptic depression in rats classified according to alcohol consumption rather than alcohol preference. These electrophysiological findings should therefore be interpreted separately from the baseline-preference analysis described above.

Paper III also examined alcohol intake during quinine adulteration. Quinine significantly reduced alcohol intake relative to baseline in saline-treated rats, whereas no significant reduction was detected in nicotine-treated rats. However, alcohol intake during quinine adulteration did not differ significantly between the treatment groups. The quinine-adulteration test is particularly relevant because aversion-resistant alcohol intake is commonly used as a measure of compulsive-like drinking, reflecting continued consumption

despite an aversive consequence (Hopf et al., 2010). Thus, the enhanced alcohol drinking behavior following repeated nicotine exposure, together with the pattern observed during quinine adulteration, may reflect more than a simple increase in consumption. Indeed, it may also indicate a shift toward a more persistent alcohol-directed behavioral pattern. This is consistent with a previous study showing that chronic nicotine can accelerate the escalation of alcohol intake and promote compulsive-like alcohol drinking in rats (Leão et al., 2015).

4.2.2 REDUCED RESILIENCE TO AUD-LIKE BEHAVIOR FOLLOWING REPEATED NICOTINE EXPOSURE

Paper IV extended the behavioral assessment by testing saline- and nicotine-exposed rats in operant alcohol self-administration boxes to evaluate the development of AUD-like behaviors following previous nicotine exposure. Results showed that previous nicotine exposure enhanced operant alcohol responding. Indeed, nicotine-exposed rats obtained a higher number of alcohol rewards and displayed an increased number of active lever presses, which was in line with previous studies (Lê et al., 2000; Lê et al., 2014; Montanari et al., 2021; Ostroumov et al., 2015). In contrast, the number of inactive lever presses was not altered between saline- and nicotine-treated rats, suggesting that nicotine exposure selectively increased alcohol-directed responding rather than producing a nonspecific increase in operant behavior. When comparing the saline and the nicotine groups for the three AUD-like behaviors used in this multisymptomatic approach, no group differences emerged in persistence in alcohol seeking when alcohol was not available, motivation for alcohol under a progressive ratio schedule of reinforcement or resistance to punishment. Although this differs from a previous study reporting increased alcohol self-administration and motivation under a progressive-ratio schedule following nicotine exposure (Domi et al., 2020), that study did not apply the full three-criterion AUD-like model or calculate a composite addiction score. It should also be noted that Paper IV used a substantially shorter period of operant alcohol self-administration than the previous study, which may have limited the emergence of group differences in the individual AUD-like behaviors. Notably, the scores obtained for each criterion were normalized to z-scores for each rat and integrated into a composite measure defined as addiction score, which has previously been shown to be highly representative of addiction-like behavior (Belin et al., 2009; Deroche-Gamonet et al., 2004). When rats were evaluated based on the addiction score, analysis revealed that all individuals

classified as resilient to AUD-like behaviors were treated with saline, whereas vulnerable rats included both saline- and nicotine-treated individuals. Specifically, six of 15 saline-treated rats and none of 15 nicotine-treated rats were classified as resilient, and the proportion of resilient animals differed between treatment groups. This finding suggests repeated nicotine exposure reduced resilience to developing an AUD-like phenotype. In this context, the absence of resilient nicotine-exposed rats suggests that repeated nicotine exposure may reduce the ability of some animals to maintain controlled alcohol-directed behavior, shifting the overall distribution of behavioral phenotypes toward vulnerability.

4.2.3 LIMITATIONS

While the multidimensional approach used in Paper IV provides a controlled experimental model to assess the development of AUD-like behavior in association with prolonged nicotine exposure, it does not fully reproduce the complexity of human nicotine use, including smoking patterns or method of administration (Matta et al., 2007). Because no resilient nicotine-exposed group was available, the design could not fully separate effects associated with nicotine exposure from those associated with the AUD-like phenotype. Moreover, increased alcohol intake after nicotine exposure may contribute to downstream behavioral and neurophysiological adaptations, which makes it difficult to fully separate direct nicotine-induced effects from alcohol-induced consequences.

4.2.4 CONCLUDING REMARKS ON NICOTINE EXPOSURE AND AUD-LIKE VULNERABILITY

Collectively, findings from Papers III and IV support the previous literature regarding repeated nicotine exposure promoting alcohol drinking while supporting the hypothesis that nicotine may facilitate the transition toward more maladaptive alcohol-directed behavior. This provides a behavioral basis for the following section, which addresses the corresponding neurobiological underpinnings within the mPFC.

4.3 NEUROBIOLOGICAL UNDERPINNINGS OF NICOTINE-ALCOHOL USE IN MPFC

The behavioral findings from Papers III and IV indicate that repeated nicotine exposure increased alcohol drinking and reduced resilience to AUD-like behavior. Further investigations aimed at identifying the associated neurophysiological correlates were performed in both Papers III and IV, focusing on mPFC using *in vivo* microdialysis and PL using *ex vivo* electrophysiology. The rationale behind the selected regions is related to the role of the mPFC in the regulation of executive control and drug seeking (Dalley et al., 2004). Impaired mPFC/PL function might therefore underlie the behavioral phenotype associated with prolonged nicotine exposure. Thus, nicotine-induced adaptations in mPFC may reduce the ability of cortical circuits to regulate alcohol use, thereby contributing to the transition toward more maladaptive alcohol-related behavior. Importantly, Paper III comprised multiple experiments and experimental cohorts. Accordingly, the behavioral, electrophysiological, and microdialysis findings are integrated here at a conceptual level.

4.3.1 PROLONGED NICOTINE EXPOSURE IS ASSOCIATED WITH ALTERED VTA–MPFC DA SIGNALING

Nicotine is well known to act on nAChRs in the VTA, thereby increasing DA release in the NAc (Nisell et al., 1994) and repeated exposure has been associated with neuroadaptations in mesocorticolimbic DA circuits, including altered VTA DA neuron excitability and downstream DA transmission (Wills & Kenny, 2021). Because mPFC receives DA projections from VTA, Paper III first assessed whether repeated nicotine exposure was associated with neurophysiological alterations in VTA DA neurons, which could influence downstream mPFC function. In this context, Paper III provided further evidence that repeated nicotine exposure altered mesocorticolimbic DA signaling. Indeed, cell attached recordings in VTA DA neurons showed increased spontaneous event frequency in nicotine-treated alcohol-consuming rats compared with saline-exposed animals. This suggests that repeated nicotine exposure was associated with enhanced spontaneous activity of VTA DA neurons. In a separate cohort, *in vivo* microdialysis was performed in the mPFC of nicotine- and saline-treated rats to assess downstream DA signaling. In line with the VTA cell attached recordings, the microdialysis data showed

increased baseline extracellular DA levels in the mPFC of nicotine-exposed rats compared with saline-exposed rats. Together, these findings support an association between repeated nicotine treatment and neuroadaptations within the VTA–mPFC circuit, which may influence how mPFC circuits respond to alcohol-related signals and contribute to nicotine-induced escalation of alcohol intake. This is of particular relevance given that mesocortical DA signaling plays an important role in modulating executive function and prefrontal control over reward-directed behavior (Seamans & Yang, 2004).

4.3.2 REPEATED NICOTINE EXPOSURE ENHANCES PL SENSITIVITY TO ACUTE ETOH

In Paper III, field potential recordings in alcohol-naive rats showed that repeated nicotine exposure can sensitize PL responses to acute 50 mM EtOH bath application. Results demonstrated a stronger EtOH-induced reduction in fEPSP amplitude in nicotine-injected rats compared with saline-injected rats. In addition, correlation analysis showed a positive association between alcohol intake and EtOH-induced depression of fEPSP amplitude in PL, as well as, in nicotine-naive rats, between alcohol intake and nicotine-induced synaptic depression. Collectively, these findings further support the literature suggesting that nAChRs are involved in mediating neurophysiological and behavioral alcohol effects (Blomqvist et al., 1996; Feduccia et al., 2012; Hendrickson et al., 2013; Söderpalm et al., 2000).

Moreover, repeated nicotine exposure was also associated with altered PL synaptic responsiveness to nicotine itself. Indeed, field potential recordings in PL from alcohol-drinking rats demonstrated a stronger depression of fEPSP amplitude following nicotine bath application in nicotine-treated rats as compared with saline-treated rats. This finding is consistent with evidence showing that chronic nicotine exposure can induce nAChR upregulation (Flores et al., 1992), therefore potentially increasing the sensitivity of PL glutamatergic transmission to acute nicotinic modulation. Additionally, given the modulatory effects of EtOH on nAChRs, these results suggest that prolonged nicotine exposure may alter acute neurobiological responses in the PL and potentially influence alcohol-related behaviors through alterations in nAChR-mediated signaling (Aistrup et al., 1999; Cardoso et al., 1999).

In a separate cohort, PL field potential recordings showed a borderline group effect on synaptic depression following acute nicotine bath perfusion when rats were classified as high or low alcohol consumers based on the 20th and 80th

percentiles of alcohol intake, respectively, with greater depression observed in high-consuming rats. This finding suggests that alcohol consumption level may be associated with differences in PL sensitivity to acute nicotine. These differences may reflect variation in nAChR-mediated functional modulation, although the underlying mechanisms were not directly assessed. In addition, while whole cell recordings in the PL showed no significant difference in baseline sEPSC frequency between nicotine- and saline-exposed rats, acute EtOH perfusion reduced sEPSC frequency in both groups, with the reduction being more pronounced in nicotine-exposed slices.

At the neurochemical level, *in vivo* microdialysis experiments under baseline conditions showed increased extracellular glutamate levels in the mPFC of nicotine-exposed rats compared with saline-exposed rats. This increase may reflect altered astrocyte-mediated regulation of extracellular glutamate, as astrocytes play a central role in glutamate clearance and nicotine exposure has been shown to induce morphological and functional changes in astrocytes, including in the PFC (Aryal et al., 2021). Importantly, although a baseline group difference was detected with *in vivo* microdialysis but not with whole cell recordings, it should be noted that these techniques examine different neurobiological aspects. Indeed, while sEPSC frequency recorded in whole cell recordings provides a measure of spontaneous presynaptic excitatory input onto recorded PL neurons, *in vivo* microdialysis evaluates extracellular neurotransmitter levels across a broader tissue region. These complementary measurements may therefore provide a more comprehensive characterization of the neurobiological state, provided that their methodological differences are considered.

4.3.3 GLUTAMATE/GLUTAMINE HOMEOSTASIS AS A POTENTIAL MEDIATOR OF ACUTE ETOH EFFECTS IN MPFC

In Paper III, *in vivo* microdialysis was also used to assess DA, glutamate and glutamine extracellular levels in mPFC following systemic EtOH (2.5 g/kg) administration in saline- and nicotine-treated rats. Results showed increased extracellular DA levels in both treatment groups following systemic EtOH administration, with no group difference between saline- and nicotine-exposed rats. However, extracellular glutamate and glutamine levels following systemic EtOH administration were selectively reduced in nicotine-exposed rats. Moreover, correlation analysis showed a positive correlation between glutamate and glutamine EtOH-induced changes. These findings suggest that

repeated nicotine exposure may alter glutamate/glutamine homeostasis in mPFC, potentially through nAChR-dependent effects on astrocyte morphology and Ca^{2+} signaling (Aryal et al., 2021). In addition, evidence from cultured astrocytes indicates that nicotine can alter glutamate uptake and glutamine synthetase activity (Lim & Kim, 2003). Current results are further supported by evidence indicating that alcohol can disrupt astrocyte-dependent glutamate homeostasis (Adermark & Bowers, 2016).

Given the microdialysis data suggesting a role for glutamine in mediating EtOH effects in mPFC, further investigations were conducted using field potential and whole cell recordings in PL. Interestingly, while glutamine itself showed no effect on fEPSP amplitude, pretreatment with glutamine prevented the synaptic depression induced by bath application of EtOH. Similarly, glutamine pretreatment blocked the acute EtOH-induced reduction of sEPSC frequency in whole cell recordings. One possible interpretation is that acute EtOH reduces excitatory transmission by interfering with glutamine-dependent replenishment of presynaptic glutamate pools. Under physiological conditions, glutamate is rapidly taken up from the extracellular environment into astrocytes, where it is converted into glutamine, which is then released again into the extracellular space. Glutamine is taken up by neurons and transformed into glutamate, which can then be loaded into presynaptic vesicles (Andersen, 2025; Tani et al., 2014). In field potential experiments, when exogenous glutamine is bath applied, this supplementation may have no detectable effect because it might not alter glutamate release per se. However, acute EtOH application might disrupt the glutamate/glutamine homeostasis, potentially impairing astrocytic function (Adermark & Bowers, 2016) and therefore limiting glutamine availability, ultimately interfering with glutamate synthesis. In contrast, when exogenous glutamine is co-administered with EtOH, higher levels of glutamine might preserve glutamate synthesis and vesicular glutamate availability, thereby preventing the EtOH-induced reduction in glutamate release. This interpretation is consistent with the role of the astrocyte–neuron glutamate/glutamine cycle in maintaining excitatory synaptic transmission (Tani et al., 2014) and with evidence that alcohol can disrupt astrocyte-dependent glutamate homeostasis (Adermark & Bowers, 2016).

4.3.4 AUD-LIKE VULNERABILITY FOLLOWING REPEATED NICOTINE EXPOSURE IS LINKED TO INCREASED PL NEURONAL EXCITABILITY AND ENHANCED GABAERGIC CONTROL

Paper IV extended these findings by examining whether PL neurophysiological alterations were associated with AUD-like vulnerability after repeated nicotine or saline exposure. Rats selected for electrophysiological assessment represented the AUD-like behavioral extremes of the population, including resilient saline-treated rats, vulnerable saline-treated rats and vulnerable nicotine-treated rats. Field potential recordings in PL following the application of a stimulus/response protocol under baseline conditions showed reduced fEPSP responses in vulnerable saline rats compared with resilient saline rats and vulnerable nicotine rats. This suggested that AUD-like vulnerability in saline-treated animals was associated with reduced excitatory synaptic transmission in PL. However, $GABA_A$ blockade with bicuculline revealed greater disinhibition in both saline and nicotine vulnerable groups compared to the resilient saline group. This finding may reflect stronger local inhibitory control over PL excitatory synaptic transmission in association with the development of AUD-like behavior. GABAergic microcircuits within PFC have been shown to play a crucial role in the regulation of pyramidal neuron function and have been suggested as a core component in mediating the neurobiological effects of alcohol and in preclinical models of AUD (Fish & Joffe, 2022). Thus, altered inhibitory control may impair PL regulation of seeking behavior (Gutman et al., 2017).

Moreover, the application of a stimulus/response protocol during bicuculline perfusion demonstrated increased fEPSP amplitude in vulnerable nicotine-treated rats compared with saline-treated rats from both the vulnerable and resilient groups. This suggests that, once inhibitory control is removed, excitatory synaptic transmission in PL might be particularly heightened in nicotine-treated vulnerable rats. In line with this, whole cell recording in current clamp mode revealed increased AP frequency in vulnerable nicotine rats compared with resilient saline rats, suggesting increased intrinsic excitability in PL neurons in association with AUD-like behavior and previous nicotine exposure. Notably, this finding was in line with current clamp results from Paper II, showing increased AP frequency in 3 crit rats. Altogether, the enhanced inhibitory control observed in PL may reflect a compensatory mechanism in response to increased neuronal excitability. This interpretation

might be further supported by *in vivo* microdialysis evidence from Paper III. Indeed, repeated nicotine exposure increased baseline glutamate levels in mPFC, which might recruit local inhibitory mechanisms to stabilize network activity. Consistent with this, further analysis of field potential data from Paper IV showed that, when rats were combined across AUD-like phenotypes, nicotine-exposed rats showed greater bicuculline-induced disinhibition in PL compared with saline-exposed rats. This last finding provides supportive evidence that repeated nicotine exposure may enhance inhibitory regulation in PL, while it should be interpreted cautiously since animals were combined across AUD-like phenotypes and the analysis cannot fully separate nicotine-dependent effects from vulnerability-related effects.

To further investigate associations between behavioral and electrophysiological measurements, correlation analyses and PCA were conducted in Paper IV. Results showed that higher addiction scores were associated with distinct PL neurophysiological parameters, including greater AP firing and enhanced bicuculline-induced disinhibition. These findings further support an association between the behavioral phenotype and neurophysiological properties in PL.

In contrast to PL, Paper IV did not detect corresponding electrophysiological alterations in NAcSh. Indeed, both field potential recordings and whole cell results showed no group differences, either under baseline conditions or when assessing bicuculline-induced disinhibition. Notably, this does not exclude a role for NAcSh in alcohol-related behavior, but suggests that neuroadaptations within PL were more evident at the measurement time point examined. It is therefore possible that alterations in NAcSh may have occurred at earlier or later stages of the experimental procedure. On the other hand, these findings support regional specificity in association with the development of AUD-like vulnerability following repeated nicotine exposure.

4.3.5 CONCLUDING REMARKS ON THE CO-USE OF NICOTINE AND ALCOHOL

Taken together, Papers III and IV suggest that repeated nicotine exposure alters mPFC function in ways that may reduce resilience to developing an AUD-like vulnerable phenotype and weaken control over alcohol-directed behavior. Across the two studies, nicotine exposure was associated with changes in VTA–mPFC DA signaling, disrupted glutamate/glutamine regulation and altered PL excitatory and inhibitory balance. In addition, the absence of

corresponding detected alterations in NAcSh further suggests that neuroadaptations associated with prolonged nicotine exposure and AUD-like vulnerability were region-specific under these experimental conditions.

4.4 ACUTE ETOH AND NICOTINE MODULATION OF PL SYNAPTIC TRANSMISSION

The findings discussed above indicate that repeated nicotine exposure altered PL responsiveness to EtOH and nicotine. To better understand the mechanisms underlying these effects, Paper III performed acute pharmacological experiments in slices from naïve rats. This approach allowed the local synaptic effects of EtOH and nicotine to be examined independently of previous alcohol or nicotine exposure. Thus, the main aim was to assess acute modulation of PL synaptic transmission by EtOH bath application and to identify potential receptor systems contributing to these effects.

4.4.1 ACUTE ETOH REDUCES PL EXCITATORY TRANSMISSION THROUGH NACHR- AND D2/D3 RECEPTOR-DEPENDENT MECHANISMS

Field potential recordings were first performed using increasing EtOH concentrations, including 20 mM, 50 mM and 100 mM. Acute EtOH bath application decreased fEPSP amplitude in PL in a concentration-dependent manner, with 100 mM EtOH exerting a more pronounced synaptic depression. For further pharmacological assessments, we selected 50 mM EtOH, a concentration previously used in acute brain slice electrophysiology studies assessing EtOH-induced effects (Adermark & Lovinger, 2006; Clarke & Adermark, 2010; Lovinger et al., 1990; Patton et al., 2016).

Notably, EtOH-induced synaptic depression in PL was also observed in whole cell recordings, which further demonstrated a reduction in sEPSC frequency following EtOH bath perfusion compared with aCSF. In contrast, no significant change was detected in spontaneous inhibitory activity following acute EtOH challenge, as sIPSC frequency was not affected. This was consistent with previous whole cell work showing no effect of EtOH bath perfusion on either spontaneous or evoked $GABA_A$-mediated IPSCs (Weitlauf & Woodward, 2008). These findings suggest that the acute depressant effect of EtOH in PL was mainly driven by modulation of excitatory synaptic

transmission, rather than by increased inhibitory synaptic activity. Thus, the whole cell findings suggest that acute EtOH may reduce PL glutamatergic transmission at least partly through a presynaptic mechanism, as sEPSC frequency is commonly interpreted as an indirect measure of presynaptic excitatory input (Choi & Lovinger, 1997).

To further extend these findings, Paper III assessed receptor systems that may contribute to acute EtOH effects in PL, including mGluR2/3, NMDA receptors, nAChRs and D2/D3 receptors. EtOH-induced synaptic depression was not prevented by previous blockade of mGluR2/3 with LY341495 or NMDA receptors with APV. Although acute EtOH has been shown to suppress NMDAR-mediated synaptic transmission (Lovinger et al., 1990; Weitlauf & Woodward, 2008), EtOH-induced synaptic depression in our experiments persisted during APV application. Thus, under the present recording conditions, the acute EtOH effect was unlikely to be mediated by direct inhibition of the NMDAR-mediated component of PL excitatory transmission.

Moreover, altered mGluR2/3 function has been reported in alcohol-dependent rats (Meinhardt et al., 2013). However, since EtOH-induced synaptic depression was not prevented by LY341495 application, our results suggest that mGluR2/3 signaling was not required for the acute EtOH effect in PL under these recording conditions. This interpretation is consistent with previous whole cell work in other circuits showing no significant effect of acute EtOH on mGluR2 agonist-evoked currents (Botta et al., 2014).

Field potential experiments from Paper III also showed that pretreatment with the nAChR antagonist mecamylamine prevented EtOH-induced depression of fEPSP amplitude. This supports the idea that nAChRs are required for the acute EtOH effect on PL glutamatergic transmission and is in line with previous evidence showing that EtOH can modulate nAChR function (Aistrup et al., 1999; Cardoso et al., 1999). Moreover, it is consistent with earlier studies showing that nAChR blockade can reduce EtOH-induced activation of mesolimbic DA signaling and decrease voluntary alcohol intake (Blomqvist et al., 1993; Blomqvist et al., 1997; Ericson et al., 1998).

In line with *in vivo* microdialysis findings from Paper III showing increased DA levels in mPFC following systemic administration of EtOH, field potential recordings further supported the involvement of DA signaling in PL. Indeed, pretreatment with the D2/D3 receptor antagonist sulpiride prevented EtOH-induced depression of fEPSP amplitude in PL, suggesting that the acute EtOH

effect was dependent on D2/D3 receptor signaling. Since mPFC receives DA projections from VTA, local D2/D3 receptor activation may be important for the regulation of excitatory synaptic transmission in PL. This interpretation is further supported by evidence indicating that DA receptors modulate glutamatergic transmission in PFC (Seamans & Yang, 2004).

4.4.2 ACUTE NICOTINE REDUCES PL EXCITATORY TRANSMISSION THROUGH NACHR- AND D2/D3 RECEPTOR-DEPENDENT MECHANISMS

To further outline the alcohol–nicotine interaction, Paper III investigated the acute nicotine effects in PL. Field potential recordings showed reduced fEPSP amplitude in PL following bath application of 1 μM nicotine. This finding is consistent with previous cortical electrophysiological work, although differences in recording configuration and nicotine concentration should be noted (Levy et al., 2006). Further field potential experiments revealed that, as expected, the acute nicotine effect on PL synaptic transmission was blocked by pretreatment with mecamylamine. Moreover, prior application of sulpiride also prevented nicotine-induced synaptic depression, suggesting that this acute modulatory effect was also dependent on D2/D3 receptor signaling.

Thus, acute EtOH and nicotine effects appear to involve nAChR- and D2/D3 receptor-dependent mechanisms regulating PL excitatory transmission. The absence of an additive effect when EtOH and nicotine were co-applied is consistent with convergent mechanisms. However, this does not prove that EtOH and nicotine act through the same pathway, as a ceiling effect may have occurred, with one drug already producing a near-maximal effect.

4.4.3 CONCLUDING REMARKS ON ACUTE ETOH AND NICOTINE MODULATION IN PL

Collectively, these acute pharmacological findings suggest a mechanistic framework for understanding the neurophysiological basis of the nicotine–alcohol interaction. Thus, Paper III showed that acute EtOH reduced PL glutamatergic transmission through a mechanism potentially requiring nAChRs and D2/D3 receptors, and acute nicotine produced a comparable synaptic depression involving the same receptor systems. These findings further support the idea that repeated nicotine exposure may enhance alcohol-related behaviors through the involvement of specific receptor systems in PL that are modulated by both nicotine and EtOH.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Although alcohol use is common in the population, only a subset of individuals transition from recreational to maladaptive use, potentially developing AUD (MacKillop et al., 2022). This thesis extends these observations to a rat model of AUD-like behavior, using an established multisymptomatic model of addiction based on DSM-IV criteria (Deroche-Gamonet et al., 2004). Consistent with the human condition and with previous studies, this thesis showed that only a fraction of animals exposed to chronic alcohol develop vulnerability to AUD-like behavior (Domi et al., 2019). In addition, this work combined behavioral studies with *ex vivo* electrophysiology and *in vivo* microdialysis to define neurophysiological profiles associated with AUD-like vulnerable and resilient phenotypes. Among the factors associated with alcohol use and abuse in humans, smoking, or more generally nicotine use, is of particular relevance (DiFranza & Guerrera, 1990). Although the neurobiological and behavioral mechanisms underlying the nicotine-promoting effect on alcohol-related behaviors have been investigated for decades, the mechanisms underlying this association are still not fully understood. In this context, nAChRs have been repeatedly shown to play a key role in mediating alcohol reward-related properties and promoting escalation of alcohol use (Blomqvist et al., 1993; Blomqvist et al., 1996; Ericson et al., 1998). Thus, the studies included in this thesis aimed to disentangle receptor-, neurotransmitter- and circuit-level mechanisms underlying the alcohol–nicotine interaction, and to further investigate the neurobiological basis of AUD-like behavior in rats.

The findings from Papers I and II indicate that AUD-like vulnerability is associated with region-specific neurophysiological alterations within BLA and PL. In BLA, vulnerable rats displayed reduced excitatory synaptic input and lower neuronal excitability, suggesting an overall hypoactive neurophysiological profile within this brain region in association with AUD-like vulnerability. Although current literature includes contrasting findings, previous studies do not fully encompass the complex behavioral repertoire that characterizes the development of AUD-like behavior in rats. Therefore, further research is needed. In PL, AUD-like vulnerability was associated with reduced spontaneous excitatory input, altered mGluR2/3-mediated regulation of glutamatergic transmission and increased intrinsic excitability. These findings suggest that AUD-like behavior is reflected by distributed and region-specific

changes across brain circuits involved in emotional processing, reward-related learning and behavioral control.

Across the included studies, the PL was a recurrent site of detectable neurophysiological alterations associated with AUD-like vulnerability, repeated nicotine exposure, and acute responses to EtOH and nicotine. However, the present experimental designs do not allow determination of whether these neurophysiological alterations represent pre-existing vulnerability factors, consequences of alcohol exposure or compensatory adaptations. Future studies should assess whether the neurophysiological alterations observed in this thesis are causes or consequences of AUD-like behavior. Therefore, longitudinal approaches, for instance including *in vivo* assessments at multiple time points, may be required.

The results from Papers III and IV further support clinical and preclinical literature indicating that nicotine exposure can promote alcohol intake and may be associated with reduced resilience to developing AUD-like behavior. At the neurobiological level, repeated nicotine exposure was associated with altered VTA–mPFC DA signaling and increased baseline extracellular DA and glutamate levels in mPFC. These findings support that prolonged nicotine exposure is associated with neuroadaptations within the mesocortical pathway that may ultimately lead to dysregulation in cortical areas. Moreover, pre-exposure to nicotine was associated with enhanced responses to local EtOH or nicotine in PL, further suggesting that nicotine exposure may increase PL sensitivity to acute alcohol- and nicotine-related synaptic modulation. Paper III also revealed a potential alteration of glutamate/glutamine homeostasis in association with repeated nicotine exposure in response to systemic EtOH. Moreover, field potential recordings showed that pretreatment with glutamine prevented the acute effect of EtOH in PL, suggesting that glutamine may exert a protective effect against EtOH-induced synaptic depression, and that its action should therefore be further investigated. Interestingly, an early study using a two-bottle choice paradigm with 10% alcohol and water reported reduced voluntary alcohol intake following oral glutamine (glutamine supplementation in food) at 100 mg/day (Rogers et al., 1955). Notably, the glutamine-induced reduction in alcohol intake appeared more pronounced in male than in female rats, suggesting a possible sex-dependent effect. Additional studies in female rats are therefore warranted. Collectively, these findings suggest that repeated nicotine exposure induces neuroadaptations in mPFC/PL that may alter how cortical circuits respond to alcohol-related signals. In particular, the data indicate that nicotine-induced neuroadaptations

may involve changes in mesocortical DA signaling, glutamatergic transmission and astrocyte-linked glutamate/glutamine homeostasis, and may therefore contribute to the increased alcohol intake and reduced resilience observed after repeated nicotine exposure.

The acute pharmacological findings further suggest that EtOH and nicotine may converge on overlapping mechanisms regulating PL excitatory transmission. Both acute EtOH and acute nicotine were associated with synaptic depression in PL, and their effects were prevented by nAChR or D2/D3 receptor blockade. Together with the lack of an additive effect during EtOH and nicotine co-application, these findings are consistent with overlapping or convergent nAChR- and D2/D3 receptor-dependent mechanisms in the PL, although alternative explanations, such as a near-maximal drug effect, cannot be excluded.

Future studies should also address circuit and cell-type specificity. Although the present findings suggest altered function in the BLA and PL, the specific afferent and efferent pathways involved remain unclear. Retrograde neuronal tracing could be combined with patch-clamp recordings to characterize PL neurons according to their projection targets. Pathway-specific stimulation of labeled afferents could further determine whether the observed alterations in synaptic transmission occur within defined circuits, such as projections from the BLA or VTA to the PL. These approaches would provide a more precise circuit-level understanding of AUD-like vulnerability and nicotine-induced neuroadaptations. In addition, cell-type-specific characterization using molecular markers to distinguish pyramidal neurons and specific interneuron subtypes, alongside morphological and electrophysiological properties, could help clarify how excitatory and inhibitory microcircuits contribute to AUD-like vulnerability and the reduced resilience associated with nicotine exposure.

The mechanisms linking nicotine exposure to altered EtOH sensitivity in PL also require further investigation. Future studies should clarify the contribution of specific nAChR subtypes to the effects of nicotine and EtOH on PL excitatory transmission. Polymorphisms in nAChR genes, particularly those affecting the α4 subunit, may be relevant in this context, as they have been shown to alter nicotine-induced receptor activation and alcohol modulation of nAChR function (Butt et al., 2003).

In addition, the role of D2 versus D3 receptors should be addressed using more selective pharmacological tools or genetic approaches. Since the present

findings suggest that both EtOH- and nicotine-induced synaptic depression require nAChR and D2/D3 receptor signaling, identifying the specific receptor subtypes involved would help clarify the receptor mechanisms underlying the effects of alcohol and nicotine in the PL. Beyond these mechanisms, future studies should distinguish the respective contributions of mGluR2 and mGluR3 to the altered regulation of PL glutamatergic transmission associated with AUD-like vulnerability.

The glutamate/glutamine findings also open important future directions. Repeated nicotine exposure altered glutamate and glutamine responses to systemic EtOH, while glutamine pretreatment prevented acute EtOH-induced depression of PL excitatory transmission. This suggests that astrocyte–neuron metabolic interactions may contribute to the regulation of acute EtOH effects under baseline conditions and may be further altered following prolonged nicotine exposure. Future studies should directly assess astrocytic mechanisms, including glutamate uptake, glutamine synthesis, glutamine transport and Ca^{2+} signaling within astrocytes.

Finally, future studies should include female rats. The present thesis was conducted in male rats, which limits the extent to which the findings can be generalized across sexes. Given that sex differences can influence alcohol intake, nicotine sensitivity, DA signaling and the development of addiction-like behaviors, it will be important to determine whether the neurophysiological signatures identified here are conserved in females or whether distinct mechanisms contribute to AUD-like vulnerability and alcohol–nicotine interactions across sexes (Becker & Koob, 2016; Moen & Lee, 2021).

From a translational perspective, the findings from this thesis suggest several mechanisms that may be investigated to develop pharmacological interventions. Potential strategies could include restoring mGluR2/3-mediated control of glutamate release, supporting astrocytic glutamate uptake and glutamine cycling or selectively modulating nAChR-dependent and D2/D3 receptor-dependent signaling. Moreover, given the association between nicotine use and AUD, more targeted treatments for individuals who co-use alcohol and nicotine should be considered.

In conclusion, this thesis identifies neurophysiological signatures in PL and BLA associated with AUD-like vulnerability, supports previous evidence that repeated nicotine exposure increases alcohol intake and further extends these

findings by showing that prolonged nicotine exposure reduces resilience to AUD-like behavior and alters mPFC/PL function. Thus, this work provides a basis for further preclinical investigation and may help guide the identification of pharmacological targets related to AUD-like vulnerability and nicotine–alcohol co-use, as well as future studies examining the relevance of these mechanisms in humans.

USE OF GENERATIVE AI

ChatGPT was used for language improvements. The author is responsible for the content and its interpretation.

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