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# TITLE OF THE Ph.D. THESIS

Alcohol-induced activation of the mesocorticolimbic dopaminergic system and its modulation by energy drinks and caffeine: neurochemical, behavioral, and immunohistochemical analysis

Scientific Disciplinary Sector(s)

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# ABSTRACT

The co-consumption of alcohol and caffeinated energy drinks among adolescents represents a growing public health concern, raising questions about its negative impact on the developing brain. Therefore, this PhD thesis aimed to investigate the effects of alcohol and caffeine, as well as alcohol and Red Bull® (AMED), on the responsivity of the mesocorticolimbic dopaminergic system, a neural circuit critically involved in reward processing and motivated behavior. During adolescence, this system is characterized by high vulnerability, which may lead to abnormal maturation in adulthood, resulting in impulsivity and an increased risk of compulsive drug use. Accordingly, this project was designed to investigate not only the acute effects of these substances, but also their long-term consequences on this system in adulthood following adolescent exposure. Notably, this study is among the first to employ a multidimensional approach across behavioral, neurochemical, and immunohistochemical levels, evaluating acute, subchronic, and chronic paradigms under both contingent and non-contingent conditions, providing new insights into the impact of these substances on neurodevelopment.

Findings demonstrate that acute pretreatment of adult rats with caffeine, the main stimulating component of Red Bull®, prevents the alcohol-induced formation of salsolinol in the posterior ventral tegmental area and the associated increase of dopamine transmission in the nucleus accumbens shell (AcbSh). Similarly, acute administration of AMED attenuates the alcohol-stimulated expression of phosphorylated extracellular signal-regulated kinase (pERK)-positive cells and of dopaminergic transmission in the AcbSh, although it did not prevent the alcohol-dependent reduction of locomotion. Subchronic AMED exposure, using an intermittent binge-like protocol designed to model adolescent patterns of consumption, revealed that its early exposure induces a reduction in the responsiveness of mesocortical dopaminergic neurons and an increased voluntary alcohol intake in adulthood. Lastly, chronic operant self-administration of AMED, from adolescence to adulthood, resulted in enhanced acquisition of the operant

response, leading to increased intake, and upregulation of mesocorticolimbic dopaminergic transmission.

These findings indicate that acute exposure to caffeine and AMED may exert protective effects against some alcohol-induced neurobiological alterations. In contrast, subchronic and chronic exposure to AMED during maturation (i.e., from adolescence to adulthood) leads to a clear dysregulation of the mesocorticolimbic dopaminergic system, accompanied by increased substance intake. Moreover, the results of the experiments under contingent conditions also suggest that additional factors, such as taste, liking, wanting, and learning processes, beyond the pharmacological effects of the substances alone, contribute to the behavioral and neurochemical outcomes.

Taken together, this study casts novel and critical insights into the developmental and context-dependent effects of AMED: highlights its potential to alter reward-related circuits and increase vulnerability to compulsive substance use, suggests that AMED consumption during adolescence may not be entirely safe, and provides a robust and neat foundation for future research on its long-term consequences and possible reversibility.

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# LIST OF ABBREVIATIONS

°C = degrees Celsius

3Rs = Replacement, Reduction, and Refinement

AADC = aromatic L-amino acid decarboxylase

AC-ALCOHOL = Experimental group receiving an acute gavage of alcohol

AC-AMED = Experimental group receiving an acute gavage of alcohol mixed with Red Bull®

AC-RB = Experimental group receiving an acute gavage of Red Bull®

AC-TW = Experimental group receiving an acute gavage of tap water

Acb = nucleus accumbens

AcbSh = nucleus accumbens shell

ADH = alcohol dehydrogenase

ADME = absorption, distribution, metabolism, and elimination

ALDH = aldehyde dehydrogenase

AMED = alcohol mixed with energy drinks

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

AP = anteroposterior

AR = aldose reductase

ATP = adenosine triphosphate

AUD = alcohol use disorder

BAC = blood alcohol concentration

BD = binge drinking

BD-ALCOHOL = binge drinking experimental group receiving alcohol by gavage, followed by voluntary alcohol consumption

BD-AMED = binge drinking experimental group receiving alcohol mixed with Red Bull® by gavage, followed by voluntary alcohol consumption

BD-RB = binge drinking experimental group receiving Red Bull® by gavage, followed by voluntary alcohol consumption

BD-TW = binge drinking experimental group receiving tap water by gavage, followed by voluntary alcohol consumption

BK = big potassium channel

BSA = bovine serum albumin

C = carbon

cAMP = cyclic adenosine monophosphate

CanEq = Can Equivalents

cm = centimeter

CNS = central nervous system

COMT = catechol-O-methyltransferases

Cyp2D = cytochrome P450 2D6

CYP2E1 = cytochrome P450 2E1

Cα = alpha carbon

DA = dopamine

DAB = 3,3'-diaminobenzidine

DAT = dopamine transporter

dL = deciliter

DOPAC = 3,4-dihydroxyphenylacetic acid

DPCPX = 8-Cyclopentyl-1,3-dipropylxanthine

DSM = Diagnostic and Statistical Manual of Mental Disorders

DV = dorsoventral

ED = energy drink

ERK = extracellular signal-regulated kinase

FDA = Food and Drug Administration

FEP = fluorinated ethylene-propylene

fmol = femtomoles

FR = Fixed Ratio

GABA = γ-aminobutyric acid

$G_i$ = inhibitory G-proteins

GIRK = G protein-gated inwardly rectifying potassium channel

GlyR = glycinergic receptors

$G_s$ = stimulatory G-proteins

H = hydrogen

HPLC = high-performance liquid chromatography

HVA = homovanillic acid

i.g. = intragastrically

i.p. = intraperitoneally

$K_M$ = Michaelis-Menten constant

L-DOPA = L-3,4-dihydroxyphenylalanine

M = moles per liter / molar

MAO = monoamine oxidase

MAPK = mitogen-activated protein kinase

mg = milligrams

min = minutes

ML = mediolateral

mL = milliliters

mm = millimeters

mM = millimolar

mPFC = medial prefrontal cortex

MSNs = medium spiny neurons

mV = millivolts

NIAAA = National Institute on Alcohol Abuse and Alcoholism

NMDA = N-methyl-D-aspartate receptor

NP = nose-poke

O = oxygen

OH = hydroxyl group

OSA = sodium octyl sulfate

PBS = phosphate-buffered saline

PE = polyethylene

pERK = ERK phosphorylation

PFC = prefrontal cortex

PHE = L-phenylalanine

PKA = protein kinase A

PND = Postnatal Day

pmol = picomoles

PST = phenolsulfotransferases

pVTA = posterior ventral tegmental area

R = radicals

RB = Red Bull®

RNA = ribonucleic acid

s.c. = subcutaneously

SA = self-administration

SA-ALCOHOL = self-administration experimental group receiving alcohol

SA-AMED = self-administration experimental group alcohol mixed with Red Bull®

SA-RB = self-administration experimental group receiving Red Bull®

SA-TW = self-administration experimental group receiving tap water

SCH 58261 = 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine

SD = Sprague-Dawley

SEM = standard error of the mean

TBS = Tris-buffered saline

TBST-X = Tris-buffered saline containing 0.1% of Triton X-100

TYR = L-tyrosine

UGT = uridine diphosphoglucuronosyltransferases

USA = United States of America

v/v = volume/volume

$VMAT_2$ = vesicular monoamine transporter 2

VTA = ventral tegmental area

# Chapter I: GENERAL INTRODUTION

## 1. ALCOHOL

### 1.1 – Chemical and physical characteristics

Alcohols are a class of organic compounds characterized by the presence of at least one hydroxyl group (OH) bonded to a carbon atom, which is called alpha carbon (Cα). The quantity of OHs present in the molecule determines an initial classification of alcohols, whose are defined as monovalent (only one OH group), divalent (two OH groups), trivalent (three OH groups), etc. However, the main classification of alcohols is determined by the number of alkyl groups or radicals (R) bonded to the saturated Cα, giving rise to primary alcohols (R-Cα-OH), secondary alcohols ($R_2$-Cα-OH) or tertiary alcohols ($R_3$-Cα-OH). The simplest alcohol, methanol, is the only exception to this latter classification, as Cα does not have an R group attached ($CH_3OH$).

All bonds between alcohol atoms are covalent, while hydrogen (H) bonds are formed between alcohol molecules. Specifically, in the OH group, oxygen (O) is more electronegative than H and attracts electrons more strongly, creating a partial negative charge on O and a partial positive charge on H. This leads to an electrostatic attraction between the positive side of one molecule and the negative side of another, allowing hydrogen bonds between the OH groups of different molecules (Kotz et al., 2009). This mechanism is also responsible for the solubility of most alcohols in water.

The characteristics described above concern all the molecules existing in the alcohol family, from the simplest (methanol) to the most complex ones (e.g., cholesterol). Although this family includes numerous organic compounds, in common parlance, the term "alcohol" is generally used to refer to ethyl alcohol or ethanol, an edible primary monovalent alcohol contained in alcoholic beverages. Consequently, from this moment on, the word *alcohol* will refer to the ethyl alcohol molecule. The condensed formula ($CH_3CH_2OH$) and the molecular formula ($C_2H_6O$)

give us information respectively on the different chemical groups and on the number of each atom that forms the alcohol molecule. This is a colorless, volatile, flammable liquid with a characteristic strong smell and a burning taste; it has a boiling point of 78.4 degrees Celsius (°C), a melting point of -114.5°C, a density of 0.785 grams (g) / milliliters (mL) at 20°C and a molecular weight of 46.07 g/mol. Alcohol and water can be mixed in any ratio to give a homogeneous mixture (Kotz et al., 2009). Alcohol's small ethyl chain also makes it completely soluble in other organic solvents, as well as in beverages often used to prepare alcoholic drinks.

## 1.2 – Alcoholic beverages

Beer, wine, and spirits are the three main alcoholic beverages, differing in their alcohol content, preparation methods, and taste. The processes through which alcohol is produced are fermentation and distillation. Alcoholic fermentation is a biological-chemical process that occurs in an anaerobic environment and exploits the ability of yeasts to transform sugars contained in fruit (e.g. wine production) or cereals (e.g. beer) into alcohol and carbon dioxide. Spirits production involves fermentation followed by distillation, a physical process that takes advantage of ethyl alcohol's lower boiling point to separate it from water, producing concentrated solutions containing up to 95 % alcohol. According to the data available on the World Health Organization's (WHO) official website, the amount of alcohol contained in a standard drink can vary widely depending on the Country (WHO, 2018). On average, it is estimated that a standard alcohol unit contains approximately 14 g of pure alcohol, equivalent to one standard drink in the United States of America (USA), which is roughly comparable to a can of beer, a glass of wine, or a shot of spirit. In cocktails, spirits like vodka, rum, gin, tequila, and others are combined with various non-alcoholic beverages, such as fruit juices, sodas, bitters, energy drinks (EDs), etc. EDs and their association with alcohol will be specifically described in Section 3 and 4 of this thesis, respectively.

Although the WHO affirmed that there is no safe level of alcohol that does not affect health (WHO, 2023), the statement "moderate alcohol consumption" is frequently used. According to the Dietary Guidelines for Americans 2020-2025, moderation is defined as no more than 1 drink in a day for women and no more than 2 drinks in a day for men. This limit, which is based on the gender differences in weight and metabolism, is meant to reflect the amount consumed on any given day rather than an average over several days. Consequently, binge drinking (BD), which is defined as consuming 4 or more drinks in roughly two hours for an adult female and 5 or more for an adult male, should be avoided (National Institute on Alcohol Abuse and Alcoholism [NIAAA], 2007; U.S. Department of Agriculture & U.S. Department of Health and Human Services, 2020).

## 1.3 – Pharmacokinetics

Pharmacokinetics is a branch of pharmacology that studies "*what the body does to a drug*" (Bereda, 2022), analyzing the absorption, distribution, metabolism, and elimination (ADME) processes. Studying the pharmacokinetics of a drug is important as it provides fundamental information to determine how much and whereby the substance enters the bloodstream, where it accumulates, in what way it is transformed, and how long it remains in the body. Therefore, it also allows us to evaluate the risks and toxicity of a substance, in order to better study the dosages, methods of administration, and the frequency of drug intake, also contributing to the safety of pharmacological therapies. Below, the pharmacokinetics of alcohol is described.

### ***1.3.1 - Absorption***

The process by which a drug enters the bloodstream is called absorption. Alcohol is a small molecule that, due to its characteristics, does not need to be digested but, instead, is rapidly absorbed into the bloodstream through biological membranes and transported throughout the tissues.

Once introduced into the body, small amounts of alcohol can be absorbed immediately through the mucous membranes of the mouth and esophagus if an alcoholic drink is held in the mouth for sufficiently long time (Jones, 2019), but the main absorption occurs once alcohol has been ingested and reaches the gastrointestinal tract: specifically, about 20% of alcohol is absorbed in the stomach, but the majority (about 80%) is absorbed in the small intestine, particularly in its first part, the duodenum, through intestinal villi and microvilli. These are small projections of the intestinal mucosa that significantly increase the surface area for absorption into the blood.

Alcohol moves down a concentration gradient because its absorption follows the laws of passive diffusion (Berggren & Goldberg, 1940), which states that substances tend to move from areas of high concentration to areas of low concentration until equilibrium is reached.

Because the rate of alcohol absorption is greater than its rate of elimination, alcohol absorption is a primary determinant of peak blood alcohol concentration (BAC), which is achieved on average within 1 hour (Mitchell et al., 2014), with shorter times (e.g., 30 minutes [min]) on an empty stomach or longer times (e.g., 90 min) on a full stomach. Besides gastric content, other important factors that influence the rate of alcohol absorption are the quantity and type of drink (higher concentrations of alcohol are absorbed more rapidly), as well as individual characteristics such as sex, age, weight, etc.

### ***1.3.2 - Distribution***

Once absorbed from the gastrointestinal tract into the blood, alcohol is rapidly distributed to all the body through the bloodstream. More specifically, after absorption into the portal venous blood, alcohol is transported to the liver and then reaches the right atrium of the heart via the hepatic veins and inferior vena cava. From the right ventricle of the heart, it travels to the lungs via the pulmonary artery, returns to the heart via the pulmonary vein, and from the left ventricle is pumped into the aorta and then distributed to all other organs (Jones, 2019). Alcohol is distributed from the blood to all tissues and fluids in proportion to their relative water content (Cederbaum, 2012). As in the case of absorption, also in this process alcohol is distributed through passive diffusion across membranes thanks to its lipophilic characteristics. This also allows alcohol to cross the highly specialized and selective blood-brain barrier and exert its effects on the central nervous system (CNS).

### ***1.3.3 - Metabolism***

Since alcohol is a toxic xenobiotic, our body has the task of metabolizing it in order to bio transform and eliminate it. A small portion of orally ingested alcohol can be metabolized before entering the systemic circulation, with first-pass metabolism occurring in the stomach, small intestine, and liver (Lee et al., 2006). However, the bioavailability of alcohol (i.e., the amount of alcohol that reaches the systemic circulation in its unchanged active form) is extremely high, meaning that first-pass metabolism is neglectable.

Alcohol provides about 7 kilocalories (kcal) per g, compared to 4 kcal from carbohydrates or protein and 9 kcal from fat. Unlike carbohydrates, stored as glycogen, or fat, stored as triglycerides, alcohol cannot be stored and circulates in body fluids until its elimination. Moreover, while the metabolism of other nutrients is tightly regulated by hormones such as insulin, glucagon, and thyroid hormones, alcohol breakdown is largely independent of hormonal control (Cederbaum, 2012). For this reason, the liver is the main organ responsible for oxidizing and clearing alcohol from the body, although some metabolism also occurs in extrahepatic tissues such as the brain (Zakhari, 2006).

Alcohol metabolism is primarily achieved by oxidative pathways involving two chemical reactions and several enzymes. The first reaction is the oxidation of alcohol to acetaldehyde and is mainly performed in the cytosol by the enzyme alcohol dehydrogenase (ADH). Although less effectively, other enzymes that can catalyze the same reaction in microsomes or in peroxisomes, are respectively cytochrome P450 2E1 (CYP2E1) and catalase. The Michaelis-Menten constant ($K_M$) is an index of enzyme-substrate affinity that helps us understand the role these enzymes play in the first reaction of alcohol metabolism. Specifically, the lower the $K_M$ value, the lower the substrate concentration that allows to reach a reaction rate equal to half the maximum rate, indicating a high enzyme-substrate affinity. Conversely, a high $K_M$ value indicates that a higher substrate concentration is required to bind half of the enzyme molecules present in solution, which means a lower affinity of the enzyme for the substrate. Among the three enzymes, catalase has the highest $K_M$ (greater than 100 millimolar [mM]). Therefore, catalase does not play a significant role in alcohol metabolism due to both its high $K_M$ and the reduced availability of peroxide in liver cells; however, it plays a more important role in alcohol metabolism in the CNS (Zimatkin & Deitrich, 1997). In contrast, ADH has a high affinity for alcohol due to its very low $K_M$, ranging from approximately 0.2 to 2 mM; consequently, at normal BAC, alcohol is entirely metabolized by this enzyme. However, when alcohol intake starts to increase, ADH tends to saturate and the Microsomal Ethanol-Oxidizing System is induced, whose key enzyme is CYP2E1 (intermediate $K_M$ of about 8-10 mM) (Matsumoto & Fukui, 2002).

The compound resulting from the activity of ADH, acetaldehyde, is more toxic than the parent drug, but is rapidly further oxidized to acetate in a second step of alcohol metabolism, catalyzed by aldehyde dehydrogenase (ALDH) in the mitochondria. Most of the acetate leaves the liver and is transported through the blood to various tissues, where it can be converted to carbon dioxide or to acetyl coenzyme A (Zakhari, 2006). The latter can then be oxidized in the Krebs cycle to produce energy, or act as a precursor for the ketone body formation and fatty acid synthesis. Although alcohol metabolism is primarily oxidative, it can also occur through nonoxidative pathways. A small portion (<0.2%) of ingested alcohol is metabolized in the liver via conjugation reactions, producing the major nonoxidative metabolites: ethyl glucuronide and ethyl sulfate. Other nonoxidative metabolites include fatty acid ethyl esters and phosphatidylethanol (Jones, 2019). Furthermore, alcohol can contribute to the formation of secondary metabolites in the brain. Among these, 6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline, better known as salsolinol, is produced in the ventral tegmental area (VTA) through a Pictet-Spengler condensation reaction between acetaldehyde and dopamine (DA). This molecule was suggested to play a role in some of the effects of alcohol. Supporting this idea, a study conducted by my research group demonstrated that the alcohol-dependent formation of salsolinol in the posterior VTA (pVTA) may represent one of the mechanisms through which alcohol exerts its reinforcing effects (Bassareo et al., 2021). This concept will be explored in Section 7 of this thesis.

### ***1.3.4 - Elimination***

The elimination phase describes how and through which pathways alcohol and its metabolites are eliminated from the body, maintaining internal balance and preventing the accumulation of toxic substances. Approximately 90-98% of ingested alcohol is not excreted unchanged but is eliminated through metabolism. Animals with small body weight metabolize alcohol at faster rates than larger animals, for instance the rate of alcohol elimination in mice is 5 times greater than the rate in humans (Cederbaum, 2012). Only a small portion (2-10%) of alcohol is eliminated unchanged through direct excretion, via the lungs (breath), kidneys (urine), sweat, and saliva.

## 1.4 – Pharmacodynamics

Pharmacodynamics, complementing pharmacokinetics, is a branch of pharmacology that studies "*what a drug does to the body*" by analyzing the effects of a substance following its interaction with its biological targets. Given its ability to influence numerous body systems, such as nervous, endocrine, immune, cardiovascular, digestive, and respiratory systems, the pharmacodynamics of alcohol is quite complex. Its effects may depend on a number of variable factors such as age, gender, diet, body composition, intrinsic alcohol elimination capacity, and interacting agents (Chan & Anderson, 2014), but its main effects on CNS are mostly dose-dependent: low doses of alcohol induce mild anxiolytic effects, euphoria, behavioral disinhibition, relaxation, etc. (Cui & Koob, 2017), while high doses lead to motor incoordination, sedation and respiratory depression. More specifically, the effects of acute alcohol intoxication, typical of CNS depression, are generally BAC-dependent. When BAC is around 50 milligrams per deciliter (mg/dL), which is close to the legal driving limit, muscle relaxation, compromised judgment, and a general feeling of arousal occur, due to the initial inhibition of the CNS inhibitory systems. When BAC reaches 80 mg/dL, motor incoordination, speech difficulties, and potential cognitive deficits are observed. BAC up to 200 mg/dL causes amnesia, emotional instability, and more severe cognitive deficits. Nystagmus and drowsiness can be seen above 200 mg/dL, while BAC equal to 300 mg/dL can lead to coma and death (Brunton et al., 2012).

Targets and effects of alcohol on the brain are described in depth in the review written by Abrahao et al. (2017). Alcohol acts as a CNS depressant, primarily by enhancing inhibitory neurotransmission and suppressing excitatory systems. Specifically, one of the primary mechanisms by which it enhances inhibitory synaptic transmission is through its action on the γ-aminobutyric acid (GABA) system, the principal inhibitory neurotransmitter system of the CNS. Alcohol is a positive allosteric modulator of the $GABA_A$ receptor; therefore, it increases the effectiveness of GABA in determining the opening of the ionic channel associated with the receptor and the entry of chloride anions, resulting in a hyperpolarization of the membrane and a reduction in neuronal excitability (Davies, 2003). Alcohol also acts

on the glycine system, by potentiating the action of glycine at glycinergic receptors (GlyR). Glycine is the main inhibitory neurotransmitter in the spinal cord, but also has an inhibitory effect in the brainstem and encephalon (Perkins et al., 2010). Beyond $GABA_A$ and GlyR, alcohol also potentiates ligand-induced activation of two other Cys-loop ligand-gated ion channels, which instead mediate neuronal depolarization: the nicotinic acetylcholine receptor and the serotonin 5-$HT_3$ ionotropic receptor (Narahashi et al., 2001). Complementary to its potentiation of inhibitory signaling, alcohol also suppresses excitatory neurotransmission, primarily by inhibiting the glutamate system, the main excitatory pathway in the CNS. This action is due to the blockade of metabotropic (Narahashi et al., 2001) but mostly to the blockade of ionotropic glutamate receptors: N-methyl-D-aspartate receptor (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate. Among these types, alcohol has a greater affinity for inhibiting cation flow of NMDA receptors, resulting in a reduction in neuronal excitability (Lovinger & Roberto, 2013). Other direct targets of alcohol are the big potassium (BK) and G protein-gated inwardly rectifying potassium (GIRK) channels, adenylate cyclase, and protein kinase C. The actions of alcohol on all of the previously mentioned targets at the level of various neurons and brain synapses also result in numerous indirect actions (Abrahao et al., 2017). One prominent example is its impact on the brain's reward system, discussed further in Section 6 of this thesis. In the aforementioned system, alcohol does not bind directly to DA or other receptors, but modulates their activity via key circuits and receptors.

## 1.5 – Alcohol-related health effects

Alcohol is defined as a toxic, psychoactive, and addictive substance. Hence, according to the WHO, the consumption of alcohol contained in alcoholic beverages can lead to three main mechanisms of harm: toxicity, intoxication, and dependence. *Toxicity* affects almost every system in the body, as alcohol is carcinogenic, hepatotoxic, teratogenic, immunotoxic, cardiotoxic, etc. Its toxicity is mainly due to its metabolites, which cause cellular damage, oxidative stress, and formation of reactive oxygen species. *Intoxication* is due to alcohol's temporary psychoactive effects which include impaired cognitive, motor, and behavioral functions. This often leads to engaging in risky activities that can be dangerous to oneself and others. Finally, alcohol induces *dependence*, a condition that often evolves into "addiction", which is better explained in Section 6 of this thesis; briefly, its repeated and long-term consumption leads to compulsive craving and reduced self-control, resulting in increased alcohol intake and greater risk of adverse health effects (WHO, 2007, 2024a).

Due to its negative consequences on the entire body, alcohol consumption is linked to more than 200 adverse health effects and it causes a huge number of deaths every year. More than 2.6 million deaths worldwide (4.7% of all deaths) in 2019 were attributable to alcohol, 13% of which were among young people aged 20 to 39 (WHO, 2024a). These deaths are caused by diseases or disorders that are entirely due to alcohol as the sole causal factor or by injuries and other medical conditions that, while not caused by alcohol itself, are aggravated or triggered by its consumption. Table 1, adapted from WHO (2024a) and based on the original data of WHO (2024b), summarizes the health conditions that were completely attributable to alcohol. The likelihood of developing these conditions depends on individual susceptibility factors, quantity and frequency of alcohol consumption. For instance, the BD condition of consuming large amounts of alcohol in short periods of time (already described in Subsection 1.2 of this thesis) increases the risk of both acute and long-term negative health consequences (Gowin et al., 2021).

| |
|---|
| **Mental, behavioural or neurodevelopmental disorders** |
| 6C40.0 Episode of harmful use of alcohol |
| 6C40.1 Harmful pattern of use of alcohol |
| 6C40.2 Alcohol dependence |
| 6C40.3 Alcohol intoxication |
| 6C40.4 Alcohol withdrawal |
| 6C40.5 Alcohol-induced delirium |
| 6C40.6 Alcohol-induced psychotic disorder |
| 6C40.7 Certain specified alcohol-induced mental or behavioural disorders (including alcohol-induced mood disorder and alcohol-induced anxiety disorder) |
| 6C40.Y Other specified disorders due to use of alcohol |
| 6C40.Z Disorders due to use of alcohol, unspecified |
| Fetal alcohol syndrome (as a part of a group of health conditions under 6A0.Y Other specified neurodevelopmental disorders) |
| **Diseases of the nervous system** |
| 6D84.0 Dementia due to use of alcohol |
| 8D44.0 Alcohol polyneuropathy |
| 8D44.1 Alcohol myopathy |
| 8D44.Y Other specified alcohol-related neurological disorders |
| 8D44.Z Alcohol-related neurological disorders, unspecified |
| **Diseases of the circulatory system** |
| Dilated cardiomyopathy due to alcoholism |
| **Diseases of the digestive system** |
| DB94.0 Alcohol fatty liver |
| DB94.1 Alcoholic hepatitis |
| DB94.2 Alcoholic liver fibrosis |
| DB94.3 Alcoholic cirrhosis of liver without hepatitis |
| DB94Y Other specified alcoholic liver disease |
| DB94.Z Alcoholic liver disease, unspecified |
| DC31.1 Acute alcohol-induced pancreatitis |
| DC32.3 Chronic alcohol-induced pancreatitis |
| **Certain conditions originating in the perinatal period** |
| KA06.2 Fetus or newborn affected by maternal use of alcohol |
| **Developmental anomalies** |
| Fetal alcohol syndrome (as a part of a group of health conditions under LD2F.0 Toxic or drug-related embryofetopathies) |
| **External causes of morbidity or mortality** |
| Alcohol poisoning (as a part of a group of health conditions under NE61 Harmful effects of or exposure to noxious substances, chiefly nonmedicinal as to source, not elsewhere classified) |
| Alcohol poisoning (as a part of a group of health conditions under PH50 Exposure to or harmful effects of undetermined intent of alcohols, and under PD00 Intentional self-harm by exposure to or harmful effects of alcohols) |
| **Factors influencing health status or contact with health services** |
| QE10 Hazardous alcohol use |

Table 1. **Health conditions completely attributable to alcohol consumption**.
Codes listed before each health condition correspond to the WHO classification system.
Taken and readapted from WHO (2024a) *(CC BY-NC-SA 3.0 IGO licence)*.

# 2. CAFFEINE

Caffeine, chemically known as 1,3,7-trimethylxanthine ($C_8H_{10}N_4O_2$), is a purine alkaloid belonging to the methylxanthine family. Caffeine is mostly found in coffee and tea; when present in tea, it has historically been called "theine". It is also present in cocoa, guaranà, and other plants, as well as in various foods and beverages, including EDs (Saraiva et al., 2023).

Caffeine is completely and rapidly absorbed in the body, with maximum plasma concentrations reported between 15 and 120 min, depending on inter-individual variability and delayed gastric emptying (Cappelletti et al., 2015). It has a stimulating effect on the CNS that begins 15-30 min after oral intake, persisting for several hours. Given its widespread global consumption, safe caffeine doses have been established. Specifically, healthy adults are advised not to exceed a caffeine intake of 400 mg/day or, more specifically, 5.7 mg/kilogram (kg) of body mass, with the exception of pregnant women, who should limit their intake to half of the adult maximum, not exceeding 200 mg/day. Furthermore, caffeine is present in cocoa drinks and chocolate, commonly ingested by children, whose maximum recommended daily intake is 3 mg/kg of body mass (European Food Safety Authority [EFSA], 2015).

Caffeine's psychostimulant effect on CNS is mainly due to its ability to antagonize the effect of adenosine at purinergic receptors. Adenosine is a fundamental nucleoside consisting of an adenine linked to a ribose sugar and is a constituent of ribonucleic acid (RNA). It plays a crucial role in cellular energy as part of adenosine triphosphate (ATP), as well as in intracellular signal transduction as a component of cyclic adenosine monophosphate (cAMP) (Layland et al., 2014). Adenosine acts on various body systems and exerts its pharmacological effects by interacting with type 1 adenosine purinergic receptors. All of them are metabotropic G-protein-coupled receptors and are divided into four subtypes: $A_1$ and $A_3$, coupled to inhibitory G-proteins ($G_i$), and $A_{2A}$ and $A_{2B}$, coupled to stimulatory G-proteins ($G_s$) (Ralevic & Burnstock, 1998). The neuromodulatory and homeostatic actions of adenosine in the CNS, including its somnogenic effects, are mainly mediated by $A_1$ and $A_{2A}$ receptors (Cunha, 2001; Lazarus et al., 2019), which are the most expressed adenosine receptors in the CNS. Specifically, $A_1$ receptors are highly expressed in

neurons of the cortex, cerebellum, and hippocampus, as well as in the dorsal horn of the spinal cord; $A_{2A}$ receptors are primarily expressed in the olfactory bulb and in striatopallidal GABAergic neurons, within brain regions such as the caudate-putamen, nucleus accumbens (Acb), and tuberculum olfactorium (Fredholm et al., 2001).

Caffeine is a non-selective antagonist of all adenosine receptor subtypes (Ribeiro & Sebastião, 2010), although its behavioral and arousal effects have been mostly attributed to the antagonism of $A_1$ and $A_{2A}$ receptors (Daly et al., 1994; Lazarus et al., 2011). By opposing the action of adenosine in the CNS, caffeine reduces the perception of fatigue and drowsiness, while enhancing alertness, concentration, attention, vigilance, and reaction time, thus promoting a general excitatory and stimulatory state that improves cognitive and physical performance (McLellan et al., 2016). Other secondary mechanisms of caffeine action in the CNS, which become significant only at concentrations much higher than physiological dietary doses, include mobilization of intracellular calcium (Bianchi, 1961), inhibition of phosphodiesterases (Choi et al., 1988), and interactions with benzodiazepine binding sites (Boulenger et al., 1982). These and other effects of caffeine on the CNS are reviewed by Nehlig et al. (1992).

# 3. ENERGY DRINKS

EDs are non-alcoholic beverages advertised and consumed for their stimulant effects. Among their ingredients, caffeine is the most important stimulant component of EDs due to its psychostimulant effects on the CNS. In fact, EDs are generally defined as beverages containing high amounts of caffeine and are marketed as enhancing energy levels, physical performance, athletic ability, and mental focus. (Alsunni, 2015; Alford et al., 2001). According to the Food and Drug Administration (FDA), EDs typically contain 41 to 246 milligrams (mg) of caffeine per 12-ounce serving, equivalent to approximately 350 mL (FDA, 2024). Besides caffeine, other ingredients generally present in EDs are taurine, glucuronolactone, B-vitamins, guaranà, ginseng, ginkgo biloba, L-carnitine, sugars and antioxidants (Higgins et al., 2010).

Despite their stimulant effects, EDs have been associated with several negative health effects, as described in detail in the review by Costantino et al. (2023). Cardiovascular effects are the most commonly reported adverse outcomes. In rats, chronic consumption of Red Bull® (RB), the most well-known ED, induces changes in cardiac function (Vargiu et al., 2021). In humans, chronic RB intake additionally affects cerebrovascular circulation, reducing cerebral blood flow velocity (Grasser et al., 2014). Vargiu et al. (2021) also investigated the abuse potential of RB, showing activation of the reward system similar to that induced by drugs of abuse. This is a particularly relevant effect, given the global growth of the EDs market following the launch of RB in 1987 (Reissig et al., 2009). Recently, Aonso-Diego et al. (2024) estimated the worldwide prevalence of EDs use and found that participants had consumed EDs at least once in their lifetime (54.7%), in the past year (43.4%), month (32.3%), week (21.6%), and daily (8.82%). Overall, the highest lifetime EDs consumption was observed among adolescents and young adults, likely influenced by RB's success attributed to its iconic slogan "RB gives you wings" and sponsorship of extreme sports. Its use is also diffused in students to enhance concentration and attention (RB Company, n.d.). As mentioned above, EDs' stimulating effects are mainly due to caffeine, with a 250 mL can of RB containing approximately 80 mg, roughly equivalent to a cup of coffee.

Below is the complete list of ingredients as indicated on the back of a RB can:

- water;
- sucrose;
- glucose;
- acidifier: citric acid;
- carbon dioxide;
- taurine (0.4 %);
- acidity regulators: sodium carbonates, magnesium carbonates;
- caffeine (0.03 %);
- vitamins: niacin (B3), pantothenic acid (B5), pyridoxine (B6), cobalamin (B12);
- flavors;
- colorings: plain caramel, riboflavin (B2).

# 4. ALCOHOL MIXED WITH ENERGY DRINKS

## 4.1 – Consumption patterns and emerging trends in youth

Nowadays, the consumption of alcohol mixed with EDs (AMED) has become increasingly common among teenagers and young people. Most of the existing literature, including the studies reported below, providing evidence for this trend, focuses on students and the general population aged around 15 to 35.

Malinauskas et al. (2007) reported that among consumers who drank more than one ED per month, 54% mixed them with alcohol during parties, with half of these drinking three or more AMED drinks per month. Binge drinkers from a high school, defined as students who had consumed five or more drinks within a couple of hours at least once in the past month, were more likely to consume AMED than occasional drinkers (49% versus 18.2%), with almost half of them reporting mixing EDs specifically with vodka (Gonzales et al., 2015). In a study of over 4000 students, 24% of alcohol drinkers reported consuming AMED at least once in the past month. AMED users also started drinking earlier (average age of first drink 15.1 versus 16 years) and had a greater frequency of alcohol consumption. Among these, some common reasons for consuming AMED included hiding the flavor of the alcohol (55%), drinking more without feeling drunk (15%), avoiding hangovers (7%), and drinking more alcohol without appearing intoxicated (5%) (O'Brien et al., 2008). Another study reported that 44.0% of 706 students had tried AMED or consumed it regularly, representing 78% of alcohol consumers. Among individuals who had assumed AMED in the last 2 weeks (9.3% of the total sample), the main motivations were reported as feeling less tired, getting drunk faster, and being able to drink more (Marczinski, 2011).

Although most of the literature focuses on the student population, similar effects have also been observed in non-student teenagers, including pre-adolescents around 13 years old (Emond et al., 2014; Kponee et al., 2014).

## 4.2 – Health and behavioral risks

The main reason why AMED consumption could be dangerous is because EDs, through their caffeine' stimulant effect, can mask the sedative effects of alcohol, a phenomenon called "wide-awake drunk" (Attwood, 2012). This means that people may not realize they are intoxicated, leading them to drink more and increasing the risk of alcohol poisoning and risky behaviors (Sefen et al., 2022). For instance, according to the study by O'Brien et al. (2008), AMED drinkers consumed more drinks per episode than non-AMED drinkers (5.8 versus 4.5 drinks), had nearly twice the number of drinking days and drunken episodes per week, and reported a greater number of drinks consumed in a single episode. They also shown more alcohol-related consequences, including a higher prevalence of being injured, experiencing sexual assault, riding with an intoxicated driver or taking advantage of another person sexually. Moreover, an increased desire to drink alcohol has been reported, suggesting that AMED modality may be more motivating than alcohol alone (Marczinski et al., 2013). A very large number of other studies have investigated the effects of AMED in humans, 42 of which were considered in the systematic review by De Giorgi et al. (2022), which is also useful to better understand AMED consumption reasons, estimate its adverse effects, and to examine associated health-related behaviors. The meta-analysis conducted within this review indicated that approximately 37% of undergraduate students reported consuming AMED, about one-third of all students.

The consumption patterns, motivations, and risks associated with AMED highlight the importance of investigating its neurobiological impact. Alcohol and EDs, alone or in combination, influence the brain's reward system, whose key mediator is DA. Therefore, the following two Sections focus on exploring DA's function and its role within this circuitry.

# 5. NEUROBIOLOGY OF DOPAMINE

## 5.1 – Discovery and general functions

DA, also known as 3,4-dihydroxyphenylethylamine or 3-hydroxytyramine, was originally considered merely a precursor to noradrenaline. Although it was first synthesized in vitro in 1910 (Barger & Ewins, 1910; Mannich & Jacobsohn, 1910), the role of DA in the CNS began to be investigated a few decades later, after the discovery of Kathleen Montagu (Montagu, 1957). In 1957, she first stated that DA is an endogenous compound inside the brain and, not long afterwards, Arvid Carlsson and his colleagues made some important observations (Carlsson et al., 1957, 1958; Carlsson & Waldeck, 1958) that would later lead to the determination of DA as a neurotransmitter and to the awarding of the Nobel Prize in Medicine to Carlsson for his research on DA. From that point on, research on the neurobiology of this important molecule increased exponentially, in order to understand and determine its roles in the body. Nowadays, we know that DA is a neurotransmitter largely distributed, involved in multiple functions in the CNS, such as cognition, reward processing, control of movement, memory, emotions, but also acts as a neurohormone in regulating the secretion of prolactin by the pituitary gland (Rangel-Barajas et al., 2015). DA also exerts important peripheral effects, regulating blood pressure, cardiac contractility, renal excretion, and immune and pulmonary functions (Amenta et al., 2002).

## 5.2 – Chemical features, biosynthesis and metabolism

DA is an organic molecule composed of carbon (C), H, nitrogen (N) and O atoms. The subscripts in its molecular formula, $C_8H_{11}NO_2$, indicate the number of atoms of each element in the molecule. In a neurochemical context, DA is a neurotransmitter belonging to the catecholamine family, together with noradrenaline and adrenaline, also known as norepinephrine and epinephrine, of which it represents the precursor. Catecholamines take their name from a characteristic functional group in their molecule consisting of a benzene ring and two OHs substituents, called catechol ($C_6H_6O_2$). In the DA molecule, an ethylamine chain (-$CH_2CH_2NH_2$) is bonded to the catechol group. In mammals, DA is the most abundant catecholamine (80%) in the brain (Costa & Schoenbaum, 2022).

Catecholamine synthesis can occur in different cells and in different parts of the body. DA is generally synthesized in the brain, particularly in the cytosol of DAergic neurons of the substantia nigra and VTA, but also in the hypothalamus and mesenteric organs (i.e., the gastrointestinal tract, spleen, and pancreas) (Eisenhofer et al., 1997). As for the other catecholamines, noradrenaline and adrenaline are the main neurotransmitters of the sympathetic nervous system and are mainly produced in the chromaffin cells of the adrenal medulla; however, their synthesis can also occur in noradrenergic and adrenergic neurons of the CNS (Khalil et al., 2024).

The starting point for catecholamine synthesis is the amino acid L-tyrosine (TYR), which can in turn be derived from the essential amino acid L-phenylalanine (PHE) through the action of the enzyme PHE-hydroxylase. The subsequent reaction involves the hydroxylation of TYR to form L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme TYR-hydroxylase, which catalyzes the rate-limiting step in the biosynthesis of the catecholamines (Kumer & Vrana, 1996). Although there is evidence that PHE may also be a substrate for TYR-hydroxylase, TYR is the preferred substrate (Fernstrom & Fernstrom, 2007). L-DOPA is rapidly decarboxylated by aromatic L-amino acid decarboxylase (AADC) to form DA. AADC is also responsible for the alternative pathway of DA synthesis: in particular, it decarboxylates PHE to phenylethylamine and TYR to tyramine, which is then oxidized to DA by cytochrome P450 2D6 (Cyp2D) (Meiser et al., 2013).

In noradrenergic neurons, DA is subsequently converted to norepinephrine by the enzyme DA-β-hydroxylase. In adrenergic neurons, but especially in chromaffin cells of the adrenal medulla, norepinephrine is methylated and transformed to epinephrine by phenylethanolamine N-methyltransferase (Khalil et al., 2024).

In DAergic neurons, DA is imported into synaptic vesicles by secondary active transport via the vesicular monoamine transporter 2 ($VMAT_2$) (Meiser et al., 2013) and stored until its release at the presynaptic terminal. In synaptic vesicles, DA is concentrated to approximately 0.1 moles per liter (M), 10-1000 times higher than its concentration in the cytosol (Elsworth & Roth, 1997).

The release of DA, as well as of all other neurotransmitters stored in synaptic vesicles, occurs through the mechanism of exocytosis following an electrical signal called *action potential* and lasting only 2 milliseconds. More specifically, the neuronal membrane has a resting membrane potential of approximately -70 millivolts (mV), which means that the inside of the neuron is electrically negative with respect to the extracellular space. Following a depolarizing stimulus strong enough to reach the -55 mV threshold, voltage-gated sodium channels first open, allowing sodium cations to rapidly enter the neuron until the membrane potential reaches +35 mV. At this point, voltage-gated potassium channels open, and the outflow of potassium cations gradually repolarizes the membrane. The action potential propagates along the axon without decrement: in unmyelinated neurons, depolarization spreads continuously along adjacent membrane regions, whereas in myelinated fibers it jumps from one node of Ranvier to the next via saltatory conduction, ultimately reaching the synaptic terminal. Due to the refractory period, previously depolarized regions cannot be reactivated immediately, ensuring unidirectional propagation of the action potential. Upon reaching the synaptic terminal, depolarization triggers a conformational change in the S4 segment of voltage-gated calcium channels, leading to their opening and allowing calcium influx down its electrochemical gradient. The resulting increase in intracellular calcium concentration activates several regulatory proteins and complexes, including the SNARE complex, promoting synaptic vesicle docking and subsequent fusion with the presynaptic membrane, mainly mediated by synaptotagmin, and ultimately leading to neurotransmitter release.

Once in the synaptic cleft, DA exerts its effects by interacting with DAergic receptors: mainly on postsynaptic ones for signal transduction, but also on presynaptic autoreceptors and heteroreceptors on non-DAergic neurons, to regulate the release of DA and other neurotransmitters, respectively. Subsequently, to avoid toxic effects or neuronal damage, the signal must be interrupted. Therefore, after carrying out its action, extracellular DA is removed from the synaptic cleft primarily through reuptake or enzymatic degradation (Meiser et al., 2013). DA reuptake is mediated by the DA transporter (DAT), a sodium-chloride-dependent, 12-transmembrane domain protein involved in DAergic transmission and also a target of numerous drugs of abuse, such as cocaine, amphetamine, and methamphetamine (Nepal et al., 2023). After being taken back into the presynaptic terminal by DAT, DA is recycled and reloaded into vesicles by $VMAT_2$ or metabolized by enzymes such as monoamine oxidase (MAO) or catechol-O-methyltransferases (COMT) (Tai et al., 2024). Surrounding glial cells also participate in DA reuptake and metabolism (Meiser et al., 2013). Besides MAO and COMT, another important enzyme for DA catabolism is ALDH, which is responsible for the final step leading to the formation of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), the main metabolites of DA. Other minor pathways are also operated by ADH and aldose reductase (AR), while phenolsulfotransferases (PST) and uridine diphosphoglucuronosyltransferases (UGT) catalyze conjugation reactions. Figure 1, taken from the study by Meiser et al. (2013), summarizes the DA biosynthetic and degradation pathway.

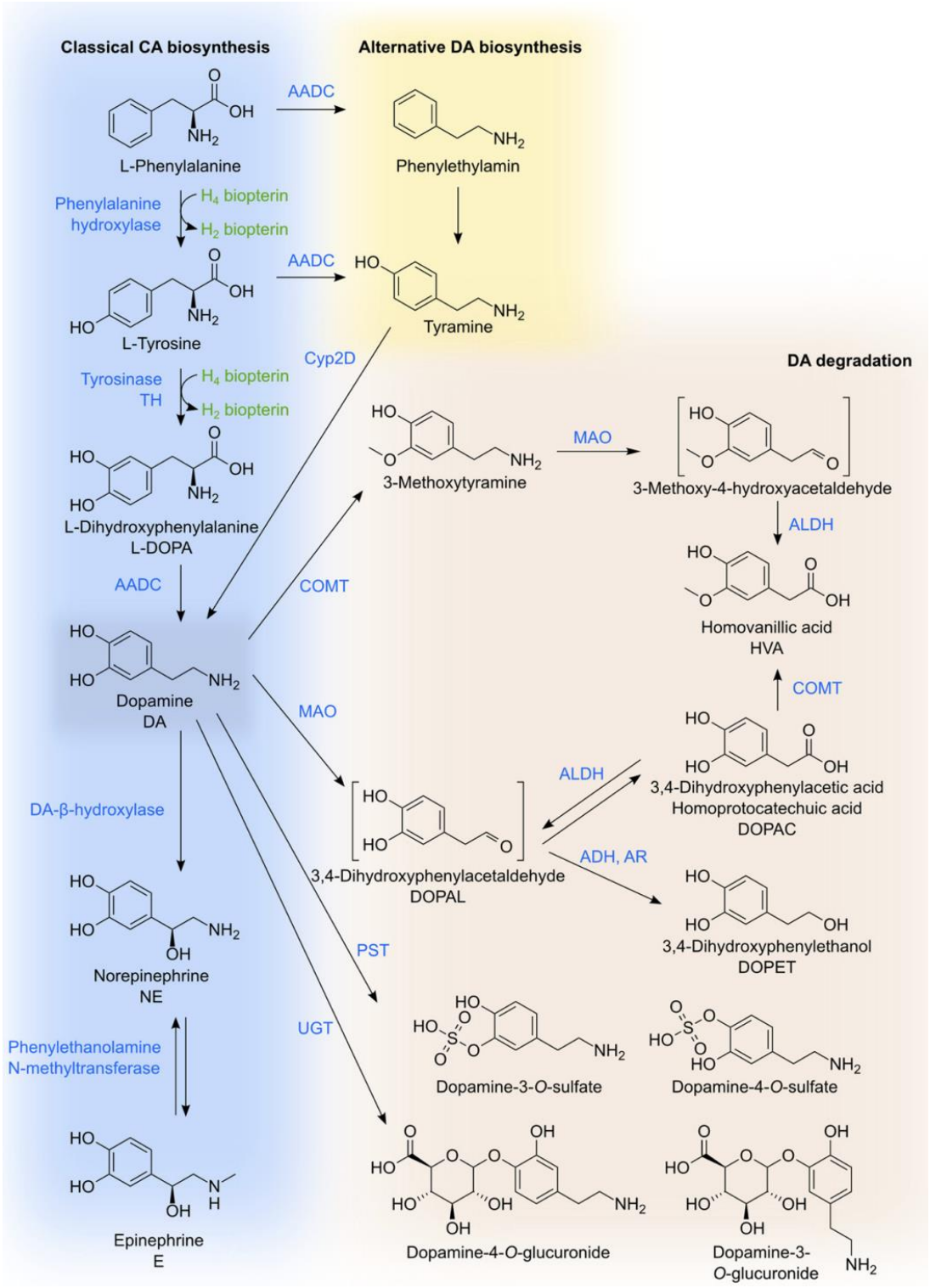


Figure 1. **Schematic representation of the main enzymes involved in DA biosynthesis and degradation**. Taken from Meiser et al. (2013) *(CC BY 2.0 licence)*.

## 5.3 – Receptors and signal transduction

As mentioned in the previous Subsection, once DA is released into the synaptic cleft, it acts on several types of receptors. All of them are metabotropic receptors, structurally characterized by seven transmembrane segments. Their extracellular portion is activated by DA, while the intracellular portion contains binding sites for G-proteins. These G-proteins are composed of three subunits (α, β, γ; with β and γ forming a dimer), which, upon receptor activation, mediate signal transduction. There are five DA receptors and they can be divided into two main families called D1-like and D2-like. The latter has a 10- to 100-fold greater affinity for DA than the D1-like family (Martel & Gatti McArthur, 2020). Therefore, the D2-like receptors are mainly involved in the modulation of the basal (or "tonic") DAergic tone, whereas D1-like family is primarily associated with phasic DA signaling (Costa & Schoenbaum, 2022).

The main difference between the two families of DA receptors is their opposite ability to modulate cAMP production, which depends on the α subunit of the associated G-protein (stimulatory in $G_s$; inhibitory in $G_i$). Another difference is their pre- or post-synaptic localization: receptors of the D1-like family are mostly post-synaptic, whereas D2-like receptors are generally found both post- and pre-synaptically on DAergic neurons (Beaulieu & Gainetdinov, 2011).

D1-like receptors, which include $D_1$ and $D_5$ subtypes, are $G_s$ protein-coupled receptors that stimulate adenylate cyclase, increasing cAMP levels and consequently activating protein kinase A (PKA), resulting in neuronal excitation. Conversely, D2-like receptors, which include $D_2$, $D_3$ and $D_4$, are $G_i$ protein-coupled receptors, therefore their activation leads to the inhibition of adenylate cyclase, a reduction of cAMP and subsequent inactivation of PKA, resulting in neuronal inhibition. Although the adenylate cyclase pathway is the most common mechanism, other signal transduction mechanisms by DA receptors have also been described, such as the activation of protein kinase C (Ma, 2025; Beaulieu & Gainetdinov, 2011; Missale et al., 1998). DA receptors are distributed throughout the nervous system and, depending on their location, are associated with different functions. The most highly expressed receptors in the CNS are those that give their names to the two families,

$D_1$ and $D_2$, while the $D_3$, $D_4$, and $D_5$ receptors are expressed in lower concentrations. Specifically, it has been reported that the relative abundance in the CNS of rats would be $D_1 > D_2 > D_3 > D_5 > D_4$ (Jaber et al., 1996). Table 2, adapted from Mishra et al. (2018), summarizes the main DAergic receptor locations and functions, while Beaulieu & Gainetdinov (2011) provide a broader and deeper understanding of the DA receptors overview.

| Receptors | D1 | D5 | D2 | D3 | D4 |
|---|---|---|---|---|---|
| Location | Striatum, nucleus accumbens. Olfactory bulb, amygdala hippocampus, substantia nigra Hypothalamus, frontal cortex | Cortex, substantia nigra, hypothalamus | Striatum, VTA Olfactory bulb, cerebral cortex | Striatum, islands of Calleja, cortex | Frontal cortex, amygdala, hypothalamus, nucleus accumbens |
| Function | Locomotion, learning and memory, attention, impulse control, sleep, regulation of renal function | Cognition, attention, decision making, motor learning, renin secretion | Locomotion, learning and memory, attention, sleep, reproductive behaviour | Locomotion, cognition, attention, impulse control, sleep, regulation of food intake | Cognition, impulse control, attention, sleep, reproductive behavior |

Table 2. **Schematic representation of the main DAergic receptor locations and functions**. Adapted from Mishra et al. (2018) *(CC BY-NC 4.0 licence).*

## 5.4 – Anatomical and functional pathways

The numbering system of areas containing DAergic neurons was originally introduced by Dahlström & Fuxe (1964) and subsequently expanded to include nine main groups, named A8 to A16, as illustrated in the adapted image taken from Björklund & Dunnett (2007) (Figure 2).

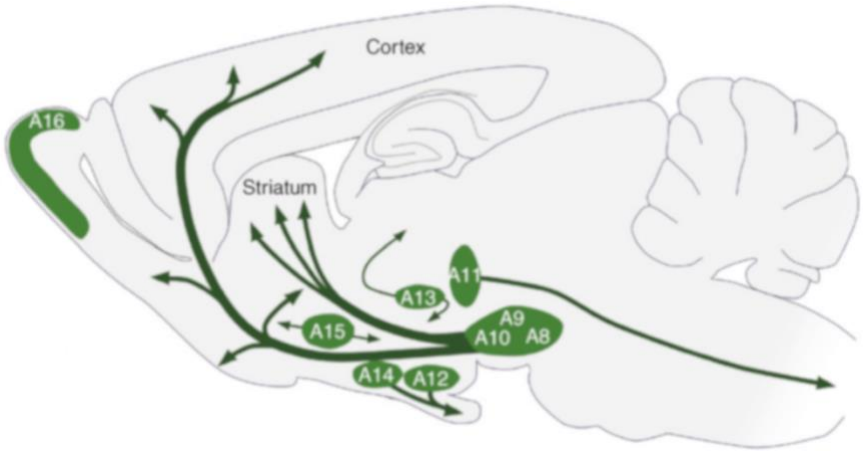


Figure 2. **DAergic areas in the rat brain**. Adapted from Björklund & Dunnett (2007).
*Degraded image for non-commercial educational use according to Art. 70(1bis).*

Among these groups of neurons, four main DAergic pathways originate: the nigrostriatal, tuberoinfundibular, mesocortical, and mesolimbic pathways. Although some of them cooperate in common mechanisms, each of these pathways has specific functions.

The *nigrostriatal pathway*, as its name suggests, consists of DAergic neurons whose neuronal cell bodies are located in the pars compacta of the substantia nigra (A9) and which project to the dorsal striatum (caudate-putamen) of the basal ganglia. This pathway is essential for voluntary movement control: it is part of the extrapyramidal system and is crucial for regulating posture, muscle tone, and motor coordination. Deficits in this pathway, particularly a selective loss of nigrostriatal DA neurons, can therefore lead to movement disorders and Parkinson's disease (Di Chiara, 1997; Costa & Schoenbaum, 2022).

In the *tuberoinfundibular pathway*, DA acts as a neurohormone rather than a neurotransmitter, since it modulates hormone secretion via the bloodstream.

Specifically, neurons of this pathway are located in the dorsomedial arcuate nucleus of the mediobasal hypothalamus (A12) and project to the median eminence. DA is released into the portal system and travels to the adenohypophysis (anterior pituitary), where it inhibits the release of prolactin (Qi-Lytle et al., 2023; Lyons & Broberger, 2014).

Finally, the last two pathways both consist of DAergic neurons whose neuronal cell bodies originate anatomically in A10 (VTA) and partly also in A9 (Di Chiara, 1997) but project to different brain areas. They are the most important DAergic pathways in the context of this thesis, as they are related to the reward system which will be described more in depth in Section 6.

The *mesocortical pathway*'s VTA DAergic neurons project to the prefrontal cortex (PFC), a crucial brain area that reaches full maturity only in young adulthood. It is considered the "center of human personality" as it controls a wide range of executive and non-executive functions, such as information processing and decision making, learning, motivation, emotional control, behavioral flexibility, cognition, complex behavioral planning, attention, inhibitory control, and different types of memory (above all, working memory) (Hathaway & Newton, 2023; Miller, 1999; Chafee & Heilbronner, 2022).

The *mesolimbic pathway* is primarily involved in reward, associative learning, reinforcement behaviors, contextual memory, and emotional processing. Its VTA DAergic neurons project to limbic areas, such as the ventral striatum (which includes olfactory tubercle and, mostly, the Acb), the central amygdala, the bed nucleus of the stria terminalis, the septal area, and the hippocampus (Di Chiara, 1997; Halbout et al., 2019; Alcaro et al., 2007).

# 6. BRAIN'S REWARD SYSTEM

## 6.1 – Key definitions and concepts

Before delving into the heart of the brain's reward system and examining the anatomical structures that regulate it, it is essential to understand some fundamental notions. This Section therefore introduces the core concepts underlying reward, motivation, and reinforcement, together with the adaptive changes that might emerge with repeated exposure to stimuli, and the pathological alterations leading to conditions such as alcohol use disorders (AUD).

### *6.1.1 - Fundamental processes: reward and its components*

Reward is a natural process in which the brain links various positive stimuli, such as needs, substances or experiences, to outcomes that are perceived as desirable (Lewis et al., 2021). A reward can be classified as primary (intrinsic) or secondary (extrinsic). Primary rewards are biologically essential for life and for the maintenance of homeostasis and reproduction; examples include eating, drinking, shelter, sex, and maternal care. Secondary rewards do not possess intrinsic biological value, are not directly necessary for survival or reproduction, and acquire significance through learning and experience, often by association with primary rewards. Examples of secondary rewards include money, social recognition, power, success, activities such as games, sports, music, or hobbies (Sescousse et al., 2013).

According Berridge et al. (2009) and Berridge & Robinson (1998), reward is not a unitary phenomenon but can instead be conceptualized as comprising three distinct psychological components: "liking", which reflects the hedonic impact of a stimulus; "wanting", which represents the incentive salience or motivational drive toward it; and "learning", which involves the formation of predictive associations and cognitive representations. *Liking* represents the core hedonic component of reward. It is essentially what most people describe as "feeling pleasure", and reflects the immediate pleasurable sensation elicited by a rewarding stimulus. *Wanting* is not a simple desire, but a stronger drive that becomes concrete rather than remaining abstract. It is a fundamental component of reward, reflecting the motivational drive

that precedes the achievement of a reward. Wanting is triggered by predictive *cues* and is guided by the psychological process of *incentive salience*, which is crucial in assigning motivational significance to a stimulus (Berridge, 2009).

Incentive salience is grounded in the predictive value of environmental cues, a property acquired through *learning*, which can therefore be described as the fundamental process through which organisms, across repeated experiences, acquire information about the relationships between stimuli, actions, and outcomes. It can be associative or cognitive: the latter uses reward as information to support goal-directed planning, whereas associative learning relies on direct reinforcement and is therefore the most extensively studied in reward research. Within associative learning, two major forms are distinguished in the context of reward. One of them is the *Pavlovian or classical conditioning*, where a previously neutral or unconditioned stimulus acquires meaning once associated with a reward and thus becomes a conditioned stimulus, or cue. Specifically, the individual learns that the stimulus predicts the reward, and can generate anticipation, behavioral habits, emotions, and motivations consistent with the nature of the original reward. On the other hand, in *operant or instrumental conditioning*, learning is based on the consequences of an action: a behavior is reinforced when it produces a positive outcome (Berridge & Robinson, 2003). One example of all these processes in the context of alcohol consumption is illustrated below.

Having a drink can be enjoyable (*liking*). Through repeated experiences, the individual learns to predict the rewarding outcomes of alcohol consumption (*learning*). Over time, especially when consumed in fun contexts, drinking alcohol, due to its disinhibiting effects, can be associated with increased socialization and positive emotions (*operant conditioning*). Likewise, even the sound of a bottle cap being opened can, itself, evoke positive emotions (*Pavlovian conditioning*). All of this could lead to actively seeking and buying a drink (*wanting*) in order to relive the reward of the experience.

### ***6.1.2 - Neuroadaptations and pathologic processes***

The mechanisms described in the previous Subsection reflect the natural and physiological processes underlying all forms of reward. However, the brain is a highly adaptive organ, and various neuroadaptations can emerge that modify the effects of rewards. One of these is the *habituation* phenomenon, which has been extensively studied and is described with at least ten well-defined characteristic features, but it can generally be defined as a temporary phenomenon in which a stimulus that initially elicits a response gradually loses its ability to evoke the same reaction when presented repeatedly (Thompson & Spencer, 1966). In the context of natural rewards, habituation refers to a progressive reduction in the mesolimbic DAergic response to repeated and predictable stimuli, as the stimulus becomes familiar and expectable. Unlike natural rewards, drugs of abuse appear not to undergo DAergic habituation. Instead, repeated drug exposure often induces *sensitization*, a process characterized by progressive neuroadaptations that enhance mesolimbic DAergic responsiveness and, consequently, motivational responses, particularly toward drug-associated cues. These processes are discussed in more detail in Subsection 6.3.1. Accordingly, as proposed by Robinson & Berridge (2000) in their incentive-sensitization theory of addiction, cues associated with drugs become progressively more effective at triggering *craving*, a pathological form of wanting. Similar to habituation to natural stimuli, *tolerance* is a neurobiological adaptation that develops after repeated administration of a drug. In this case, a previously effective dose no longer produces the same response, requiring an increased dose to achieve the original effect, although the dose-response curve is not as predictable (Peper, 2009). Tolerance reduces liking, but DAergic sensitization maintains excessive wanting, driving craving and relapse even in the absence of hedonic pleasure (Berridge & Dayan, 2021). In summary, drug use typically begins as an attempt to achieve a desired pleasurable effect. In some individuals, due to certain neuroadaptations, this behavior can become compulsive, occurring regardless of the reward. All these changes in brain circuitry could lead to the development of disorders; regarding alcohol, the focus of this thesis, they are manifested as AUD, described below.

#### *6.1.2.1. - Alcohol use disorder and cycle of addiction*

AUD is a medical condition introduced in 2013 with the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). It is diagnosed when at least two of the 11 symptoms related to alcohol use (which are described below) have occurred in the previous year and it can be classified as mild (presence of 2-3 symptoms), moderate (4-5 symptoms), or severe (6 or more symptoms). AUD also encompasses two disorders that were previously distinguished in the DSM-IV: alcohol abuse (roughly corresponding to mild AUD) and alcohol dependence (moderate to severe AUD) (National Institutes of Health, National Institute on Alcohol Abuse and Alcoholism, 2014; Tyler & Leggio, 2024). The following are the diagnostic criteria for AUD, as reported in the DSM-5, Revised Text (American Psychiatric Association, 2022):

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.*

As previously mentioned, the term AUD encompasses a broad spectrum of conditions, ranging from mild to severe. In the scientific literature, however, the term *alcohol addiction* is often used to denote the more severe, relapsing, and compulsive forms of the disorder, even though it does not appear in the official terminology of the DSM-5 (American Psychiatric Association, 2022). This severe form follows a cyclical pattern, in which episodes of compulsive alcohol intake are followed by periods of aversive emotional states, leading to pathological wanting during which individuals are particularly vulnerable to relapse, resulting in a continuation of the cycle. This cycle can be divided into three distinct stages: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (craving), reflecting a progressive, spiralling dysregulation of brain reward systems that promote compulsive alcohol use and progressively impair self-control (Koob & Le Moal, 1997; Koob & Volkow, 2010).

The first phase of *binge/intoxication* consists of acute alcohol consumption that produces immediate rewarding effects. Specifically, BD (already mentioned in Subsection 1.2) is defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as consuming four or more standard drinks for women and five or more for men within about two hours, resulting in a BAC of approximately 0.08 g-percent or above (NIAAA, 2007). This BAC, equivalent to 80 mg/dL, not only produces the desired rewarding effects, such as euphoria and disinhibition, but is also associated to other less desirable effects including motor incoordination, speech difficulties, and potential cognitive deficits, as described in Subsection 1.4.

In the periods following episodes of heavy alcohol consumption, individuals with severe AUD enter the next phase, called *withdrawal/negative affect*, with symptoms that can appear as early as 4-12 hours after a BD episode and include emotional symptoms, such as reduced motivation, decrease in mood, anxiety, stress, sadness, and irritability, as well as physical symptoms, such as sleep disorders and

general malaise. In this phase, the individual may drink not to achieve positive effects, but to alleviate negative ones (negative reinforcement) (American Psychiatric Association, 2022; Koob & Volkow, 2010; Tyler & Leggio, 2024).

In the *anticipation/preoccupation (craving)* stage, the individual begins to think of alcohol as a positive reinforcer, then becomes obsessed with it and actively seeks it, planning their next binge experience. Therefore, this stage is a key element in relapse (Koob & Volkow, 2010).

A person can enter the addiction cycle at any of the three stages, and the duration and frequency of the cycle are variable (Semaan & Khan, 2023; Tyler & Leggio, 2024), which means that relapse, often triggered by cues, can occur many years later.

The processes described so far are complex phenomena involving multiple brain structures. In the following Sections, the organization of the brain's reward system will be described.

## 6.2 – Early insights

The idea of a "reward system" first arose in 1954 following the well-known intracranial self-stimulation experiment in rats conducted by Olds & Milner (1954). In their important study, electrodes were permanently implanted in different brain areas and animals were tested in Skinner boxes where they had the opportunity to stimulate their own brains by pressing a lever. The authors discovered that there are neutral brain areas, areas where the animals avoided self-stimulation, and areas where the animals exhibited strong self-stimulation. Specifically, the latter was measured when the electrode had been implanted in the septal area and the tegmentum, which they called "reinforcing structures" and were subsequently identified, together with other structures, as "pleasure centers" (Olds, 1956). Thus, by the mid-1950s, several rewarding areas of the brain had already been identified, and the idea that a reward system in the brain existed was beginning to take shape. Furthermore, in the same decade, as anticipated in Subsection 5.1, the discoveries of Kathleen Montagu (Montagu, 1957) and Arvid Carlsson et al. (1957, 1958) paved the way for characterizing the role of DA in the brain and its central role in the reward system.

We currently know that the brain's reward system is implicated in controlling the pleasure feeling derived from both primary and secondary stimuli, as well as from addictive substances. Therefore, it is a fundamental system that influences our daily choices, as it plays a crucial role in processing pleasure, associative learning linked to rewarding experiences, and regulation of goal-oriented behaviors.

## 6.3 – Neuroanatomical and functional organization

The brain's reward system is a complex network of cortical and subcortical structures that work together to evaluate stimuli and direct behavior toward both natural and pharmacological rewarding experiences. Within these circuits, distinct types of neurons and their associated neurotransmitter systems play specialized roles in learning which stimuli are gratifying and guiding decision making based on these rewards (Hikosaka et al., 2008). In this Section, the main structures of the reward system are described, with particular attention to the central role of the DAergic system.

### *6.3.1 - Mesocorticolimbic dopaminergic system*

As previously described in Section 5.4, the two DAergic systems primarily involved in reward processing are the mesolimbic and mesocortical pathways, both originating mainly from the A10 region. They are often considered together and referred to as the *mesocorticolimbic system*, which has long been recognized as having a central role in the reward system. It is widely agreed that addictive drugs act mainly, though not entirely, on the brain's mesocorticolimbic DAergic system (Kelley & Berridge, 2002; Di Chiara & Imperato, 1988). The key anatomical structures forming these pathways include the VTA, the Acb, and the PFC. Highly simplified, VTA DAergic neurons project to the Acb, where DAergic transmission mediates reward, and to the PFC, which modulates behavior. As seen in Figure 3, redrawn from Russo & Nestler (2013), the VTA is the primary source of DAergic neurons in this system, projecting not only to the Acb and PFC but also to other areas, including the amygdala, which is crucial for forming associative memories related to fear and reward, and to the hippocampus, which enhances memory encoding based on the emotional valence of a stimulus.

Some brain structures, such as Acb, are critically involved in the effects of various classes of drugs of abuse, which, although they act through distinct molecular mechanisms (including depressants such as alcohol, stimulants such as amphetamine and cocaine, and opioids such as heroin, morphine, methadone, and fentanyl), increase DA levels preferentially within the Acb (Di Chiara & Imperato, 1988).

Accordingly, the Acb serves as a central hub for the processing of pleasurable experiences and is often referred to as the brain's "pleasure center" (Ebrahimi et al., 2024). It is in turn functionally divided into two subregions called *core* and *shell*, which play different roles in motivated behavior. Bassareo & Di Chiara (1999) examined the differential responsiveness of DA transmission to food-stimuli in these two subareas and showed that the Acb shell (AcbSh) is more selective than Acb core, reacting mainly to unexpected natural rewards, and that DA activity in this subarea decreases when stimuli are repeated or predictable (i.e., habituation). In another of their studies, it was observed that food-induced increases in DA occurred not only in the Acb but also in the medial PFC (mPFC). However, habituation emerged exclusively in the AcbSh, and this effect was reversible, as it disappeared after five days (Bassareo & Di Chiara, 1997). By contrast, although it has been proven that both addictive drugs and palatable food increase extracellular DA in AcbSh, as anticipated before, drugs bypass normal adaptive mechanisms (such as habituation), enabling drug-associated cues to maintain or even potentiate DA release in the AcbSh, which progressively enhances motivational responses (i.e., sensitization) and contributes to the onset of addiction (Di Chiara, 1999; Bassareo et al., 2003; Di Chiara & Bassareo, 2007). Thus, the mesolimbic component plays a fundamental role in attributing motivational value to stimuli and in reinforcing behavior, mainly through DAergic adaptations within the AcbSh that modulate incentive salience and reward-related learning.

In parallel, the mesocortical component fulfills a complementary but distinct function, integrating reward-related information with higher-order executive processes, such as decision-making, behavioral regulation, and outcome evaluation, thereby guiding goal-directed behavior. Part of this modulatory function involves projections from the PFC to the Acb, where networks of PFC neurons expressing $D_1$ receptors regulate reward-seeking behavior. These networks promote choices that lead to higher or uncertain rewards (therefore also risky, but potentially more advantageous), enhance the repetition of rewarded actions, and reduce the impact of non-rewarded outcomes. Other PFC neurons expressing $D_2$ receptors and projecting to the basolateral amygdala, a limbic structure involved in encoding memories

associated with emotional events, are essential for behavioral flexibility, allowing decision-making to be adjusted when reward probabilities change (Jenni et al., 2017).

In summary, mesocorticolimbic DAergic system enhance the incentive salience of reward-predicting stimuli and modulate certain aspects of reinforcement-related learning, making these stimuli motivationally attractive and able to drive approach behavior, which is a mechanism that can become pathologically amplified in addiction. Accordingly, DAergic transmission is thought to primarily mediate the "wanting" component of reward, partially contribute to reward-related "learning", but not the hedonic pleasure associated with consuming rewards (i.e., "liking") (Berridge & Robinson, 1998; Schultz, 1998).

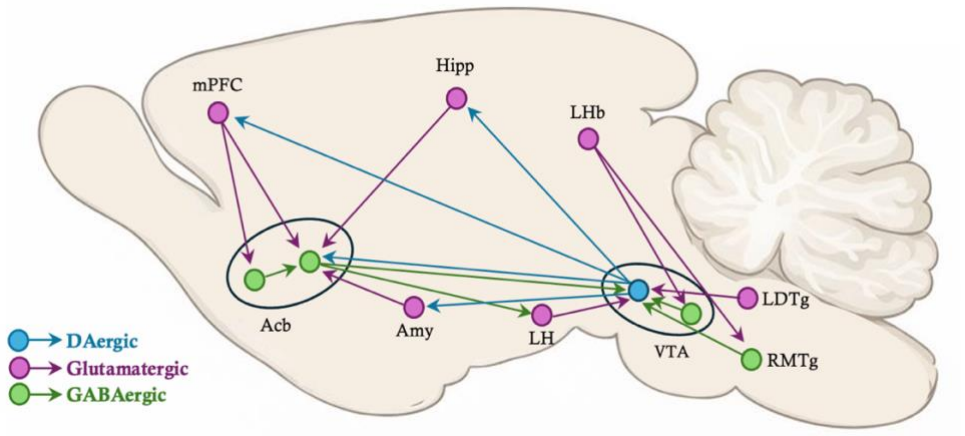


Figure 3. **Main DAergic, glutamatergic, and GABAergic projections of the mesocorticolimbic system**. *Abbreviations: Hipp = hippocampus; Amy = amygdala; LDTg = lateral dorsal tegmentum, LHb = lateral habenula; LH = lateral hypothalamus; RMTg = rostromedial tegmentum.* Redrawn from Russo & Nestler (2013).

### *6.3.2 - Modulatory and integrative circuits*

Although the DAergic system, with its VTA-Acb and VTA-PFC projections, is considered the main reward circuit, other anatomical brain structures and neurotransmitter systems play crucial modulatory roles. The connections between these structures are characterized by feedback loops that regulate DA release and influence behavioral responses to rewarding stimuli. Figure 3 provides a very simplified overview; it highlights, however, not only DAergic but also glutamatergic and GABAergic pathways, which establish additional levels of regulation within the mesocorticolimbic system. For instance, it has been observed that glutamatergic and GABAergic neurons of the ventral pallidum can activate in response to reward stimuli, influencing the VTA in opposing ways and promoting either approach or avoidance responses (Faget et al., 2024). Moreover, VTA GABAergic neurons can locally modulate DAergic transmission influencing behavior, while their long-range projections to the Acb specifically inhibit cholinergic interneurons, another neurotransmitter system involved, thereby modulating associative learning (Creed et al., 2014). The system is further complicated by the presence in the VTA of glutamatergic neurons, DA neurons that co-release glutamate or GABA, and neurons co-releasing both glutamate and GABA, which modulate motivated behavior in distinct ways (Root et al., 2020; Morales & Margolis, 2017).

Neurons in the Acb are predominantly GABAergic medium spiny neurons (MSNs), categorized into two subpopulations expressing $D_1$ or $D_2$ DAergic receptors. Soares-Cunha et al. (2020) demonstrated that both types of MSNs can mediate either aversion or reward, depending on the pattern of stimulation. Brief activation of these neurons induces reward, associated with increased DA release in the VTA, whereas prolonged stimulation leads to aversive responses.

As previously noted, DA primarily mediates "wanting" rather than "liking"; by contrast, hedonic reactions are mainly mediated by the opioid endogenous system, the endocannabinoid endogenous system, and the orexin system within dedicated hedonic hotspots (Berridge & Dayan, 2021). Among these systems, the μ-opioid receptors play a fundamental role both in liking and in wanting (Meier et al., 2021) and are central to the rewarding properties of non-opioid drugs, such as alcohol (Bassareo et al., 2021). μ-opioid driven "liking" and "wanting" arise from distinct

regions within the Acb: a small rostro-dorsal portion of the AcbSh mediates "liking", whereas "wanting" is more broadly represented and strongly mediated throughout the entire medial AcbSh (Peciña, 2008). Complementing the hedonic and motivational functions of μ-opioids, the endocannabinoid system plays a key role in reward processing and motivated behavior by modulating mesolimbic DA transmission, preferentially enhancing signaling in the AcbSh rather than the Acb core (De Luca et al., 2014). Furthermore, the orexin system plays a central role in addiction, modulating drug-seeking and relapse (Capó et al., 2025). Other neurotransmitters and neuropeptides, such as serotonin, norepinephrine, adenosine, galanin, and histamine, also modulate various aspects of the brain's reward system (Arias-Carrión et al., 2014; Zhang et al., 2023), highlighting the complexity and interconnected nature of its regulation.

At the molecular level, reward system activity is also reflected in the engagement of specific intracellular signaling pathways. A key effect of administering drugs of abuse is the activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway. Specifically, increased ERK phosphorylation (pERK) in brain areas such as the Acb, with the AcbSh often showing stronger pERK responses than the core, reflects neuronal plasticity and drug-induced reward behaviors (Cahill et al., 2014; Remus & Thiels, 2013).

# GENERAL AIM

The combined consumption of alcohol and EDs (AMED) has been increasing among adolescents and young adults, together with growing concerns regarding the potential negative consequences and associated risk-taking behaviors, as discussed in Section 4. This trend therefore represents a current public health issue that warrants further understanding including, in particular, investigations on neural circuits and mechanisms from a preclinical perspective. In fact, although an extensive body of epidemiological evidence has been well documented and extensively discussed in this thesis introduction, the literature suffers a lack of preclinical studies investigating the neurobiological alterations associated with the effects of the combined intake of alcohol and EDs. Elucidating these mechanisms is therefore essential to improve our understanding of AMED-related effects and to support the development of effective preventive strategies and public health policies.

Within this framework, the overall aim of this PhD project is to investigate, from a preclinical point of view, the neurobiological, behavioral, and molecular effects of the most prevalent ED commonly associated with alcohol consumption among young people in recreational settings, RB, alone and in combination with alcohol (AMED) under various experimental protocols and dosing regimens.

This aim will be initially pursued (Chapter II, Sections 7 and 8) by examining the acute, non-contingent effects of RB, its primary psychoactive component (caffeine), alcohol, and their interaction, in both adolescent and adult rats, in order to characterize their discrete pharmacological effects in drug-naïve animals at different stages of life and, therefore, at different phases of neurodevelopment. The rationale of planning these experiments is that they would provide, in AMED-naïve animals, the behavioral and neurochemical bases to support the understanding of the effects arising from subchronic non-contingent and chronic contingent exposure paradigms, from adolescence to adulthood (Chapter II, Sections 9 and 10).

Under this perspective, evaluating in adulthood the long-term consequences of adolescent exposure to these substances, i.e. assessing the behavioral and neurobiological adaptations that develop over time, also considering processes

related to context-dependent behavior such as liking, wanting, and learning, may provide critical evidence for understanding the neurobiological mechanisms underlying the AMED phenomenon and its long-term potentially harmful consequences. Consistently, the study will mainly focus on the mesocorticolimbic DAergic system, which is responsible for processing the acute and adaptive responses to addictive substances as well as for regulating the processes related to context-dependent behavior. These aims will be pursued by measuring, in particular, in vivo DA release from the AcbSh and the mPFC, together with a DA-related post-synaptic molecular endpoint and a range of behavioral outcomes.

In summary, by adopting a multidisciplinary approach, together with experimental protocols and dosing regimens with high translational relevance to model consumption patterns in adolescents, this work aims to deepen the current knowledge on the effects of caffeine, RB, alcohol and AMED at the molecular, behavioral and neurochemical levels in specific brain regions critically involved in the reward processing and behavioral control.

Chapter II presents in detail the experimental studies conducted in this thesis, highlighting for each study their rationale, methodologies, and findings, with the goal of addressing the aims outlined above.

# Chapter II: EXPERIMENTAL STUDIES

## 7. Receptor and Metabolic Insights on the Ability of Caffeine to Prevent Alcohol-Induced Stimulation of Mesolimbic Dopamine Transmission

The content of this Section is based on research conducted by our group and is accordingly adapted from our recent publication (Bassareo et al., 2024).

### 7.1 – Background and aim

The consequences of the combined use of caffeine and alcohol have been extensively studied as they are among the most widely consumed psychoactive substances in the world (Mehta, 2016; Nehlig, 1999). As mentioned in Section 2, caffeine is the main psychoactive component of EDs, which in turn are often consumed in combination with alcohol. Although the effects of caffeine-alcohol interactions have been explored, the literature presents conflicting results. It has been previously shown that caffeine, at doses near the threshold for eliciting arousal (Acquas et al., 2002; Hasenfratz et al., 1993) and locomotor activity (López-Cruz et al., 2013; Dar, 1988), can counteract the reinforcing effects of alcohol as it prevented the development of alcohol-induced conditioned place preference and aversion (Porru et al., 2020), suggesting that this behavioral effect may be mediated through DA dependent associative learning mechanisms (Di Chiara et al., 2004). This hypothesis is supported by evidence showing that alcohol-induced place conditioning is blocked by DA receptor antagonists (Spina et al., 2010; Walker & Ettenberg, 2007), and that caffeine exerts an inhibitory effect on the firing of DA neurons in the pVTA through its antagonistic action on $A_{2A}$ receptors (Valenti et al., 2021; Stoner et al., 1988). Moreover, administration of caffeine before alcohol prevents the DA-dependent increase in pERK levels in the AcbSh, a DA receptor-dependent marker of mesolimbic DA activation by alcohol (Ibba et al., 2009; Porru et al., 2020, 2021; Sanna et al., 2002), as well as a mechanism underlying DA-dependent associative

learning (Di Chiara et al., 2004; Beninger & Gerdjikov, 2004; Marotta et al., 2014). Regarding the mechanism by which alcohol activates mesolimbic DA transmission, this role has been attributed first to the metabolic conversion of alcohol into acetaldehyde (Correa et al., 2012; Peana et al., 2017), and subsequently to the condensation of acetaldehyde with DA to form salsolinol, which is therefore thought to play a key role in alcohol's reinforcing and addictive properties (Deehan et al., 2013; Hipólito et al., 2012; Quintanilla et al., 2014; Quintanilla et al., 2016). Previous findings obtained in our laboratory showed that alcohol-dependent salsolinol formation in the pVTA and the consequent increase in DA transmission in the AcbSh were mediated by μ-opioid receptors and are both prevented by inhibition of brain catalase (Bassareo et al., 2021). Taken together, these findings indicate that caffeine can interact with some of alcohol's effects, likely through a DAergic mechanism. However, despite some evidence, the mechanisms underlying these interactions remain poorly understood. Moreover, considering that not only the combined intake of caffeine and alcohol but also the consumption of AMED is increasingly common, it is crucial to investigate the effects of the main psychoactive component of caffeinated beverages, independently of other EDs ingredients.

Based on this rationale, the aim of this first part of the study was to investigate, in adult male rats, whether and how an acute, non-contingent treatment of caffeine can modulate the neurochemical mechanisms underlying alcohol's reinforcing properties. Adult male rats were used to ensure full maturation of the mesolimbic DAergic system, allowing for a reliable assessment of the neurochemical effects of alcohol and caffeine. In particular, we sought to evaluate, using caffeine at different doses and via different routes of administration, its potential effect on alcohol-induced DAergic transmission in the AcbSh and alcohol-dependent salsolinol formation in the pVTA. An additional objective was to determine whether these effects are primarily mediated by one of the adenosine receptors, using selective $A_1$ and $A_{2A}$ receptor antagonists. Finally, another aim was to assess whether caffeine could influence mesolimbic DAergic transmission independently of alcohol-induced salsolinol formation, through exogenous administration of salsolinol.

## 7.2 – Methods

Figure 4 provides an overview of the experimental and analytical procedures used in this study.

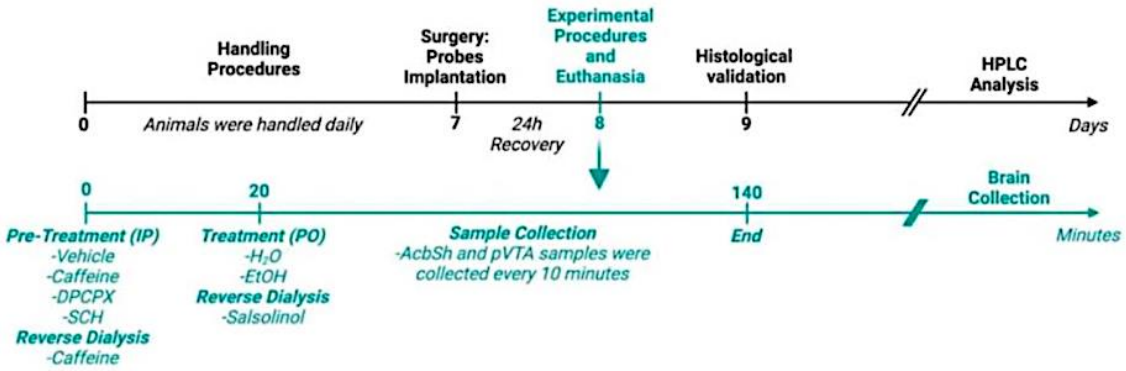


Figure 4. **Schematic overview of experimental design**.
*Abbreviations: IP = intraperitoneal; PO = per os/intragastrical; DPCPX = 8-Cyclopentyl-1,3-dipropylxanthine; SCH = 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; $H_2O$ = tap water; EtOH = alcohol; HPLC = high-performance liquid chromatography.*
Taken from Bassareo et al. (2024) *(CC BY-NC-ND 4.0 licence).*

### *7.2.1 - Animals*

Adult male Sprague-Dawley (SD) rats, weighing around 300 g and with an average age of PND 70, were used in this part of the study (total n = 77 animals; Charles River, Calco, Italy). Subjects had access to tap water and food *ad libitum*. Prior to the beginning of the experiments, rats were habituated to handling for one week and randomly assigned to the experimental groups. All procedures were designed to limit animal suffering and to use the minimum number of animals necessary, in accordance with the principles of Replacement, Reduction, and Refinement (3Rs), and were conducted in accordance with the authorization of the Italian Ministry of Health (No. 371/2020-PR).

### *7.2.2 - Drugs*

Alcohol (Sigma-Aldrich, Milan, Italy) was administered intragastrically (i.g.) at a dose of 1 g/kg, corresponding to a volume of 5.8 mL/kg, using a 20% volume/volume (v/v) solution in water. Caffeine (Sigma-Aldrich, Milan, Italy) was dissolved in saline at a volume of 3 mL/kg and was administered intraperitoneally (i.p.) at doses of 3 or 15 mg/kg, 20 min prior to water or alcohol administration. In a separate set of experiments, caffeine was dissolved in normal Ringer's solution (147 millimolar [mM] sodium chloride, 4 mM potassium chloride, 2.2 mM calcium chloride) to a final concentration of 10 micromolar (μM) and delivered into the pVTA by reverse dialysis beginning 30 min before tap water or alcohol exposure.

The adenosine receptor antagonists DPCPX (8-Cyclopentyl-1,3-dipropylxanthine) and SCH 58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) (Tocris, Bristol, UK) were suspended in saline with 0.3% Tween-80 and in 0.5% methylcellulose, respectively, and administered i.p. at a dose of 2 mg/kg, 20 min before water or alcohol. The racemic compound (±)-salsolinol (Santa Cruz Biotechnology, Dallas, TX, USA) was dissolved in normal Ringer's solution to a concentration of 10 nanomolar (nM) and infused into the pVTA via reverse dialysis. Doses and concentrations of alcohol (Melis et al., 2015; Bassareo et al., 2021; Howard et al., 2008; Brodie et al., 1990; Brodie & Appel, 1998; Xiao et al., 2009), caffeine (Acquas et al., 2002; Porru et al., 2020; Acquas et al., 2010), DPCPX (De Luca et al., 2007), SCH 58261 (Simola et al., 2004), and salsolinol (Hipólito et al., 2011; Melis et al., 2015; Hipólito et al., 2009) were chosen according to previously published studies.

### *7.2.3 - Microdialysis experiments*

#### *7.2.3.1 - Acute microdialysis probe preparation*

The acute microdialysis vertical probes (Figure 5) were prepared as previously described (Vargiu et al., 2021), according to the method of Di Chiara et al. (1993) as modified by Tanda et al. (1996).

Briefly, microdialysis probes were assembled using 22G hypodermic needles and silica capillaries (140 micrometers [μm] outer diameter, 50 μm inner diameter; Polymicrotechnologies Inc., Arizona, USA), subsequently inserted into an AN69 membrane composed of a sodium-meta-allyl-sulfonate acrylic copolymer (external diameter 310 μm, internal diameter 220 μm; Hospal Dasco, Italy) to form the active dialyzing portion. The final probe configuration allowed selective targeting of the AcbSh and the pVTA, with the entire membrane sealed except for the active dialyzing portion left exposed, measuring 1.5 mm for AcbSh probes and 0.8 mm for pVTA probes.

Figure 5. **Microdialysis vertical probe for "acute" sampling**.

#### *7.2.3.2 - Surgery and probe implantation*

On the day of surgery, the anesthetic Equithesin (sodium thiopental 1.2 g, magnesium sulfate 2.1 g, chloral hydrate 4.25 g, propylene glycol 42.8 mL, 90% alcohol 11.5 mL, brought to 100 mL with distilled water) was prepared and administered i.p. at a dose of 5 mL/kg. Once anesthetized, the animal's head was shaved and positioned in a stereotaxic apparatus (Kopf Instruments), a high-precision instrument that immobilizes the skull using ear bars and a nose-mouth clamp.

After proper positioning of the rat's head, a 2 cm incision was made in the scalp to expose the cranial surface. The stereotaxic apparatus features three graduated axes that provide three-dimensional coordinates of the skull. Using the bregma (the intersection of the parietal and temporal sutures) as a reference point (Paxinos & Watson, 1998), two small holes were drilled in the skull to allow the simultaneous stereotaxic implantation of two vertical probes, one into the pVTA and one into the AcbSh, according to the rat brain atlas of Paxinos & Watson (1998): anteroposterior (AP) –5.8 mm and mediolateral (ML) ±0.5 mm from bregma, and dorsoventral (DV) –8.0 mm from the dura mater for the pVTA; AP +1.8 mm and ML ±1 mm from bregma, and DV –7.6 mm from the dura mater for the AcbSh (Figure 6). Probes were implanted ipsilaterally, with left or right hemisphere assignment randomized. After securing the fibers to each other and to the skull using dental cement (GlasIonomer Cement CX-Plus, Shofu®), the incision was sutured anteriorly and posteriorly around the microdialysis implant. After surgery, animals were housed individually for post-operative recovery.

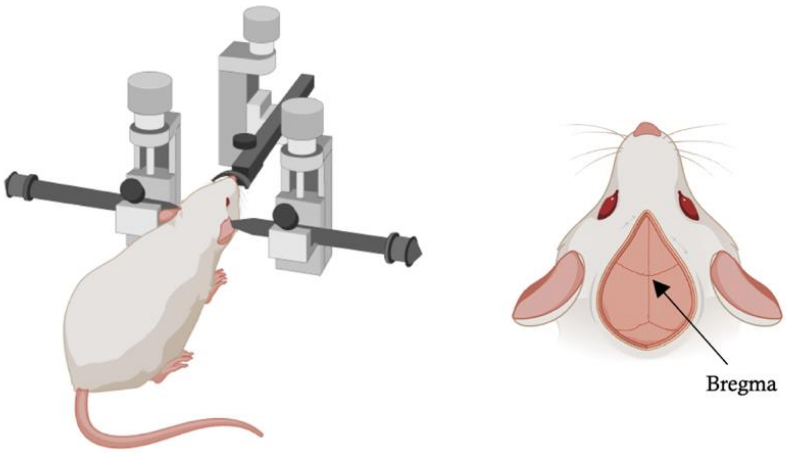


Figure 6. **Animal positioned in a stereotaxic apparatus (left) and exposed skull showing bregma (right).** Image created with BioRender (2025).

#### *7.2.3.3 - Experimental day*

The day after surgery, experiments were conducted in awake, freely moving animals. The pVTA and AcbSh probes were connected to an infusion pump (CMA/100, Carnegie Medicine, MA, USA) equipped with 1 mL Hamilton syringes and perfused with normal Ringer's solution at a flow rate of 1 µL/min. For the reverse dialysis experiments, Ringer's solution was either supplemented with caffeine to a final concentration of 10 µM, or with salsolinol at a concentration of 10 nM. The syringe needle was connected to the probe inlet via polyethylene (PE) tubing, and a separate two-way swivel was used for each probe, preventing any obstruction and allowing the animal to move freely. Dialysate samples were collected from the probe outlet using fluorinated ethylene-propylene (FEP) tubing. Dialysate samples (10 µL each) consisted of Ringer's solution, which mimics the ionic composition of the extracellular fluid, enriched with molecules diffusing from the surrounding extracellular space with a molecular weight below the 40 kilodaltons (kDa) membrane cutoff.

A total of 15 dialysate samples were collected per experiment over a 140-minute period. Typically, a pretreatment was administered at time 0, followed by the treatment 20 min later (or 30 min later in the caffeine reverse dialysis protocol), after which sample collection continued for an additional 2 hours. Collected dialysate samples were stored at –80°C until chromatographic analysis.

At the end of the microdialysis experiments, animals were euthanized with an overdose of anesthetic and decapitated using a rodent guillotine. The brains were then removed, rinsed in saline at 8°C, and stored at 4°C in 4% paraformaldehyde until histological analysis.

#### *7.2.3.4 - Sample analysis*

Dialysate samples (10 µL) were injected without purification into a high-performance liquid chromatography (HPLC) system (Figure 7) for the simultaneous quantification of salsolinol from the pVTA and DA from the AcbSh (Figure 8), as previously described (Bassareo et al., 2021).

HPLC analyses were performed using a reverse-phase column, Supelcosil LC-18 DB (15 cm length, 5 µm particle size; Supelco), optimized for the separation of polar compounds such as DA and salsolinol. Detection was carried out with a coulometric detector, Coulochem II (ESA, Bedford, MA, USA), fitted with a high-sensitivity analytical cell (model 5014B, ESA). The mobile phase consisted of a phosphate buffer containing 50 mM sodium dihydrogen phosphate, 0.5 mM sodium octyl sulfate, 0.1 mM ethylenediaminetetraacetic acid, 15% v/v methanol. For DA analysis, the mobile phase pH was adjusted to 5.5 using disodium hydrogen phosphate, whereas for salsolinol analysis the pH was adjusted to 3.7 using phosphoric acid. The mobile phase was degassed prior to use and slowly and continuously mixed using a magnetic stirrer. The flow rate was maintained at 1 mL/min by a Jasco PU-1580 pump, ensuring stable chromatographic conditions throughout the analysis. With these conditions, the sensitivity of the assay was 5 femtomoles (fmol) per sample.

Upon injection, samples were conveyed to the analytical cell of the coulometric detector, which operates with two electrodes set at +125 mV (oxidation) and –175 mV (reduction). Electrochemical oxidation and reduction of the analytes generated electrical currents that were recorded and converted into chromatograms, with each peak corresponding to a specific compound present in the sample. Chromatographic data acquisition and analysis were performed using ESA CDS software for compound quantification.

At the beginning of each analytical session, standard solutions of DA and salsolinol were used as references for identification (retention time) and quantification (peak area or peak height). They were prepared by weighing the respective powders and preparing individual $10^{-2}$ M stock solutions. Serial dilutions were then performed to obtain working standards of 200 fmol/20 µL, 100 fmol/20 µL, 50 fmol/20 µL, and 25 fmol/20 µL. Basal levels were defined as the mean

concentration of the last consecutive samples showing less than 10% variability, collected during the 30-min period preceding treatment.

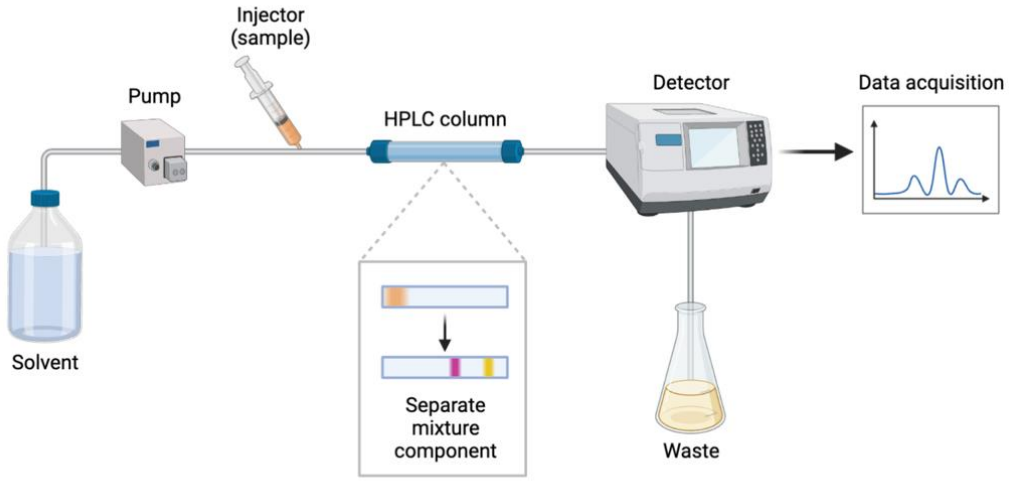


Figure 7. **Representation of the main components of an HPLC system**.
Image created with BioRender (2025).

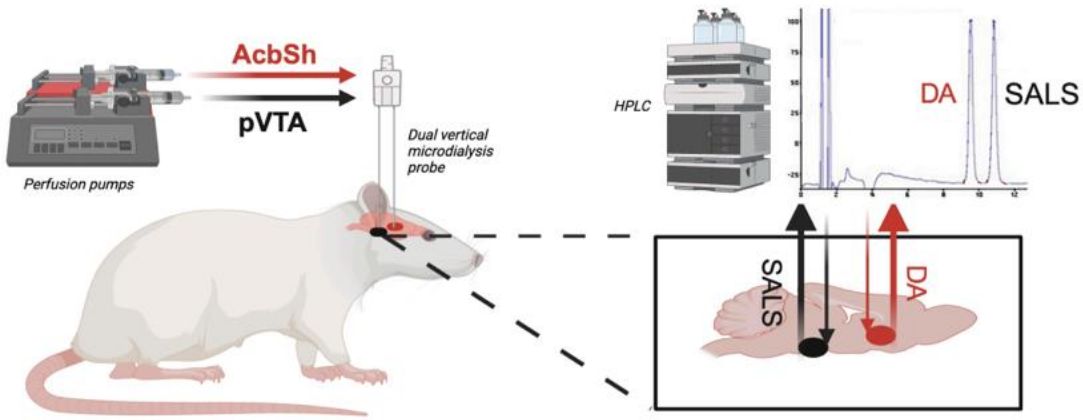


Figure 8. **Schematic representation of dual probe in-vivo brain microdialysis procedures, sampled areas and neurotransmitters recorded**. *Abbreviations: SALS = salsolinol*.
Taken from Bassareo et al. (2024) *(CC BY-NC-ND 4.0 licence).*

Histological analysis to verify probe implantation was performed after the brains had been stored for at least one week at 4°C in 4% paraformaldehyde. Coronal sections (100 μm) were cut using a vibratome (Technical Products International, St. Louis, MO, USA) and examined under a stereomicroscope to confirm the correct localization of the dialysis probes in the pVTA and AcbSh, with reference to the rat brain atlas of Paxinos & Watson (1998). Representative images of probe placement are shown in Figure 9.

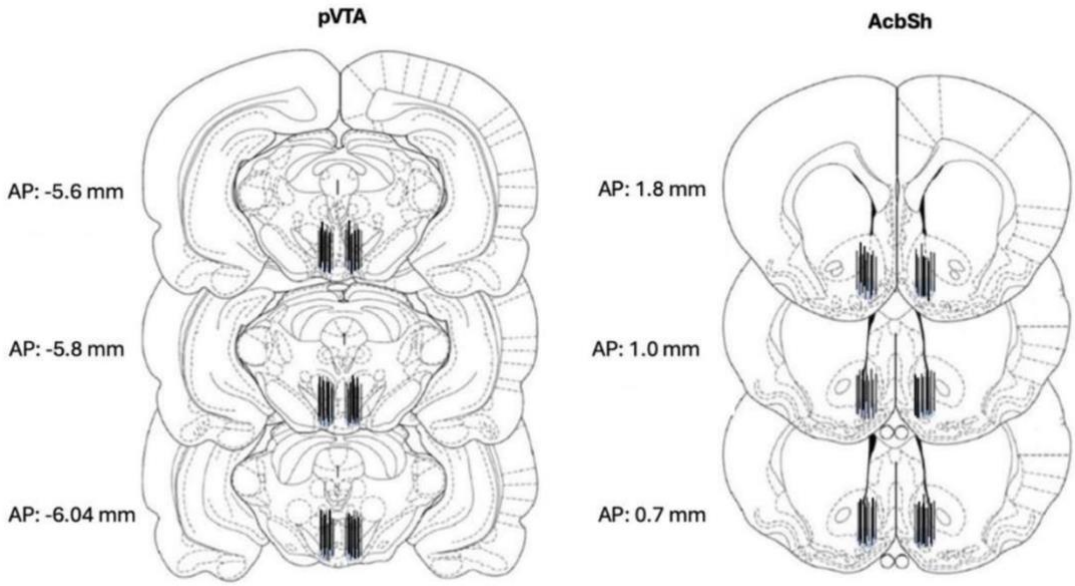


Figure 9. **Representative images showing the dialyzing portion of microdialysis probes after histological verification**. Taken from Bassareo et al. (2024) *(CC BY-NC-ND 4.0 licence).*

## 7.3 – Results

Three-way ANOVA of pVTA salsolinol concentrations after tap water or alcohol administration revealed significant effects of pre-treatment (caffeine 3 mg/kg) ($F_{1,21} = 69.74$; $p < 0.001$), treatment (alcohol) ($F_{1,21} = 91.6$; $p < 0.001$), time ($F_{12,252} = 53.71$; $p < 0.001$) and the following time × pre-treatment × treatment ($F_{12,252} = 40.9$; $p < 0.001$) interaction. Tukey's post hoc test revealed that alcohol elicits the formation of salsolinol in the pVTA and that caffeine (3 mg/kg) significantly prevents this effect ($p < 0.01$). Three-way ANOVA of AcbSh DA data points after tap water or alcohol administration showed a significant effect of time ($F_{12,252} = 23.47$; $p < 0.001$) and the following significant pre-treatment × time ($F_{12,252} = 20.33$; $p < 0.001$), treatment × time ($F_{12,252} = 17.36$; $p < 0.001$) and pre-treatment × treatment × time ($F_{12,252} = 15.48$; $p < 0.001$) interactions. Tukey's post hoc analysis revealed that caffeine pre-treatment (3 mg/kg) completely prevents the increase of DA in the ipsilateral AcbSh ($p < 0.01$) (Figure 10A).

Three-way ANOVA of salsolinol levels after treatment (tap water or alcohol) revealed significant effects of pre-treatment (caffeine 15 mg/kg) ($F_{1,22} = 145.08$; $p < 0.001$), treatment (alcohol) ($F_{1,22} = 180.72$; $p < 0.001$), time ($F_{12,264} = 80.09$; $p < 0.001$) and the following pre-treatment × treatment ($F_{1,22} = 144,36$; $p < 0.001$), time × pre-treatment ($F_{12,264} = 64.44$; $p < 0.001$), time × treatment ($F_{12,264} = 78.38$; $p < 0.001$), time × pre-treatment × treatment ($F_{12,264} = 64.39$; $p < 0.001$) interactions. Tukey's post hoc test revealed a significant formation of salsolinol only in the pVTA of saline+alcohol treated rats ($p < 0.01$) and showed that caffeine pre-treatment (15 mg/kg) completely prevented the pVTA salsolinol formation. Three-way ANOVA of DA data shows a significant effect of time ($F_{12,264} = 11.19$; $p < 0.001$) and the following significant pre-treatment × time ($F_{12,264} = 8.45$; $p < 0.001$), treatment × time ($F_{12,264} = 8.35$; $p < 0.001$) and pre-treatment × treatment × time ($F_{12,264} = 6.46$; $p < 0.001$) interactions. Tukey's post hoc analysis revealed that caffeine pre-treatment (15 mg/kg) prevented the increase of ipsilateral AcbSh DA after alcohol administration ($p < 0.01$) (Figure 10B).

Two-way ANOVA of salsolinol concentration after saline or caffeine administration during salsolinol perfusion showed a significant effect of time ($F_{12,72} = 224,118$; $p <$

0.001) but Tukey's post hoc test revealed that caffeine (15 mg/kg) failed to significantly affect pVTA salsolinol concentrations ($p>0.05$). Two-way ANOVA of AcbSh DA concentrations during salsolinol perfusion shows significant effects of pre-treatment ($F_{1,6} = 7.73$; $p = 0.03$) and time ($F_{12,72} = 8.6$; $p < 0.001$) and a significant pre-treatment x time ($F_{12,72} = 2.82$; $p < 0.01$) interaction. Tukey's post-hoc test revealed that caffeine pre-treatment (15 mg/kg) significantly reduced the increase of AcbSh DA induced by ipsilateral pVTA salsolinol perfusion ($p < 0.01$) (Figure 10C).

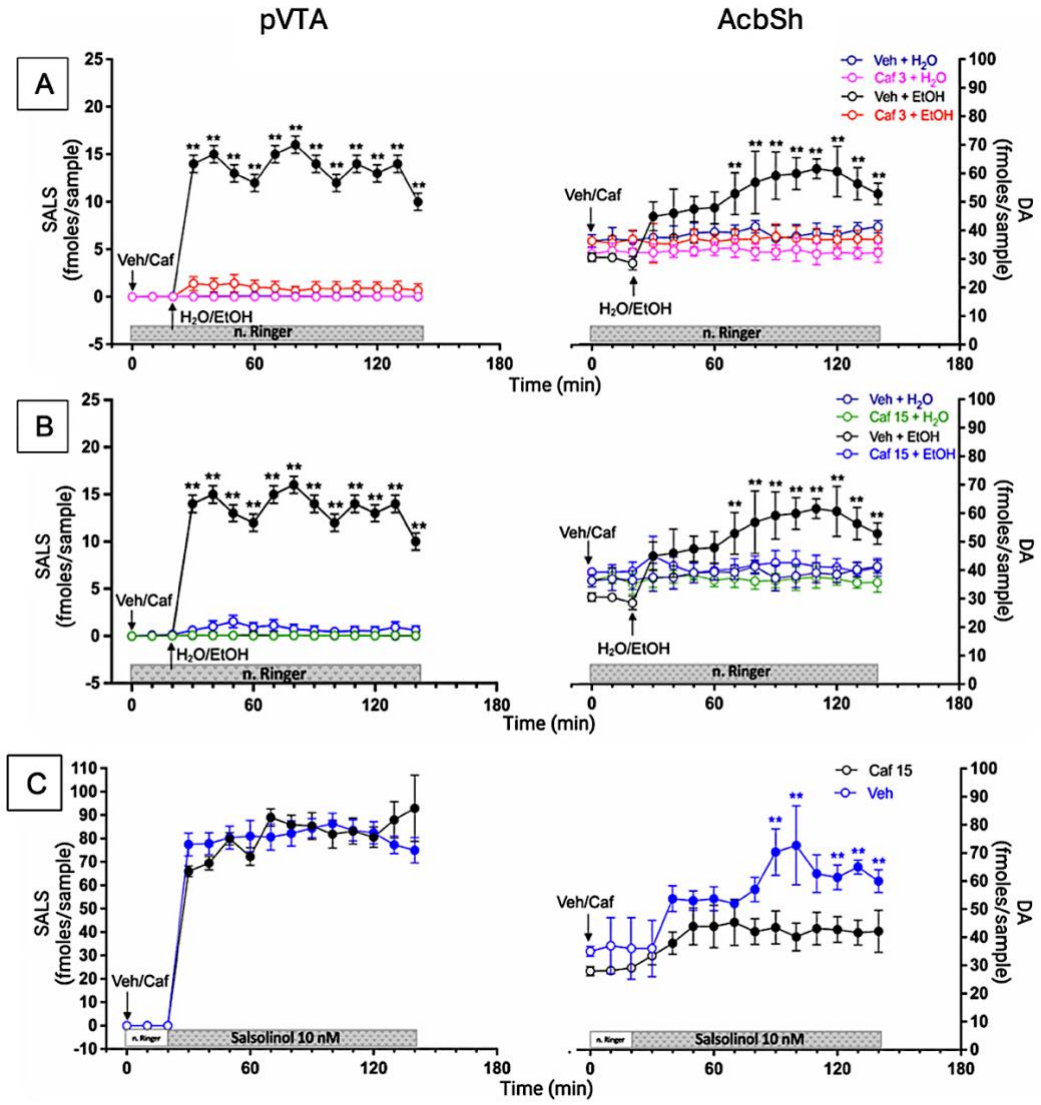


Figure 10. **Effects of i.p. caffeine pre-treatment on alcohol-induced pVTA salsolinol formation and AcbSh DA increase**. **A**: caffeine 3 mg/kg in normal Ringer; **B**: caffeine 15 mg/kg in normal Ringer; **C**: caffeine 15 mg/kg in Ringer containing 10 nM salsolinol.
*Abbreviations: Veh = saline; Caf = caffeine; $H_2O$ = tap water; EtOH = alcohol; SALS = salsolinol*. Filled symbols indicate samples representing $p < 0.001$ versus basal; **$p < 0.01$ versus Caf (3 mg/kg) + EtOH, versus Caf (15 mg/kg) + EtOH, and versus Caf (15 mg/kg) + SALS.
Taken from Bassareo et al. (2024) *(CC BY-NC-ND 4.0 licence).*

Three-way ANOVA of salsolinol concentrations, monitored for 20 min after saline or DPCPX or SCH 58261 administration, failed to reveal any significant effect ($p>0.05$). Three-way ANOVA of AcbSh DA data after tap water or alcohol administration, shows a significant effect of time ($F_{12,204} = 1.99$; $p = 0.027$) and a significant treatment × time ($F_{12,204} = 1.92$; $p = 0.034$) interaction. Tukey's post-hoc test revealed that SCH 58261 pre-treatment significantly prevented the increase of ipsilateral AcbSh DA after alcohol administration ($p>0.05$ SCH 58261 + tap water versus SCH 58261 + alcohol). Tukey's post-hoc test revealed that pre-treatment with DPCPX failed to significantly affect the increase of DA in the ipsilateral AcbSh ($p<0.05$ DPCPX + tap water versus DPCPX + alcohol) (Figure 11A).

Two-way ANOVA of salsolinol concentrations during caffeine perfusion of the pVTA and two-way ANOVA of salsolinol concentrations after alcohol administration in the presence of caffeine failed to reveal any significant effect ($p > 0.05$). Two-way ANOVA of DA concentrations during caffeine perfusion of the pVTA and two-way ANOVA of DA concentrations after tap water or alcohol administration in the presence of caffeine failed to reveal any significant effect ($p > 0.05$) (Figure 11B).

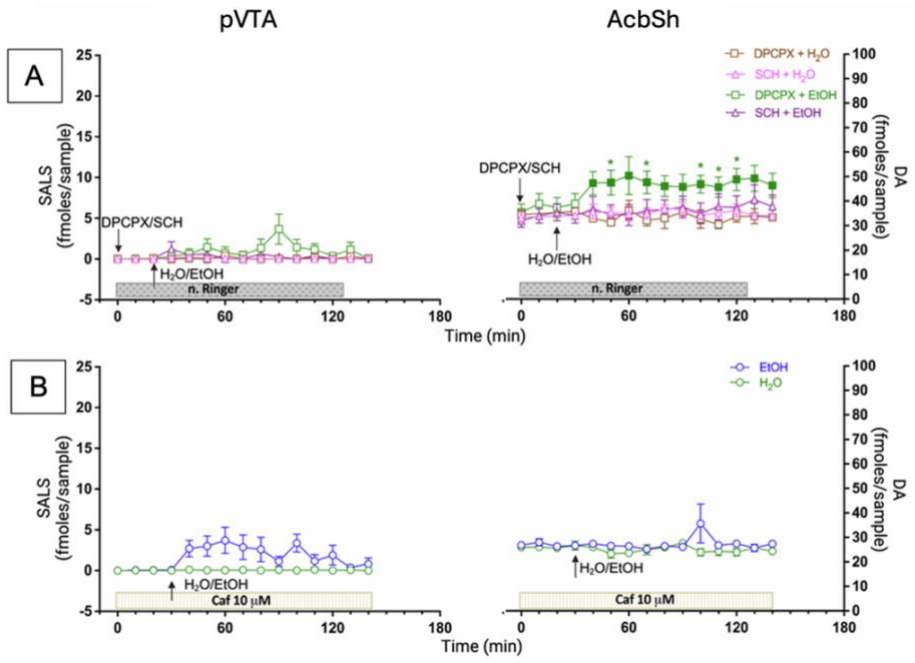


Figure 11. **Effects of i.p. DPCPX and SCH 58261 pre-treatment (A) and caffeine perfusion (B) on alcohol-induced pVTA salsolinol formation and AcbSh DA increase**.
*Abbreviations: SCH = SCH 58261; $H_2O$ = tap water; EtOH = alcohol; Caf = caffeine SALS= salsolinol.*
Filled symbols indicate samples representing $p < 0.001$ versus basal; *$p<0.05$ versus DPCPX+$H_2O$.
Taken from Bassareo et al. (2024) *(CC BY-NC-ND 4.0 licence).*

## 7.4 – Discussion

Based on the present in vivo brain microdialysis data, the study demonstrates that acute alcohol administration induces salsolinol formation within the pVTA and a concomitant increase in DA transmission in the AcbSh. These neurochemical effects were consistently prevented by a caffeine pre-treatment, indicating that caffeine interferes with the ability of alcohol to activate the mesolimbic DA pathway at an early neurochemical level. Specifically, this interference appears to involve mechanisms related to salsolinol generation within the pVTA, rather than alterations in its extracellular bioavailability, as salsolinol levels remained unchanged during pVTA perfusion with exogenous salsolinol in the presence of caffeine. On this basis, we hypothesized that caffeine counteracts alcohol-induced activation of pVTA DA neurons, as well as alcohol-dependent salsolinol generation and AcbSh DA transmission, through an adenosine receptor-mediated mechanism. Consistent with this hypothesis, blockade of both $A_1$ and $A_{2A}$ adenosine receptors by DPCPX or SCH 58261, respectively, prevented the formation and detection of salsolinol in the pVTA following alcohol administration. In contrast, differential effects were observed on alcohol-evoked DA release in the AcbSh. While antagonism of $A_{2A}$ receptors with SCH 58261 prevented the alcohol-induced increase in DA transmission, blockade of $A_1$ receptors with DPCPX was ineffective. Under these conditions, it is conceivable that blockade of $A_1$ receptors, resulting in the removal of endogenous adenosine tone, may facilitate DA release in the AcbSh despite the absence of locally generated salsolinol. This dissociation suggests that alcohol-induced salsolinol formation and alcohol-driven activation of mesolimbic DA transmission are regulated by partially independent mechanisms. Further support for this hypothesis comes from the observation that caffeine attenuated the increase in AcbSh DA elicited by exogenous salsolinol delivered by reverse dialysis. This finding indicates that caffeine can prevent mesolimbic DA activation even when salsolinol formation is bypassed, suggesting the involvement of additional modulatory processes.

Complementary electrophysiological and metabolomic analyses performed in the same study of Bassareo et al. (2024) support the interpretation of the microdialysis results. For example, consistent with the notion that caffeine does not directly

interfere with salsolinol generation, it has been demonstrated that caffeine does not prevent salsolinol formation in vitro. This finding rules out the possibility that caffeine directly affects salsolinol production by inhibiting catalase, an enzyme whose activity has been shown to be required for salsolinol formation (Melis et al., 2015; Bassareo et al., 2021). In vitro patch-clamp recordings further showed that caffeine suppresses the modulatory effect of alcohol on the firing rate of pVTA DA neurons. Importantly, the same effect was reproduced by the $A_{2A}$ receptor antagonist SCH 58261, but not by the $A_1$ receptor antagonist DPCPX, suggesting that caffeine's ability to suppress alcohol-induced modulation of DA firing is mediated via $A_{2A}$ receptors. Additionally, caffeine also prevented the excitatory effects of salsolinol on pVTA DA neurons, reinforcing the results obtained in vivo. Lastly, untargeted metabolomics of pVTA lysates showed that both alcohol and caffeine alter lipid profiles, with caffeine preventing most alcohol-induced changes, including in oleamide, a metabolite known to inhibit DA tone and reduce alcohol intake in rats (Hryhorczuk et al., 2018; Alen et al., 2018). Caffeine also increased carnitine and acyl-carnitine levels under alcohol exposure. Since carnitine has been shown to inhibit catalase activity and block catalase-mediated alcohol effects in mice (Manrique et al., 2006; Gülçin, 2006), this increase may reduce catalase-mediated alcohol oxidation and prevent salsolinol formation. This mechanism could explain why caffeine prevents salsolinol formation in vivo but shows no effect in vitro. For further details on these complementary studies supporting the microdialysis experiments, see the full version of Bassareo et al. (2024).

In conclusion, the present work provides, to our knowledge, the first direct evidence that caffeine prevents alcohol-induced activation of the mesolimbic DA pathway. Our results indicate that this effect is primarily mediated by inhibition of salsolinol formation in the pVTA, with $A_{2A}$ receptor signaling playing a predominant role in mediating alcohol-evoked DA release in the AcbSh, while $A_1$ receptor blockade affects salsolinol generation without significantly altering DA transmission. Moreover, caffeine also attenuates DA responses elicited by exogenous salsolinol, suggesting the involvement of additional mechanisms independent of salsolinol formation. On the other hand, further studies are needed to clarify the precise mechanisms underlying $A_{2A}$ receptor-mediated inhibition of alcohol-induced

mesolimbic activation and the dissociation between $A_1$ and $A_{2A}$ receptor contributions to salsolinol generation and DA release. One limitation of the present part of this study is that it focuses exclusively on the mesolimbic DA pathway in alcohol-naïve rats, which reflects only the initial phase of alcohol exposure and early vulnerability to AUD. Although this system represents a critical entry point in the neurobiology of addiction, as discussed in Section 6, the onset and maintenance of AUC involve a broader network of brain regions, including the mesocortical system. Moreover, the experiments rely on an acute, non-contingent administration of alcohol, which does not fully capture the complexity of voluntary intake, learning processes, and neuroadaptations associated with chronic alcohol exposure. Consequently, future investigations should aim to extend these findings to later stages of AUD, including repeated exposure, withdrawal, craving, and relapse, and to examine potential modulatory factors that may influence caffeine's effects within the brain circuits implicated in AUD.

# 8. Phosphorylation of Extracellular Signal-Regulated Kinase and Dopamine Transmission in the Nucleus Accumbens Shell Following Acute Administration of Red Bull®, Alcohol, and Their Combination

The following Section reports unpublished data from recent research conducted by Puliga et al. (manuscript in preparation).

## 8.1 – Background and aim

It has been shown that acute exposure to various drugs of abuse leads to ERK activation in several brain structures, with the AcbSh being one of the most strongly activated regions (Girault et al., 2007). This effect is thought to be driven, at least in part, by the activation of post-synaptic D1 receptor signaling, following drug-induced increases in DAergic transmission, preferentially within the AcbSh (Acquas et al., 2007; Di Chiara, 1999; Di Chiara, 2002). Consistently, Ibba et al. (2009) indicate that DA D1-mediated ERK activation may be a key mechanism through which alcohol modulates both drug-taking and drug-seeking behaviors, with the mesolimbic pathway emerging as critically involved in the primary reinforcing effects of alcohol.

While previous studies have primarily focused on classical drugs of abuse and alcohol, little is known about the effects of EDs, such as RB, either alone or in combination with alcohol, on ERK phosphorylation in the AcbSh. Evidence for a possible modulation of the ERK signaling pathway by EDs comes from Porru et al. (2020), who investigated the effects of caffeine, the main psychoactive component of these beverages. In that study, pretreatment with caffeine was shown to prevent the alcohol-induced ERK activation in the AcbSh. In line with these findings, Section 7 of this thesis demonstrated that caffeine pretreatment also prevents the alcohol-induced increase in DA levels in the AcbSh (Bassareo et al., 2024).

However, caffeine represents only one of the many components of RB; therefore, given the increasing tendency to consume AMED, it is necessary to evaluate the effects of the complete formulation. Accordingly, the present study aimed to investigate the effects of acute administration of RB, alcohol, or their combination on ERK phosphorylation and DA transmission in the AcbSh, while

also assessing locomotor activity immediately following treatment. In particular, to address this aim, two distinct cohorts of naïve rats were used: young adults and adults. This choice of age was intended to better characterize the acute effects of these substances in a fully developed mesolimbic system, avoiding the use of adolescent animals, whose mesolimbic system is not yet mature. Moreover, this approach allowed for a more direct comparison of the DAergic responses observed in this acute study with those obtained in subsequent subchronic and chronic studies.

The overall experimental design is illustrated in Figure 12.

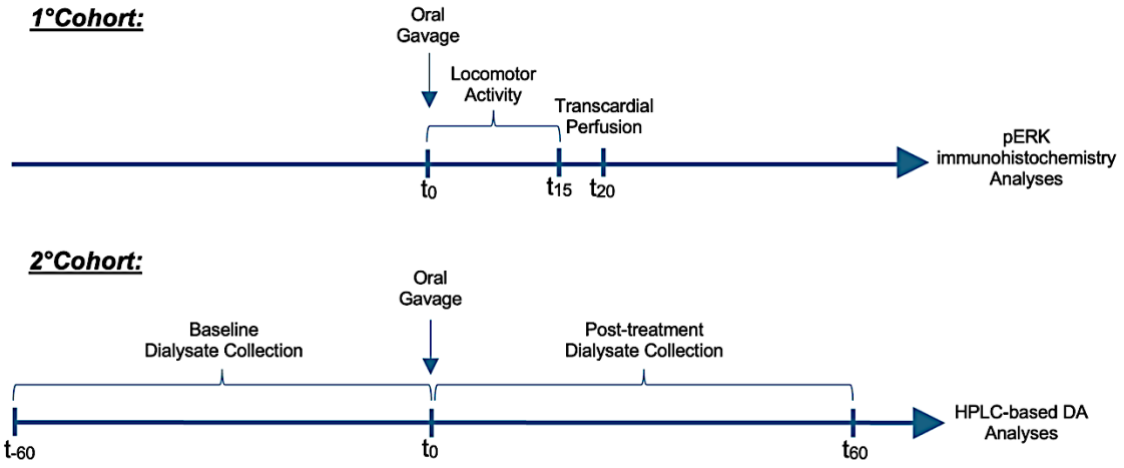


Figure 12. **Schematic representation of the experimental designs pursued with two independent cohorts of animals**.

### *8.2.1 - Animals*

Two independent cohorts of 24 male young adults (200 g) and adults (300 g) SD rats were used in this part of the study (total n = 48; Charles River, Calco, Italy). Rats were housed under controlled environmental conditions, with a constant temperature of 22 °C and relative humidity of 60%, on a 12 hours light / 12 hours dark cycle (lights on from 08:00 to 20:00 hours). Standard laboratory food and tap water were available ad libitum throughout the study. All experimental procedures were carried out in compliance with the authorization of the Italian Ministry of Health (No. 169/2024-PR). Every effort was made to minimize animal suffering and to reduce the number of animals used, in accordance with the principles of the 3Rs.

### *8.2.2 - Drugs*

Solutions were administered by oral gavage (volume of administration: 20 mL/kg of body weight, i.g.). Alcohol was prepared as a 20% v/v solution in tap water and administered at a dose of 3.2 g/kg. RB was diluted to 35% and administered at a dose corresponding to 2 Can Equivalents (CanEq)/kg, defined as the equivalent of two 250 mL cans for a 70 kg human subject. Alcohol and RB were administered either alone or in combination (AMED), while control animals received tap water only. AMED solution contained the same percentage of alcohol (20% v/v) and RB (35% v/v) as the individual treatments. The alcohol dose was selected based on the protocol described by Coleman et al. (2011) and adapted for rats. According to the dose conversion method proposed by Nair & Jacob (2016), the dose of 3.2 g/kg of alcohol used in rats corresponds to a human equivalent dose of 0.52 g/kg, equal to 36.4 g of pure alcohol (2.6 standard USA drinks) for a 70 kg individual. Similarly, using the same approach, the RB dose of 2 CanEq, containing 160 mg of caffeine for a 70 kg human, corresponds to approximately 14 mg/kg in rats.

### *8.2.3 - Locomotor activity*

The first cohort consisted of 24 naïve young adult male rats. One week after arrival, animals were weighed daily to monitor body weight. During this period, each rat was handled daily and habituated to the gavage procedure by administering 1 ml/kg of tap water via oral gavage. Once they reached approximately 200 g, they were randomly assigned to four experimental groups based on the treatment they would receive via acute oral gavage: AC-TW (tap water); AC-ALCOHOL (alcohol); AC-RB (RB); AC-AMED (AMED). Immediately after administration, animals were placed in a locomotor activity chamber to record spontaneous locomotion for 15 minutes, in order to assess differences between experimental groups. Locomotor activity was measured using the Opto-Varimex Mini system (Columbus Instruments), a device designed to detect movement in rodents via infrared beam interruptions. Twenty minutes after the oral gavage, animals were deeply anesthetized and subjected to transcardial perfusion.

### *8.2.4 - Immunohistochemistry: free-floating sections protocol*

After transcardial perfusion with ice-cold phosphate-buffered saline (PBS; composed of 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium dihydrogen phosphate, and 2 mM potassium dihydrogen phosphate; pH 7.4), followed by ice-cold 4% paraformaldehyde, brains were removed and post-fixed overnight in 4% paraformaldehyde at 4 °C. The following day, brains were washed in PBS (3 times × 20 min) and subsequently sectioned in PBS maintained at a 4 °C constant temperature. Coronal sections of 40 µm in thickness were obtained using a Leica VT1000 S vibratome (Leica Microsystems, Germany), covering the entire rostro-caudal extent of the AcbSh. Free-floating sections were stored at -20 °C in a cryoprotective solution until their immunohistochemical processing.

Immunohistochemistry was carried out on free-floating sections following a two-day protocol, with all steps performed at a constant temperature of 4 °C under gentle agitation, in agreement with our previously published protocol (Ibba et al., 2009; Porru et al., 2021). On day 1, sections were placed two per well in 24-well plates, and sections were washed in Tris-buffered saline (TBS, 400 µL per well, 3 times × 10 min), followed by TBS containing 0.1% of Triton X-100 (TBST-X, 400 µL per well, 3 times × 10 min). Sections were then incubated in a just prepared 1% hydrogen peroxide solution (400 µL per well, 1 time × 30 min), after which they were washed in TBST-X (400 µL per well, 3 times × 30 min). Non-specific binding sites were blocked by incubating the sections in TBST-X containing 3% bovine serum albumin (TBST-X/BSA 3%, 400 µL per well, 1 time × 60 min). Finally, sections were incubated overnight with the primary antibody against phospho-p44/42 MAPK (ERK1/2) at a 1:350 dilution in TBST-X/BSA 3% (400 µL per well, 1 × overnight). Negative controls (blanks) were incubated in TBST-X/BSA 3% without the primary antibody. The following day, sections were washed in TBST-X (400 µL per well, 3 times × 10 min) and then incubated with a biotinylated anti-rabbit secondary antibody diluted 1:800 in TBST-X/BSA 1% (400 µL per well, 1 time × 60 min). After washing in TBST-X (400 µL per well, 3 times × 10 min), sections were incubated with the avidin biotin peroxidase complex (400 µL per well, 1 time × 60 min) and then washed in TBST-X (400 µL per well, 3 times × 10 min). Sections were incubated in a freshly prepared 3,3'-diaminobenzidine (DAB) working solution composed of

0.5 mg/mL DAB, 0.015 mg/mL glucose oxidase, 0.4 mg/mL ammonium chloride, and 1.25 mg/mL D-(+)-glucose in TBS. Once optimal staining was achieved, the reaction was stopped by removing the DAB solution and washing the sections in TBS (1 mL per well, 3 × 20 min). Lastly, brain sections were mounted onto positively charged glass slides using a Petri dish with TBS, and they were left to dry overnight in a clean, dry environment. On the following day, sections were dehydrated through a graded alcohol series. Slides were subsequently immersed in: deionized water (1 time × 10 min), 80% alcohol (2 times × 5 min), 95% alcohol (2 times × 5 min), 100% alcohol (2 times × 5 min), and 100% xylene (2 times × 5 min). At this stage, coverslips were mounted using Eukitt, and after drying for at least one hour, images of the slides at different magnifications were captured under a Zeiss Axioskop 40 microscope equipped with a Nikon D-5000 digital camera (Figure 13). Images were then analyzed for the quantification of pERK-positive neurons using ImageJ software, applying a manually verified threshold. All analyses were conducted in a double-blind manner with respect to the experimental groups.

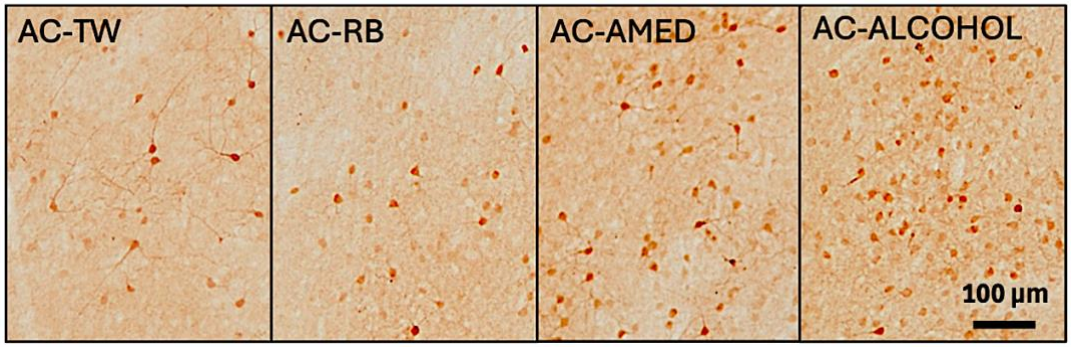


Figure 13. **Representative images of pERK-positive cells in the AcbSh.** Panels show selected regions of the medial AcbSh at 10× magnification; cell counts were performed on the entire AcbSh.

### *8.2.5 - Microdialysis experiments*

Microdialysis experiments were performed as described in Section 7.2.3 in a new cohort of 24 naïve adult male rats. Following the collection of baseline samples, animals received acute oral gavage according to their experimental group, and a total of 6 post-treatment dialysate samples were collected. Samples were analyzed for DA content using an HPLC system, and histological analyses were carried out to confirm probe placement, following the procedures described in Subsection 7.2.3.4.

## 8.3 – Results

A one-way ANOVA performed on locomotor activity immediately after oral gavage revealed a significant main effect of treatment for both total ($F_{3,20} = 3.9$; $p = 0.0232$) and ambulatory activity ($F_{3,20} = 6.3$; $p = 0.0036$). Post hoc Tukey's multiple comparisons test showed significant differences between AC-ALCOHOL and AC-RB and between AC-AMED and AC-RB in both measures, whereas no significant differences were observed among the remaining comparisons (Figure 14).

A one-way ANOVA conducted on pERK levels in the AcbSh revealed a highly significant effect of treatment ($F_{3,173} = 49$; $p < 0.0001$). Post hoc Tukey's multiple comparisons test indicated significant differences between AC-ALCOHOL and all the other groups, as well as between AC-AMED and all the other groups. No significant difference was observed between AC-TW and AC-RB (Figure 15).

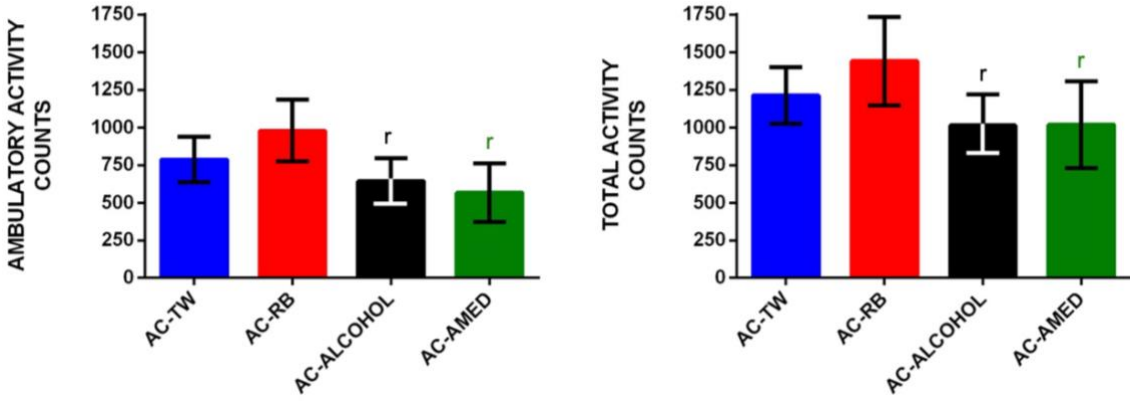


Figure 14. **Ambulatory (left) and total (right) locomotor activity during the 15 min following i.g. treatment.** Activity is expressed as counts of infrared beam interruptions. $^{r}p < 0.05$ versus AC-RB.

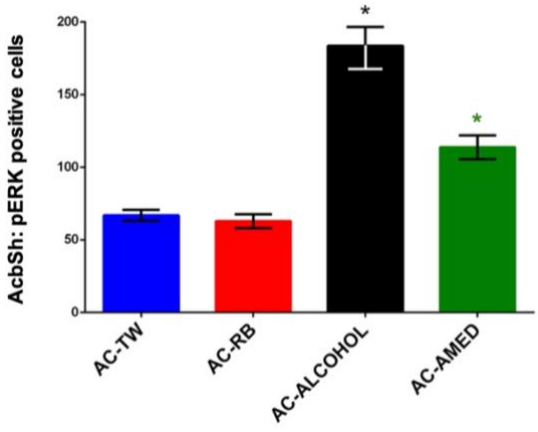


Figure 15. **Number of pERK positive cells in the AcbSh 20 minutes after i.g. treatment.** $^{*}p < 0.05$ versus all other groups.

A two-way repeated measures ANOVA of AcbSh DA concentrations following i.g. treatment showed significant main effects of treatment ($F_{3,17} = 20.66$; $p < 0,0001$) and time ($F_{6,102} = 7.53$; $p < 0,0001$), as well as a significant treatment × time interaction ($F_{18,102} = 2.36$; $p = 0.0037$). Post hoc Tukey's multiple comparisons tests showed that AC-ALCOHOL significantly differed from AC-TW at all time points over the 60 minutes following treatment. Significant differences were observed between AC-ALCOHOL and AC-RB at minutes 10, 40, and 50, and between AC-ALCOHOL and AC-AMED at minutes 10, 30, 40, 50, and 60 (Figure 16).

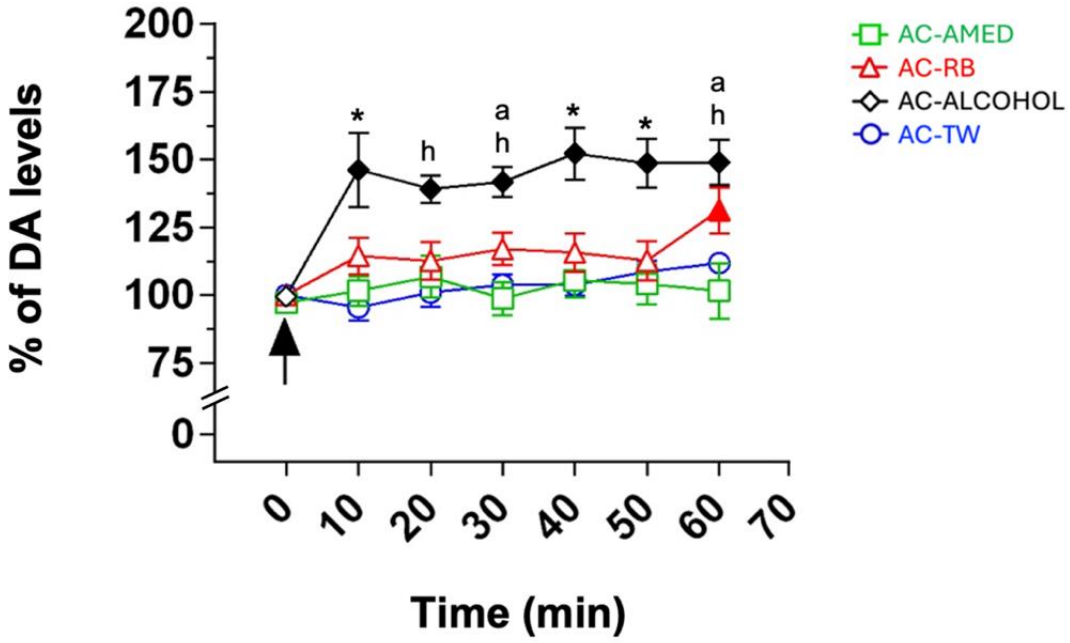


Figure 16. **Extracellular DA concentrations in the AcbSh measured over 60 minutes following i.g. treatment.** Data are expressed as percentage of basal values ($t_0$ = 100%, calculated as the mean of six consecutive basal samples) and reported as mean ± SEM. Filled symbols indicate samples representing $p < 0.05$ versus basal values. *$p < 0.05$ versus all other groups; $^{h}p < 0.05$ versus AC-TW; $^{a}p < 0.05$ versus AC-AMED. The color of each significance symbol indicates the group showing the significant difference: black = AC-ALCOHOL.

## 8.4 – Discussion

In this part of the study, we investigated the effects of acute oral gavage administration of alcohol, RB, and their combination (AMED) on spontaneous locomotor activity, AcbSh DA transmission and pERK-positive cell activation.

As expected, results showed that RB administration induced an increase in locomotor activity during the first 15 minutes following treatment, consistent with its stimulant properties (Ferreira et al., 2024). This locomotor stimulating effect is likely attributable to caffeine, which was present at approximately 14 mg/kg in the administered RB solution, a dose within the range previously reported to enhance locomotor activity in both young and adult rats (Marin et al., 2011). By contrast, alcohol (3.2 g/kg) produced a decreased locomotor activity, consistent with the sedative effects observed at higher alcohol doses. When RB was co-administered with alcohol (AMED), the stimulatory effect was no longer observed, indicating that the AMED combination was unable to prevent the sedative effect of alcohol. Importantly, AC-AMED and AC-ALCOHOL received equivalent alcohol doses, allowing a direct comparison between treatments in which the only differing variable was whether alcohol was administered alone or co-administered with the addition of RB. Moreover, 20 minutes after i.g. treatment, AC-AMED prevented the alcohol-induced stimulation of mesolimbic DAergic transmission and significantly reduced the alcohol-induced ERK phosphorylation in the AcbSh compared with AC-ALCOHOL, whereas RB alone did not show differences in ERK activation relative to control animals.

Taken together, these results indicate that, under acute administration, even when the alcohol dose is equivalent, AMED does not trigger the same rewarding response as alcohol alone, suggesting that increasing the AMED dose may be required to match the effect. This reduction may be attributed to the action of caffeine, the main psychoactive stimulant in RB, as previous studies have demonstrated that acute caffeine administration prevents the alcohol-induced increase in ERK activation in the AcbSh (Porru et al., 2020). Consistently, as described in Section 7 of this thesis, caffeine pretreatment was also found to prevent the alcohol-induced increase in DA levels in the AcbSh (Bassareo et al., 2024).

Therefore, these preventive effects could be attributable, at least in part, to the action of caffeine. However, it is important to consider that other compounds present in RB may also contribute to these effects. Consequently, further studies are needed to clarify the specific role of acute AMED administration in modulating alcohol-induced mesolimbic signaling.

# 9. Binge-Like Administration of Alcohol Mixed to Energy Drinks to Male Adolescent Rats Severely Impacts on Mesocortical Dopaminergic Function in Adulthood

This Section presents research carried out by our group and adapted from our recent publication (Dazzi et al., 2024).

## 9.1 – Background and aim

As stated earlier, alcohol is often combined with EDs, which contain substantial amounts of caffeine together with other compounds, due to the belief that stimulants can counteract alcohol's sedative effects or accelerate the onset of its effects (O'Brien et al., 2008; Higgins et al., 2010). AMED use is particularly common on weekends and is often driven by the misconception that EDs are safe, which ultimately promotes heavier drinking of AMED and results in higher levels of alcohol-related impairment (Marczinski & Fillmore, 2006, 2014; Arria et al., 2011). This is particularly concerning during adolescence, a critical developmental period during which the mPFC is still maturing and therefore more susceptible to the effects of substances of abuse. In this age group, AMED consumption is frequently characterized by BD-like episodes, during which EDs are combined with high-alcohol beverages, further increasing the risk of acute alcohol intoxication (Petribu et al., 2023). Furthermore, longitudinal studies indicate that AMED consumption during adolescence may contribute to greater alcohol intake and an elevated risk of developing AUD in adulthood (Arria et al., 2011; Miller, 2008a, 2008b).

As outlined in Section 6 of the present thesis, the mesocorticolimbic DAergic pathway is a key component of the brain's reward and motivation circuitry and has been shown to play a critical role in regulating impulsive behavior and mediating all stages of addiction (Kalivas & Volkow, 2005; Koob & Volkow, 2010; Jentsch et al., 2014). Dysfunctions within this system have been linked to heightened impulsivity and loss of control, contributing to the development of addictive behaviors (Jentsch et al., 2014; Koob et al., 2014). Within this framework, the mPFC is believed to be central in driving the maladaptive changes characteristic of addiction. Specifically,

these changes involve an increased responsiveness to drug-related cues combined with impaired regulation of the mesolimbic system, which underlies drug-seeking behavior (Kalivas & Volkow, 2005). Consequently, altered mPFC function may serve as an early marker of neuroadaptation in alcohol addiction (Koob et al., 2014). Although substantial evidence exists in humans, animal research remains limited, leaving unclear how adolescent exposure to AMED affects reward-related circuits and behavior into adulthood. Understanding these effects is essential for assessing the potential long-term consequences of early exposure to these substances.

Based on the previous considerations, the aim of the present part of my study was to investigate the long-term impact of adolescent exposure to alcohol, RB, or AMED on alcohol consumption and brain function in adulthood, in order to clarify how such exposure may influence behavioral and neurochemical changes related to reward. Specifically, the study aimed to assess how non-contingent administration of these substances during adolescence, following a typical adolescent BD pattern of intake, affects voluntary alcohol consumption in young adulthood and DAergic responsivity of the mPFC during reward-related processes.

Figure 17 describes the experimental design carried out in this study.

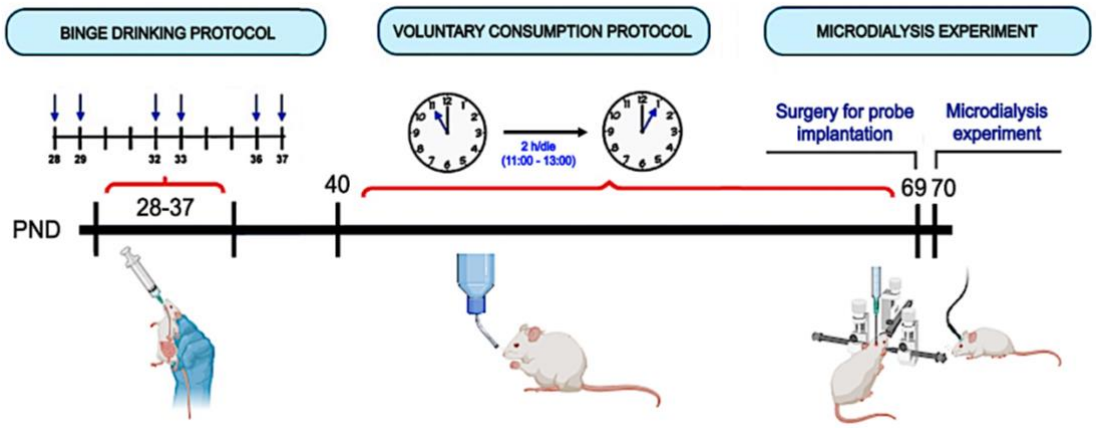


Figure 17. **Schematic overview of the procedures conducted in this study**.
*Abbreviations: PND = Postnatal day; h/die = hours per day*.
Image created with BioRender (2025).

### *9.2.1 - Animals*

30 male SD adolescent rats (Charles River, Como, Italy) were used in this study. Animals were housed under controlled environmental conditions, with a 12 hours light / 12 hours dark cycle (lights on from 08:00 to 20:00 hours), a constant temperature of 22±2°C, and 65% relative humidity. Rats had ad libitum access to standard laboratory food and tap water. All efforts were made to minimize animal suffering and reduce the number of animals used, in accordance with the 3R principles of animal experimentation. All experimental procedures were carried out in compliance with the authorization of the Italian Ministry of Health (No. 371/2020-PR) .

### *9.2.2 - Drugs*

During the BD protocol, alcohol (3.2 g/kg), RB (2 CanEq), their combination (AMED: 3.2 g/kg alcohol + 2 CanEq RB), or tap water were prepared and administered by oral gavage as reported in Subsection 8.2.2.

In the subsequent voluntary alcohol consumption phase, alcohol was made available as a 5% v/v solution, with a progressively decreasing sucrose concentration as described by Lallai et al. (2016).

### *9.2.3 - Binge-like protocol of administration*

At weaning on Postnatal Day (PND) 21, animals were housed in groups of four per cage and randomly assigned to one of four experimental groups, which received a BD protocol of administration with tap water (BD-TW), alcohol (BD-ALCOHOL), RB (BD-RB), or AMED (BD-AMED), as previously described. Treatments were administered once daily via oral gavage from PND 28 to 37 following an intermittent schedule of two consecutive days of administration (PND 28-29, 32-33, 36-37) followed by two days without treatment (PND 30-31, 34-35, 38-39). This protocol was designed to mimic a validated adolescent BD model described by Coleman et al. (2011).

### *9.2.4 - Alcohol voluntary consumption protocol*

The day after the last administration of the BD protocol, from PND 40, all experimental groups underwent a four-week period of voluntary alcohol consumption. During this phase, animals were temporarily separated from their cage mates and individually housed for the 2-hour daily alcohol access period (between 11:00 and 13:00 hours), with no food or water available during this period, which were instead provided ad libitum during the remaining 22 hours. To promote alcohol intake without using food or fluid deprivation, the alcohol solution was sweetened with sucrose, following the procedure described by Lallai et al. (2016). Specifically, the solution contained a constant 5% alcohol v/v, with sucrose starting at 5% and reduced by 1% every two days until reaching 1%, which was then maintained from the 9$^{th}$ day for the rest of the treatment. Alcohol intake was measured in each animal at the end of each session.

### *9.2.5 - Microdialysis experiments*

The microdialysis experiments were conducted similarly to those described in the previous Sections; therefore, only the main differences, compared to the previous ones described in Subsection 7.2.3, are outlined below.

#### *9.2.5.1 - Acute microdialysis probe preparation*

In the present study, probes were specifically designed for insertion into the mPFC. The active length of the dialysis membrane was 4 mm, allowing sampling from both the infralimbic and prelimbic cortices, as previously described (Dazzi et al., 2014). Before use, probes were tested in vitro to determine their recovery rate using a DA solution of known concentration. Only microdialysis probes with a recovery value within a mean range of 15 ± 3% were used in the experiment, while probes with recovery values outside this range were excluded. DA values in the dialysis samples were not corrected for the 'in vitro' recovery.

#### *9.2.5.2 - Surgery and probe implantation*

On PND 69, immediately after the end of the voluntary alcohol consumption protocol, rats were deeply anesthetized with isoflurane, and a concentric dialysis probe was implanted in the mPFC (AP +3.2 mm and ML +0.8 mm from bregma, DV –5.3 mm from the dura mater), according to the rat brain atlas of Paxinos & Watson (1998). By convention, the microdialysis probe was implanted in the right hemisphere. A small screw was carefully inserted into the lower quadrant of the left hemisphere, with care taken to avoid skull penetration, to provide additional stabilization for the implant. The probe was then secured to both the screw and the skull using dental cement (GlasIonomer Cement CX-Plus, Shofu®), and the incision was sutured in a manner that allowed the animal to move freely upon recovery.

#### *9.2.5.3 - Experimental day*

Experiments were performed in freely moving rats, 24 hours after probe implantation, on PND 70. Ringer's solution (3 mM potassium chloride, 125 mM sodium chloride, 1.3 mM calcium chloride, 1 mM magnesium chloride, 23 mM sodium bicarbonate, 1.5 mM potassium phosphate, pH 7.3) was continuously perfused through the dialysis probe at a rate of 2 μL/min. Dialysate samples were collected every 20 min from 9:00 to 15:00 hours. To prevent potential variability in extracellular DA levels due to differences in voluntary alcohol intake, all experimental groups were presented with an empty bottle between 11:00 and 13:00 hours, the period when alcohol would normally be made available. At the end of the experiment, the correct placement of each probe was verified histologically.

#### *9.2.5.4 - Sample analysis*

Samples were immediately analyzed for DA in the mPFC as previously described (Dazzi et al., 2002). Dialysate samples (40 μL) were injected into a HPLC system equipped with a reverse-phase column (LC18, 2.1 mm × 750 mm, 5 micrometers (μm) particle size, 90 Angstrom pore size), optimized for DA separation from other sample components. The detection limit for DA was 2 fmol per injection. The mobile phase consisted of a phosphate buffer at pH 2.3 (acidic pH, which ensured DA was protonated for cationic analysis) containing 73.4 mM sodium dihydrogen phosphate, 4.4 mM sodium chloride, 3 mM sodium octyl sulfate (OSA), 0.1 mM ethylenediaminetetraacetic acid, and 10% v/v methanol. OSA acted as a counter-ion, competing with sample analytes for interaction with the charged groups on the column. By adjusting its concentration, the retention times of different compounds, particularly DA, could be modulated to improve chromatographic resolution. In addition, methanol plays an important role in reducing peak width and thereby minimizing overlap between analytes. A volume of 40 μL was injected into the HPLC for both DA standards ($10^{-6}$ M) and dialysate samples, ensuring linearity between peak area and analyte concentration. Injection was carried out using a sample loop system, which was opened to fill with sample (load position) and then closed to transfer the sample onto the column (inject position). The mobile phase, under constant piston pressure, then conveyed the sample through the column, where

compounds are retained according to their affinity for the charged groups on the stationary phase, resulting in characteristic retention times. After elution, analytes reached the glassy carbon working electrode (6 mm diameter). An auxiliary block connected to the column outlet, together with a 16 μm-thick plastic gasket, ensured proper 180° alignment of the electrode, allowing the eluted compounds to reach the electrode in extremely small volumes, thereby enhancing detection sensitivity. DA, separated from other sample components, was oxidized at the working electrode (+650 mV). The flow rate was set at 0.3 mL/min, resulting in a DA retention time of approximately 15 minutes. The resulting current, generated by electron transfer during oxidation, was detected by the electrochemical detector and recorded as a chromatographic peak. The average DA concentration measured in the first consecutive samples was taken as 100% (basal), and all subsequent values were expressed as mean ± standard error of the mean (SEM) relative to the basal value.

## 9.3 – Results

Following exposure to the BD protocol with the various substances, animals' voluntary alcohol consumption was evaluated, as shown in Figure 18.

During the training period (Figure 18A), two-way ANOVA revealed a significant main effect of the experimental group ($F_{3,95} = 10.55$; $p < 0.0001$), a significant effect of time ($F_{2.234,212.2} = 34.78$; $p < 0.0001$), and a significant interaction between these factors ($F_{27,855} = 2.716$; $p < 0.0001$). Across the 10-day training, all experimental groups exhibited their highest alcohol consumption, likely influenced by the sugar content of the solution. However, drinking patterns differed among groups: BD-ALCOHOL consumed significantly less alcohol from day 2 to day 10 (1.18 ± 0.34 g/kg) compared to the average intake of the other groups (2.05 ± 0.30 g/kg; p values ranging from 0.0438 to < 0.0001). In contrast, BD-RB displayed the highest intake during the first four days, when sugar concentration was at its peak (3.48 ± 0.52 g/kg; $p = 0.0117$ on day 1), with significant differences observed on days 1, 2, and 4. Finally, BD-AMED showed a significantly higher intake only on day 3.

After training (Figure 18B), two-way ANOVA revealed significant main effects of the experimental group ($F_{3,95} = 5.735$; $p = 0.0012$) and time ($F_{8.252,783.9} = 3.817$; $p = 0.0002$), as well as a significant interaction between factors ($F_{51,1615} = 2.223$; $p < 0.0001$). Alcohol intake became more stable across days in all groups. BD-ALCOHOL still tended to consume less alcohol than BD-TW, though differences were statistically significant only on the first two days ($p = 0.0045$, day 1; $p = 0.0067$, day 2; day 3–10: $p = 0.2797$ to $> 0.999$). Notably, BD-AMED exhibited a significantly higher voluntary alcohol intake compared to BD-TW from day 13 until the end of the experiment, with the exception of days 22 and 23 (1.87 ± 0.23 g/kg versus 0.89 ± 0.10 g/kg in controls; $p = 0.0002$–$0.112$), becoming significant compared with all experimental groups from day 24 to 28.

Following the voluntary alcohol consumption protocol, extracellular DA levels in the mPFC were measured, as shown in Figure 19. Measurements were taken under basal conditions, during the two-hours period typically preceding alcohol presentation, during the two hours of empty-bottle availability, and in the two hours after bottle removal.

One-way ANOVA revealed no significant differences between groups under basal conditions ($F_{3,28} = 0.4946$; $p = 0.6889$), suggesting that prolonged voluntary alcohol intake did not significantly modify basal extracellular DA levels in the mPFC. Specifically, basal DA concentrations were 1.640 ± 0.195 picomoles (pmol)/sample in BD-TW, 1.465 ± 0.282 pmol/sample in BD-ALCOHOL, 1.516 ± 0.405 pmol/sample in BD-RB, and 1.559 ± 0.577 pmol/sample in BD-AMED. Data are expressed as pmol of DA per 20-min sample (mean ± SEM, n = 8 rats/group).

In contrast, two-way ANOVA revealed no significant effect of experimental group ($F_{3,28} = 0.4454$; $p = 0.7225$), a significant main effect of time ($F_{18,504} = 8.611$; $p < 0.005$), and a significant interaction between factors ($F_{54,504} = 4.806$; $p < 0.0001$) when analyzing the sensitivity of mesocortical DAergic neurons to alcohol presentation. Specifically, BD-TW displayed an increase in extracellular DA as early as 80 min before alcohol presentation (+90% over basal values), which remained elevated when the bottle was available (+100%), and returned to basal levels 60 min after bottle removal ($p = 0.035–0.02$). BD-ALCOHOL showed increased extracellular DA concentration when the empty-bottle was presented (+50%), with a further increase 60 min after presentation, and an even more pronounced increase following bottle removal (+200% and +350%, respectively; $p = 0.0366–0.0050$). BD-RB showed a non-significant increase (+30%) during alcohol anticipation ($p = 0.2207–0.6407$), with no significant changes observed during bottle availability. Finally, BD-AMED did not show significant changes in DA extracellular concentration during anticipation, presentation, or after removal of the bottle (all $p > 0.9999$).

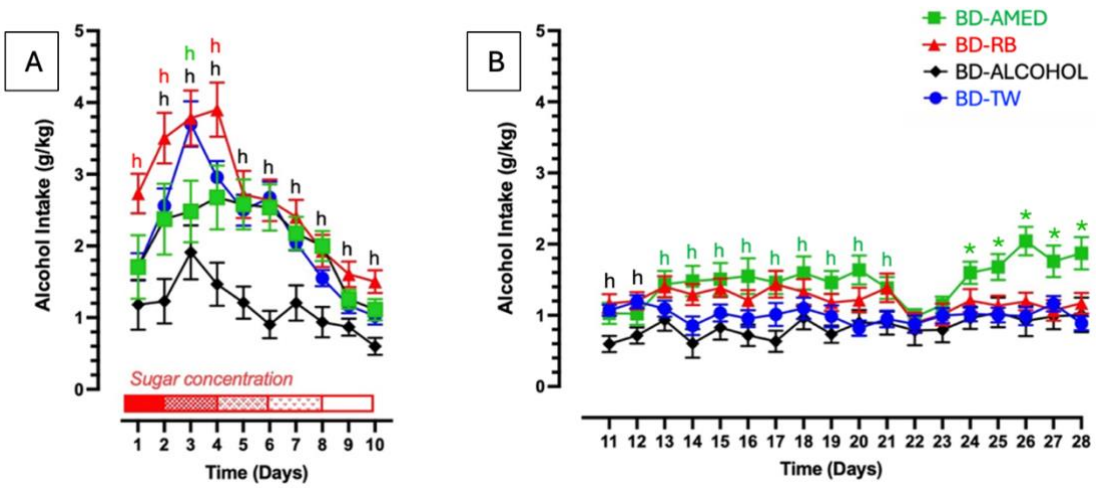


Figure 18. **Effects of BD protocol during adolescence on voluntary alcohol consumption in adulthood during training (A) and after training (B)**. Data are expressed in g of alcohol/kg of body weight and are mean ± SEM of 7–8 rats/group. *p < 0.005 versus all other groups; $^{h}$p < 0.05 versus BD-TW. The color of each significance symbol indicates the group showing the significant difference: green = BD-AMED; black = BD-ALCOHOL; red = BD-RB.
Adapted from Dazzi et al. (2024) *(CC BY 4.0 licence).*

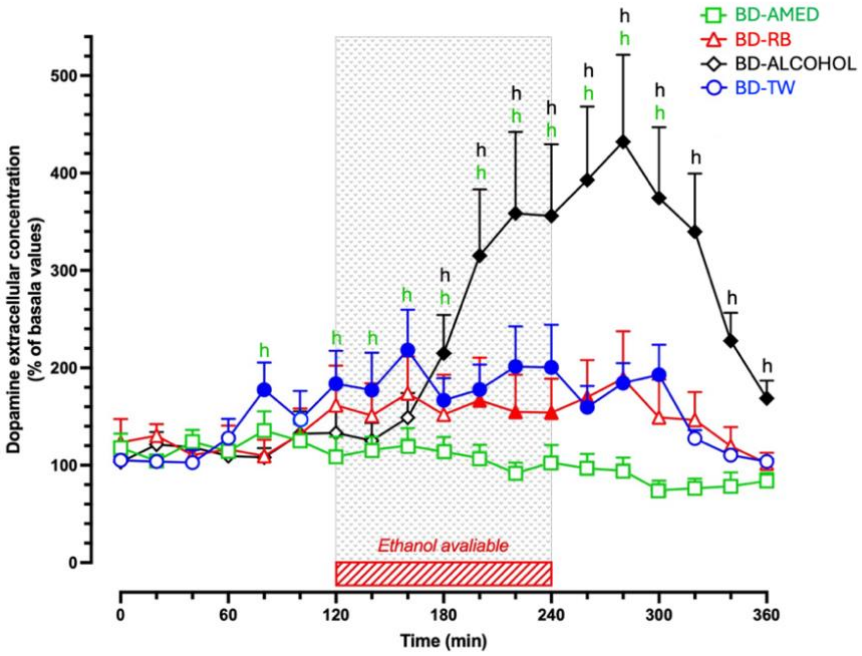


Figure 19. **Effects of BD-like adolescent protocol and voluntary alcohol consumption in adulthood on mPFC DA transmission before, during, and after exposure to an empty bottle in the familiar drinking context**. Data are expressed as percentage of basal values and are mean ± SEM of 7–8 rats/group. Filled symbols indicate samples representing p < 0.05 versus basal values. $^{h}$p < 0.05 versus SA-TW. The color of each significance symbol indicates the group showing the significant difference: green = SA-AMED; black = SA-ALCOHOL.
Adapted from Dazzi et al. (2024) *(CC BY 4.0 licence).*

## 9.4 – Discussion

Our findings demonstrate that previous intermittent, BD-like adolescent exposure to AMED during youth leads to a reduced DAergic response in the mPFC upon alcohol presentation in adulthood, despite similar basal extracellular DA levels across experimental groups. In contrast, BD-ALCOHOL showed a significant and sustained increase in mPFC DA, even though their total intake was lower than BD-AMED during the 4-week voluntary alcohol consumption protocol. This observation aligns with anecdotal reports suggesting that some substances, such as caffeine, can modulate some effects of alcohol. The reduced responsiveness of mesocortical DAergic neurons in the AMED group is also in line with previous findings (Lallai et al., 2016) showing that increased voluntary alcohol intake in socially isolated rats is associated with decreased sensitivity of these neurons to alcohol presentation. Specifically, mesocortical DA responsiveness to alcohol seems to be inversely correlated with consumption: the higher the intake, the lower the DA response. In BD-AMED, which voluntarily consumes the most alcohol, extracellular DA in the mPFC remains stable across anticipation, consumption, and satiety phases. This inverse relationship reinforces the critical role of a dysfunctional mPFC in addiction development, highlighting DAergic hypofrontality as a key factor in disrupting the balance between mesocortical and mesolimbic pathways and potentially triggering the transition from use to abuse. The reduced mPFC DA response in BD-AMED may thus underlie the loss of inhibitory control and contribute to the development of compulsive alcohol use (Jentsch & Taylor, 1999; Jentsch et al., 2014).

The mPFC is essential for higher-order cognitive processes such as decision-making, behavioral control and flexibility, and goal-directed planning (Goto & Grace, 2005). Binge-like AMED exposure during adolescence, which combines alcohol and RB components, could disrupt mPFC neuroplasticity during this critical developmental period. Alcohol alone is known to impair brain maturation, particularly in the mPFC, causing changes in neurotransmission, neuroplasticity, inflammation, myelin integrity, and glial function (Seemiller & Gould, 2020; Tetteh-Quarshie & Risher, 2023). Our findings suggest that alcohol combined with RB may produce long-lasting alterations in the mPFC, reflected in increased voluntary

alcohol consumption and reduced DAergic sensitivity. Notably, the caffeine present in RB may also contribute to these effects, as chronic adolescent caffeine exposure has been shown to affect mPFC DAergic function (Boeck et al., 2009), potentially altering dendritic plasticity and natural pruning processes (Christensen et al., 2020; Juárez-Méndez et al., 2006). Overall, these results indicate that adolescent exposure to alcohol and AMED induces distinct adaptive changes in mPFC DAergic function, with AMED leading to decreased sensitivity to external stimuli. This altered DA responsiveness may contribute to heightened vulnerability to alcohol addiction. Electrophysiological and behavioral data performed in the same study of Dazzi et al. (2024) corroborate these results. For instance, consistent with the DAergic hyporesponsiveness observed in the mPFC, complementary behavioral analyses revealed an age-dependent reduction in prepulse inhibition in BD-AMED during adulthood, a sensorimotor gating measure commonly associated with impaired mPFC function (Tóth et al., 2017). Electrophysiological data further support these findings, showing that adolescent AMED exposure suppresses the acute alcohol-induced increase in firing rate in VTA slices, potentially leading to reduced DA release from neurons projecting to the mPFC. For further details, see the full study (Dazzi et al., 2024).

# 10. Chronic Intake of Red Bull® Alone or in Combination with Alcohol Alters Operant Behavior and Mesocorticolimbic Dopamine Transmission

## 10.1 – Background and aim

Adolescence is characterized by extensive physiological, emotional, cognitive, and behavioral transformations that drive brain maturation; therefore, it represents a particularly vulnerable phase, often marked by increased impulsivity, reduced inhibitory control, and a greater tendency to seek risks and intense sensations. Consequently, this is the period during which individuals often begin experimenting with drugs, including alcohol. Moreover, nowadays alcohol is increasingly consumed together with EDs, a behavior that is even more hazardous, as AMED consumers have been reported to engage in riskier behaviors compared to alcohol-only consumers (Mallett et al., 2013; Miller, 2012; Thombs et al., 2010; Snipes & Benotsch, 2013). Despite the potentially serious consequences for individual health, a deep analysis of the consequences of the assumption of a full ED with all its components is lacking in the literature, with the majority of the studies focused on the effects of the combination of alcohol with caffeine alone. Only a few studies using animal models have focused on the effects of AMED (Petribu et al., 2023). Among the limited available data, operant self-administration (SA) of AMED has shown that this combination is particularly reinforcing, driving rats to consume larger quantities compared to alcohol alone (Roldán et al., 2018). In line with the lack of a consistent literature, even less is known about the effects of these substances on the mesocorticolimbic system, which plays a key role in balancing inhibitory control and reward-driven pleasure. In this context, our group has previously investigated the effects of RB on this system, showing that DAergic transmission was increased through a non-adaptive mechanism, exhibiting a pattern similar to that observed with drugs of abuse (Vargiu et al., 2021).

Despite some preliminary findings, much remains unclear regarding how AMED specifically affects the neural mechanisms underlying reward-driven behavior. Given the existing gap in the literature regarding the abuse liability of ED

and their potential to modulate the neurobiological effects of alcohol when consumed together as AMED, the present part of the study aimed to clarify how this combination affects reward-related behaviors and their underlying neural mechanisms in early adulthood following exposure since adolescence. The project was designed to investigate how operant SA of alcohol alone, RB alone or their combination influences motivation and brain circuits involved in goal-directed behavior in young adult rats previously trained from adolescence to self-administer these solutions. Specifically, the study aimed to determine whether SA of AMED alters motivational responses and alcohol consumption compared with SA of alcohol or RB alone, and to characterize differences in mesocorticolimbic DA transmission, monitored in vivo during SA, between AMED-exposed animals and controls. In summary, the aim of this part of the study was to deepen the knowledge on the consequences of chronic voluntary AMED intake by investigating its effects on impulsive behavior from adolescence to early adulthood, as well as on neurochemical activity within key brain regions involved in reward.

Figure 20 illustrates the experimental timeline used in this study.

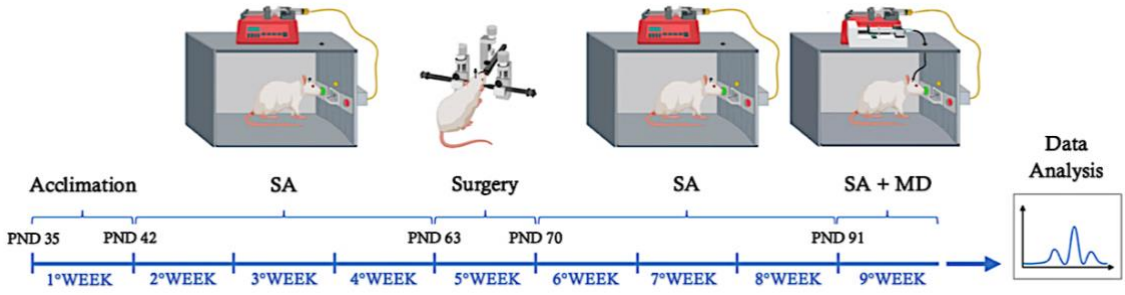


Figure 20. **Timeline of the experimental design**.
*Abbreviations: MD = microdialysis.* Image created with BioRender (2025).

### *10.2.1 - Animals*

36 male adolescent SD rats were used in this study (Charles River, Calco, Italy). Upon arrival (PND 35), rats were housed in the animal facility and allowed to acclimate for 7 days before the start of the experimental procedures. Rats were maintained under controlled environmental conditions, with a constant temperature of 22°C and relative humidity of 60%, on a 12 hours light / 12 hours dark cycle (lights on from 08:00 to 20:00 hours). Standard laboratory food and tap water were provided ad libitum throughout the study.

All experimental procedures were carried out in accordance with the authorization of the Italian Ministry of Health (No. 169/2024-PR). Every effort was made to minimize animal suffering and reduce the number of animals used, in accordance with the principles of the 3Rs.

### *10.2.2 - Drugs*

Alcohol and RB were used in the present study, and tap water was used as control. 96% alcohol was diluted to 10% (v/v in tap water). AMED was prepared with 10% alcohol in standard RB. All solutions were freshly prepared daily before SA sessions.

### *10.2.3 - Self-administration*

On PND 42, animals were randomly assigned to one of four experimental groups based on the solution available during SA: SA-RB (standard RB), SA-ALCOHOL (alcohol in tap water), SA-AMED (alcohol in standard RB), or SA-TW (tap water, control group). The SA training lasted for 3 weeks, followed by a one-week break for surgery and recovery, and then resumed from PND 70. In total, animals underwent 6 weeks of SA training, followed by two additional days of SA in combination with microdialysis.

SA protocol consisted of 30-minute daily sessions, five times per week. Animals were placed in soundproof operant chambers (Skinner boxes) equipped with two lateral nose-poke (NP) devices, fitted with lights of different colors, and a central drinking spout. Animals were trained to insert their nose into the active NP, signaled by a green light serving as a highly salient visual cue. Initially, animals were trained under a Fixed Ratio (FR) 1 schedule during the first week, which was increased to FR3 in the second week and then maintained at FR5 from the third week until the end of training. Responding at the inactive NP, marked by a non-salient cue red light, had no consequence. In contrast, inserting the nose into the active NP, one (FR1), three (FR3), or five (FR5) consecutive times, activated the photocell sensors, and the response was recorded by a computer-controlled system (Graphic State 2 Software, Coulbourn Instruments, PA, USA). The system in turn triggered an infusion pump (Razel, Scientific Instruments, CT, USA) which delivered 0.2 mL of the assigned solution to the drinking spout. Simultaneously, a yellow light above the spout was illuminated to facilitate the association between the reward and a visual cue. Through this setup, animals learned to perform an operant behavior based on their motivation to work for a reward.

Prior to each training session, rats were placed in the operant chamber for 5 minutes with the software turned off (all lights off, no reward delivered). This adaptation period was necessary for subsequent microdialysis experiments, allowing baseline dialysate samples to be collected in the absence of external stimuli and establishing reference DA levels.

After the adaptation period, the 30-minute SA session began, consisting of several alternating phases. In the work phase (S1), the NP devices were illuminated, and animals had two possible responses. If the active NP was triggered, the session proceeded to the reward phase (S2), during which the yellow light was turned on and the 0.2 mL solution was delivered through the drinking spout. This phase lasted 5 seconds and allowed the animal to consume the solution assigned to its experimental group. On the other hand, if the active NP was not triggered within 10 seconds during S1, the session entered the time-out phase (S3), during which all lights were turned off for 5 seconds. Following S3, the session returned to S1, and the cycle repeated for the 30-min SA session.

#### ***10.2.4 - Microdialysis Experiments***

Microdialysis experiments were conducted in a similar manner as described in Subsection 7.2.3, with one important difference: unlike the previous acute experiments, microdialysis experiments were performed three weeks after surgery, allowing for complete post-operative recovery and ensuring that animals did not lose their training in performing the operant behavior. This timing was also chosen to eliminate any confounding effects of surgical stress and to obtain stable neurotransmitter measurements during SA. Below, the procedure for conducting a chronic microdialysis experiment is described.

##### ***10.2.4.1 - Probe preparation for chronic microdialysis***

Chronic microdialysis probes (Figure 21D) were prepared using the same materials and general approach described in Subsection 7.2.3.1, with some modifications based on the method of Lecca et al. (2006) to ensure compatibility with the guide cannula (Figure 21A). The use of chronic guide cannulae is necessary to combine brain microdialysis with the SA protocol, which requires that animals have

not undergone stereotaxic surgery recently, in order to allow full recovery and restoration of SA behavior. Briefly, the push-pull connector (Figure 21B) was used as support to build the microdialysis probe and to fit with guide cannulas. The dialyzing surface measured 1.5 mm for AcbSh probes and 3 mm for mPFC ones.

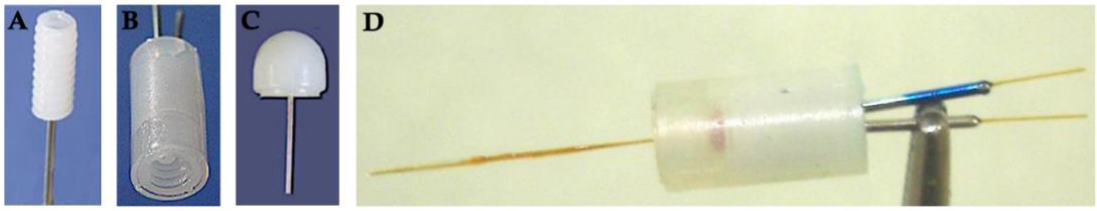


Figure 21. **Guide cannula (A), push-pull (B), dummy (C), chronic microdialysis probe (D)**.

#### *10.2.4.2 - Surgery and guide cannula implantation*

After three weeks of SA, animals were deeply anesthetized with Equithesin (5 mL/kg, i.p.; see Section 7.2.3.2 for its composition). Once the depth of anesthesia was verified by checking the corneal reflex and paw pressure response, a microdialysis probe guide cannula (Push–Pull guide cannula, Plastics One, Roanoke, VA, USA) was stereotaxically implanted as described by Lecca et al. (2006) either in the AcbSh (AP +1.8 mm and ML ±1.0 mm from bregma, DV –3.6 mm from dura mater) or in the mPFC (AP +3.7 mm and ML ±0.8 mm from bregma, DV –2.0 mm from dura mater), according to coordinates from the atlas of Paxinos & Watson (1998). The final vertical position of the microdialysis probe (inserted only on the day of the experiments) was –7.6 mm and –5.0 mm from the dura mater for the AcbSh and mPFC, respectively. The chronic guide cannula was secured to the skull using a glass ionomer dental cement (GlasIonomer Cement CX-Plus, Shofu®; total weight of cannula + probe + cement: < 0.8 g). A dummy cannula (Figure 21C) was then inserted to maintain patency of the guide cannula until the day of the experiment. Unlike acute microdialysis experiments, chronic microdialysis protocols involve several weeks of experimentation after surgery; therefore, dedicated post-operative pharmacological support is required to ensure full recovery. Specifically, an anti-inflammatory drug with analgesic properties (meloxicam, 1 mg/kg) was administered subcutaneously (s.c.) for 3 days, while betadine (povidone-iodine) was

applied as a topical antiseptic. At the end of surgery, animal received 2 ml of 5% glucose solution s.c. to facilitate post-operative recovery, and antibiotic therapy (gentamicin sulfate, 40 mg/kg, i.p.) was administered for 5 days. After surgery and until the end of the study, animals were housed in single cages. During the one-week recovery period without SA, a second bottle containing the group-specific experimental solution was added to the home cage, to facilitate the subsequent recovery of operant behavior, which resumed the following week and continued for additional three weeks.

#### *10.2.4.3 - Experimental days and sample analysis*

During the ninth week, microdialysis experiments coupled with SA were performed over two consecutive days. SA was conducted in the same Skinner boxes and under the same experimental conditions as before, except for the adaptation phase, which was extended to 30 minutes to obtain the necessary baseline samples. Prior to each session, the dummy cannula was removed and the microdialysis probe compatible with the guide cannula was inserted, a procedure that does not cause any discomfort to the animal. The probe had been previously connected to the microdialysis pump under the same conditions described in Subsection 7.2.3.3, and the tubing was passed through a hole in the top of the Skinner box to reach the animal's head. Sample collection occurred in three phases: a 30-min pre-session phase to establish baseline levels of DA, a 30-min phase during SA to study changes in the experimental groups during operant behavior for reward, and a 30-min post-session phase following SA. Across all three phases, a total of nine samples were collected, rapidly frozen, and subsequently analyzed. At the end of the second day of microdialysis experiment, animals were sacrificed and their brains collected for appropriate histological analyses, as described in Subsection 7.2.3.3.

Samples were analyzed for their DA content using an HPLC system and histological analyses were performed to verify probe placement, in the same manner as described in Subsection 7.2.3.4.

## 10.3 – Results

Figure 22 illustrates the cumulative number of active and inactive NP recorded across the training weeks for the acquisition of operant behavior during SA. While the number of inactive NP remained consistently low across all experimental groups, active nose poke responses increased progressively in SA-RB and SA-AMED, reflecting successful acquisition of the operant behavior. During the first three weeks of training, a three-way ANOVA revealed a significant main effect of treatment ($F_{3,54}$ = 3.89; p = 0.014) and response ($F_{1,54}$ = 21.27; p = 0.000025). Significant interactions were also observed between treatment and response ($F_{3,54}$ = 4.30; p = 0.008), treatment and session ($F_{42,756}$ = 1.96; p = 0.0003), response and session ($F_{14,756}$ = 2.38; p = 0.003), and treatment, response and session ($F_{42,756}$ = 2.08; p = 0.0001). Post hoc analyses indicated that the number of active NP during operant sessions was significantly higher in SA-RB and SA-AMED compared with SA-ALCOHOL and SA-TW. During the three weeks of SA post-surgery, a three-way ANOVA showed a significant main effect of treatment ($F_{3,54}$ = 7.42; p = 0.0003), response ($F_{1,54}$ = 27.20; p = 0.000003), and session ($F_{14,756}$ = 2.11; p = 0.01). Significant interactions were observed between treatment and response ($F_{3,54}$ = 6.90; p = 0.0005), treatment and session ($F_{42,756}$ = 1.54; p = 0.02), response and session ($F_{14,756}$ = 1.79; p = 0.04), and treatment, response and session ($F_{42,756}$ = 1.47; p = 0.03). Consistent with the first three weeks, post hoc analyses indicated that the number of active NP during operant sessions was significantly higher in SA-RB and SA-AMED compared with SA-ALCOHOL (except during the 5th and 6th sessions) and SA-TW, with SA-RB exhibiting significantly more active NP than SA-AMED.

Figure 23 shows the total intake of alcohol, RB, AMED and tap water during operant sessions throughout the training SA weeks. In the first three weeks of training, a two-way ANOVA revealed a significant main effect of treatment ($F_{3,24}$ = 3.91; p = 0.02) and session ($F_{14,336}$ = 18.8; p = 0.00001). Post hoc analyses indicated that intake was higher in SA-RB compared with SA-ALCOHOL and SA-TW. Intake in SA-AMED was lower than SA-RB, except during the last two days of the third week, when intake reached levels comparable to SA-RB. During the three weeks of SA post-surgery, a two-way ANOVA showed a significant main effect of treatment

($F_{3,10}$ = 3.9; p = 0.05) and session ($F_{14,140}$ = 2.8; p = 0.05). Post hoc analyses revealed that intake in SA-RB remained higher than in SA-ALCOHOL and SA-TW throughout the sessions. Intake in SA-AMED group was lower than in SA-RB one from the 1st to the 8th session, except for the last sessions, when it reached levels comparable to SA-RB, except during the 13th session. Overall, SA-RB and SA-AMED were the preferred solutions across the SA training period.

Figure 24 illustrates variations in DAergic transmission in the AcbSh before, during, and after FR5 SA sessions over two consecutive days in rats previously trained for 6 weeks. SA induced increases in extracellular DA concentrations on both the first and second day in SA-RB, SA-AMED and SA-ALCOHOL, compared with SA-TW. A three-way ANOVA revealed a significant main effect of treatment ($F_{3,26}$ = 38.36; p = 0.000001), time ($F_{6,156}$ = 40.46; p = 0.00001), and a significant interaction between treatment and time ($F_{18,156}$ = 4.51; p = 0.000001). Tukey's post hoc test indicated statistically significant differences between SA-TW and all other groups on both the first and second day. SA-AMED showed a significant increase in DA concentrations compared with SA-RB from minute 20 to minute 60 on the first day; additionally, SA-AMED differed from SA-ALCOHOL at minutes 20, 50, and 60 on the first day and at minute 10 on the second day.

Figure 25 illustrates variations in DAergic transmission in the mPFC before, during, and after FR5 SA sessions over two consecutive days in rats previously trained for 6 weeks. SA induced increases in extracellular DA concentrations on both the first and second day only in SA-RB and SA-AMED compared with SA-TW, whereas no increase was observed in SA-ALCOHOL. A three-way ANOVA revealed a significant main effect of treatment ($F_{3,27}$ = 27.79; p = 0.000001), time ($F_{6,162}$ = 15.56; p = 0.000001), and a significant interaction between treatment and time ($F_{18,162}$ = 6.49; p = 0.000001). Tukey's post hoc test indicated no statistically significant differences between SA-RB and SA-AMED on DAergic transmission in the mPFC on the first day. However, on the second day, SA-AMED elicited a greater increase in DA concentrations than SA-RB during the first 20 minutes of the SA session.

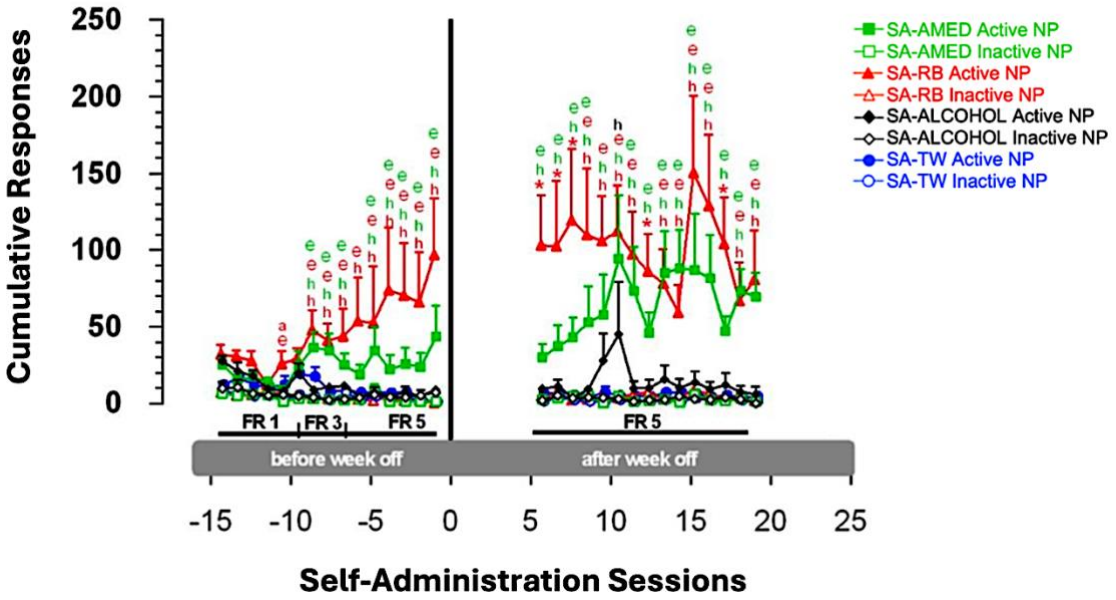


Figure 22. **Cumulative responses for active and inactive NP during operant SA sessions across 6 weeks of training.** Data are expressed as mean ± SEM. Filled symbols = active NP; empty symbols = inactive NP. *p < 0.005 versus all other groups; [a]p < 0.05 versus SA-AMED; [c]p < 0.05 versus SA-ALCOHOL; [h]p < 0.05 versus SA-TW. The color of each significance symbol indicates the group showing the significant difference: red = SA-RB; green = SA-AMED; black = SA-ALCOHOL.

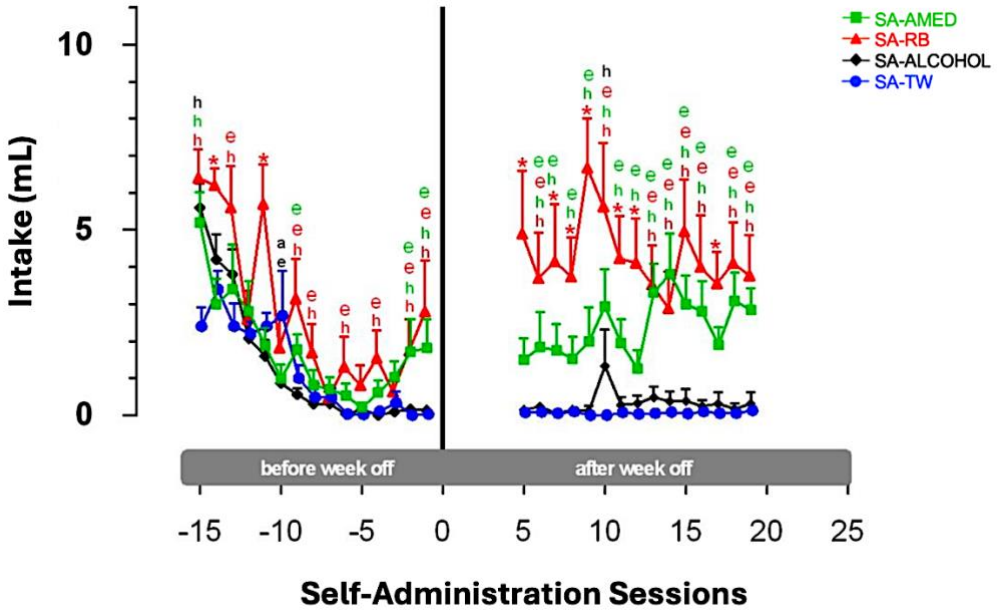


Figure 23. **Total intake of alcohol, RB, AMED and tap water during operant SA sessions across 6 weeks of training.** Data are expressed as mean ± SEM. *p < 0.05 versus all other groups; [a]p < 0.05 versus SA-AMED; [c]p < 0.05 versus SA-ALCOHOL; [h]p < 0.05 versus SA-TW. The color of each significance symbol indicates the group showing the significant difference: red = SA-RB; green = SA-AMED; black = SA-ALCOHOL.

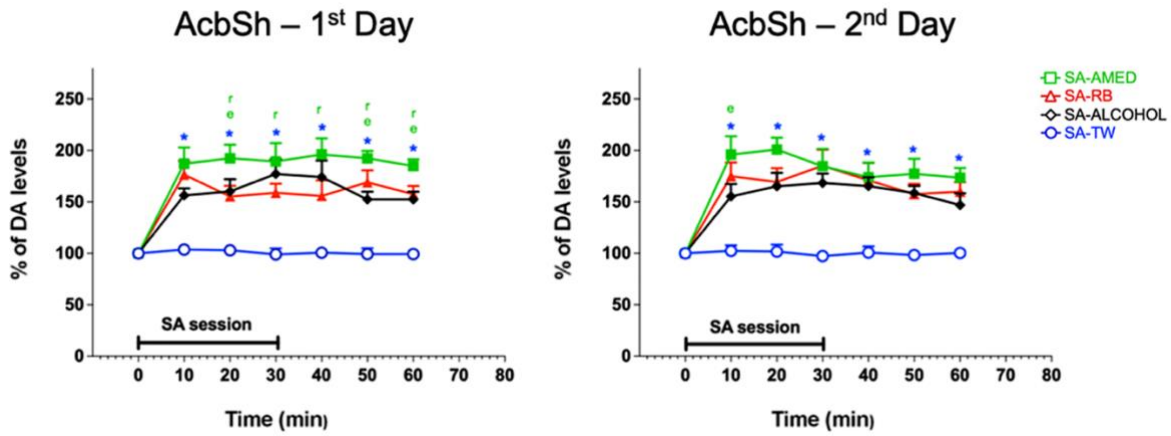


Figure 24. **Variations in DAergic transmission in the AcbSh before, during, and after FR5 SA sessions over two consecutive days in rats previously trained for 6 weeks.** Data are expressed as percentage of basal values ($t_0$ = 100%, calculated as the mean of three consecutive basal samples) and reported as mean ± SEM. Filled symbols indicate samples representing $p < 0.05$ versus basal values. *$p < 0.05$ versus all other groups; $^{e}p < 0.05$ versus SA-ALCOHOL; $^{r}p < 0.05$ versus SA-RB. The color of each significance symbol indicates the group showing the significant difference: green = SA-AMED; blue = SA-TW.

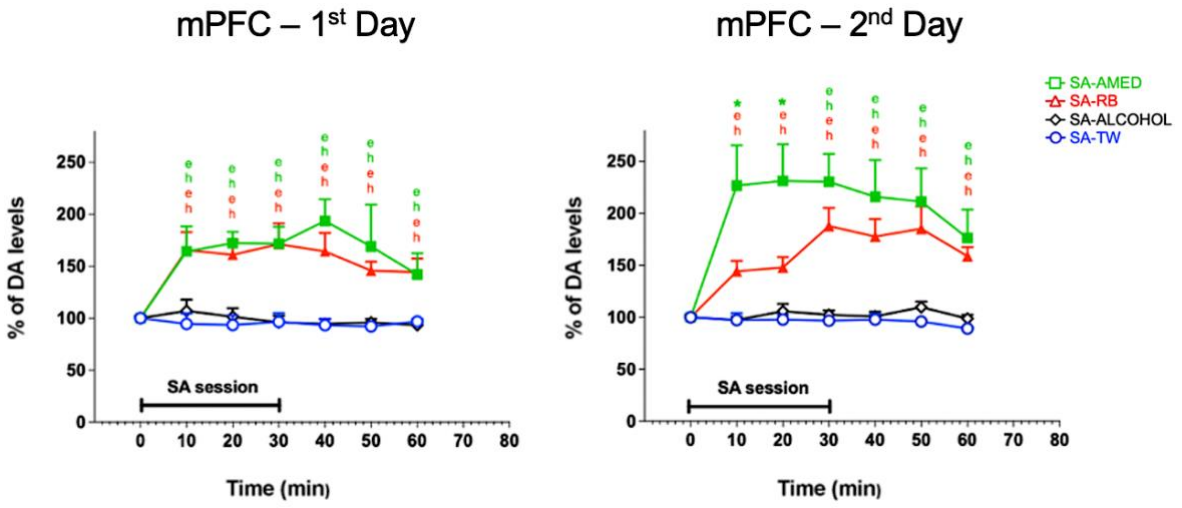


Figure 25. **Variations in DAergic transmission in the mPFC before, during, and after FR5 SA sessions over two consecutive days in rats previously trained for 6 weeks.** Data are expressed as percentage of basal values ($t_0$ = 100%, calculated as the mean of three consecutive basal samples) and reported as mean ± SEM. Filled symbols indicate samples representing $p < 0.05$ versus basal values. *$p < 0.05$ versus all other groups; $^{e}p < 0.05$ versus SA-ALCOHOL; $^{h}p < 0.05$ versus SA-TW. The color of each significance symbol indicates the group showing the significant difference: green = SA-AMED; red = SA-RB.

## 10.4 – Discussion

The present study investigated the acquisition and maintenance of operant SA behavior for alcohol, RB, and AMED, whose difficulty increased over time, and their impact on DAergic transmission in the AcbSh and the mPFC. Specifically, the SA sessions began during mid-adolescence (PND 42), a period roughly corresponding to 16–18 years of age in humans (Spear, 2015), and continued into adulthood, during which mesocorticolimbic DAergic transmission was assessed. Increasing the effort required for the animals to obtain the reward from FR1 to FR5 allowed us to evaluate their motivation, which reflects the reinforcing properties of the self-administered beverages.

One of the main findings of this part of my thesis work is that SA-RB and SA-AMED displayed progressively improving performance and sustained operant behavior throughout all experimental sessions. In contrast, in the SA-ALCOHOL and SA-TW groups, animals showed almost no active NP or intake, particularly when the schedule was shifted from FR1 to FR3. Specifically, 10% alcohol in tap water induced a weak operant responding; however, when the same concentration of alcohol was mixed with RB (AMED), it acquired rewarding properties that allowed animals to consume it in substantial amounts. This indicates that RB enhances the rewarding properties of alcohol, making it appealing even to subjects that would not normally seek it out. The persistence of these effects after surgery demonstrates the stability of the learned operant behavior and excludes the possibility that surgical procedures interfered with motivation or performance.

Another important finding concerns the differences in DAergic increases across the two brain regions of interest. SA-RB, SA-AMED and SA-ALCOHOL showed a significant increase in DA transmission in the AcbSh compared with SA-TW, both on the first and second day of SA coupled with microdialysis. Moreover, our data show that SA-AMED exhibited significantly higher DA increases compared with both SA-RB and SA-ALCOHOL, suggesting a synergistic interaction between alcohol and RB that enhances DAergic AcbSh transmission. Regarding DAergic transmission in the mPFC, we observed a potentiation of it in SA-AMED and SA-RB groups, with a greater increase in the first one compared to the second one on the

second day, during the first 20 minutes of SA coupled with microdialysis. Notably, although SA-AMED intake was lower than that of SA-RB, it still elicited a potentiated both AcbSh and mPFC DAergic response, suggesting that the combination of alcohol and RB enhances DA release beyond what would be expected from the amount consumed alone. These potentiating effects of AMED on DA transmission in both regions may result from the integration of the neurobiological effects of RB and alcohol.

Another interesting point to take into account is the effect of the taste of these beverages on DAergic transmission in both AcbSh and mPFC. In fact, it is well known that novel and palatable taste is able to increase DA in these two areas, with a higher rise in mPFC (Bassareo & Di Chiara, 1997). Considering this aspect we can hypothesize that also the palatable taste of RB and AMED may contribute to inducing a stimulation of DA transmission.

Taken together, these findings indicate that AMED may combine the reinforcing properties of both alcohol and RB, producing robust operant responding, high intake, and pronounced activation of DAergic systems. While effects in the AcbSh of SA-AMED suggest enhanced reward processing, the selective and potentiated activation of DA transmission in the mPFC could be ascribed to the effects of the particular and palatable taste of AMED and of caffeine that it is widely recognized to stimulate prefrontal DAergic transmission (Acquas et al., 2002; De Luca et al., 2007).

# GENERAL DISCUSSION

The overall goal of this thesis was to investigate how alcohol and RB, administered alone or in combination, affect the brain reward system. This objective was addressed through a series of complementary experimental approaches integrating neurochemical, behavioral, and molecular outcomes, with the aim of providing a multidimensional characterization of the effects induced by these substances. Importantly, these investigations were conducted across distinct developmental stages, with a particular focus on adolescence and emerging adulthood, which are considered periods of heightened neurobiological vulnerability. This increased sensitivity is largely dependent on the maturational state of the brain, especially the ongoing development of mesocorticolimbic circuits involved in reward processing. Although direct comparisons between age stages in rats and humans are not straightforward, it is generally accepted that early-to-mid adolescence in humans (approximately 10–18 years of age) roughly corresponds to PND 25–42 in rats, whereas human adolescence and emerging adulthood (18–25 years) approximately corresponds to the rat developmental period between PND 42 and 65 (Spear, 2015; Vetter-O'Hagen & Spear, 2012). The study was further specifically designed to evaluate the impact of different drug administration timing (adolescent versus adult, adult from naïve adolescents and adults from adolescent-administrered animals) and paradigms (acute versus chronic; voluntary versus involuntary), allowing a more extensive evaluation of the resulting neurobiological responses and potentially greater translational power.

Given the increasing trend of mixing alcohol with EDs, we first aimed to evaluate the effects of the main stimulating component of RB, caffeine. Specifically, we investigated the effects of acute caffeine pretreatment, administered at different doses and through different routes (i.p. and reverse dialysis), on alcohol-induced responses. The results showed that caffeine is able to interfere with alcohol-induced activation of the mesolimbic system. In particular, caffeine prevented the alcohol-induced formation in the pVTA (Bassareo et al., 2024) of salsolinol, a molecule implicated in the rewarding effects of alcohol (Deehan et al., 2013; Hipólito et al.,

2012; Quintanilla et al., 2014; Quintanilla et al., 2016), and also prevented the alcohol-induced increase in DA levels in the AcbSh (Bassareo et al., 2024). Similar results were obtained with the selective adenosine $A_{2A}$ receptor antagonist SCH 58261, whereas the $A_1$ receptor antagonist DPCPX did not prevent the alcohol-induced increase in DA levels, despite preventing the increase in salsolinol (Bassareo et al., 2024). These findings indicate that caffeine exerts these effects primarily through its action on $A_{2A}$ receptors. Moreover, caffeine was also able to prevent the increase in DA levels induced by exogenous salsolinol, without reducing salsolinol levels themselves, suggesting the existence of at least one additional mechanism, independent of salsolinol generation, through which caffeine may indirectly reduce mesolimbic DAergic transmission (Bassareo et al., 2024). These neurochemical effects are consistent with previous behavioral studies showing that caffeine, administered at doses sufficient to induce mild arousal (Acquas et al., 2002; Hasenfratz et al., 1993) and increase locomotor activity (López-Cruz et al., 2013; Dar, 1988), can attenuate certain reinforcing effects of alcohol, such as conditioned place preference and aversion (Porru et al., 2020).

After assessing some of caffeine's individual effects, and to better model the way RB is typically consumed with alcohol in humans, we administered non-contingent oral solutions of RB and alcohol via gavage, either alone or in combination. As described in Section 8.2.2, the doses of RB and alcohol administered to rats were approximately equivalent to the consumption of respectively two cans of RB and 2.6 U.S. standard drinks for a human, in order to reproduce a plausible intake scenario among adolescents. Moreover, the dose of caffeine contained in the RB and AMED solutions corresponds to 14 mg/kg in rats, which is comparable to the 15 mg/kg dose used in the first study, in which caffeine alone was tested. Given this comparability, the lack of an increase in mesolimbic DA levels in the AcbSh in the AC-AMED group was expected and is consistent with the preventive effects of acute caffeine, in contrast to the alcohol-mediated increase in AcbSh DA levels observed in AC-ALCOHOL. Importantly, the amount of alcohol was identical in the AC-ALCOHOL and AC-AMED administered rats. This indicates that, at an equivalent alcohol dose, the presence of RB reduces the ability of alcohol to elicit the same rewarding effect as when administered alone, suggesting that a higher AMED dose

might be required to achieve a comparable level of reward. The attenuation of the alcohol-induced increase in DA in the AcbSh following AMED is consistent with the previous experiment, in which a comparable dose of caffeine, administered alone, prevented the alcohol-mediated DA increase (Bassareo et al., 2024). In this regard, it would be critical in future studies assessing whether AMED fails to determine conditioned place preference or, in other words, whether RB, similarly to caffeine (see Porru et al., 2020), may prevent alcohol-elicited place conditioning. In a separate cohort of animals, locomotor activity and ERK activation were also assessed using identical dosing (Puliga et al., in preparation). As expected, RB stimulated locomotor activity in AC-RB, likely due to the caffeine dose contained in this solution, which falls within the range known to stimulate locomotor activity in both young and adult rats (Marin et al., 2011). In contrast, alcohol at this relatively high dose produced a sedative effect, reducing locomotor activity in AC-ALCOHOL. This effect was also observed in AC-AMED, indicating that caffeine's locomotor-stimulating effect is not monitored when it is combined with a sedative dose of alcohol. Furthermore, ERK phosphorylation in the AcbSh was markedly increased in AC-ALCOHOL, consistent with Ibba et al. (2009), and significantly different from AC-AMED. Thus, after acute administration, AMED was found to prevent the alcohol-induced DAergic transmission and the activation of pERK-positive neurons in the AcbSh, without reducing alcohol's sedative locomotor effects. The alcohol-induced increase in pERK in the AcbSh is a well-established finding (Ibba et al., 2009), whereas the reduction of pERK observed in AC-AMED may be explained by the presence of caffeine in the mixture, which has been shown to prevent alcohol-induced increases in pERK levels in the AcbSh (Porru et al., 2020, 2021) and is overall in agreement with prevention of caffeine (Bassareo et al., 2024) and RB (Puliga et al., in preparation) to affect alcohol-stimulated AcbSh DA release.

Subsequently, we administered the same doses to investigate in adulthood the effects of repeated exposure along adolescence. Specifically, animals received intermittent, non-contingent oral administrations designed to model a BD pattern in early adolescent rats (PND 28–37). Voluntary alcohol intake was subsequently assessed during late adolescence–emerging adulthood (PND 40–68), and DAergic transmission was evaluated in young adulthood (PND 70) in the mPFC, a brain

region critically involved in reward-related behavior and impulse control. Voluntary alcohol intake measurements revealed that BD-AMED animals consumed more alcohol than all other groups. After four weeks, during microdialysis, animals were exposed to the drinking context in the absence of alcohol, allowing the assessment of mesocortical DAergic responses to contextual cues previously associated with voluntary alcohol consumption. Under these conditions, BD-AMED animals exhibited reduced responsiveness of mesocortical DAergic neurons, whereas BD-ALCOHOL animals displayed a marked increase in DA release in the mPFC, despite similar basal extracellular DA levels across experimental groups. These results are consistent with previous findings showing that increased voluntary alcohol intake is associated with decreased sensitivity of mPFC DAergic neurons in socially isolated rats (Lallai et al., 2016). Therefore, although BD-AMED consumed more alcohol, they exhibited an altered mesocortical DAergic transmission, likely reflecting a disruption in mPFC function that may underlie the loss of inhibitory control and contribute to the greater alcohol consumption, which, from a translational perspective, may suggest a neurochemical substrate of vulnerability to the development of compulsive alcohol use (Jentsch & Taylor, 1999; Jentsch et al., 2014). Overall, these results suggest that co-exposure to RB and alcohol during adolescence interfere with the proper development and long-term functioning of the mPFC.

Finally, in order to more closely mimic human behavior, we allowed animals to take these substances contingently using an oral operant SA protocol to evaluate their reinforcing properties from mid-adolescence to adulthood (PND 42–89), and we assessed both mesocortical and mesolimbic DAergic systems during SA sessions in adulthood over two consecutive days (PND 91–92). Operant responding aimed at obtaining the reward solution, as well as the resulting intake, was significantly higher in SA-RB and SA-AMED compared with SA-ALCOHOL. However, SA-AMED exhibited increased mesocortical and mesolimbic DAergic transmission, showing synergistic effects compared with both SA-ALCOHOL and SA-RB. This result suggests that contingent administration of AMED from adolescence to adulthood, in a context where animals work to obtain the substance, may enhance operant

responding and DAergic activation both in the mPFC and in the AcbSh, potentially making the mesocorticolimbic pathway more sensitive in adulthood.

The increased mesocortical transmission observed in the last experiment, as opposed to the reduction observed in the previous one (Dazzi et al., 2024), may be attributable to several factors. In our previous experiment, all animals that had been previously exposed to the different substances in a non-contingent manner voluntarily consumed only alcohol, and during the microdialysis session an empty bottle was presented as a conditioned stimulus, without any substance intake. In contrast, during microdialysis coupled with SA, animals actively worked to obtain the different solutions, and thus the pharmacological effects of the substances themselves must also be taken into account. Consequently, these two conditions cannot be directly compared. In this context, altered mPFC function has been proposed as an early marker of neuroadaptation in alcohol addiction (Koob et al., 2014), suggesting that the differential mesocortical responses observed across experimental conditions may reflect distinct expressions of an underlying vulnerability rather than contradictory outcomes.

Similarly, the increase in DA levels observed in the AcbSh of SA-AMED in this protocol cannot be directly compared with the lack of increase observed in AC-AMED condition, as the latter involved a single non-contingent administration, whereas the former reflects a chronic operant behavioral paradigm in which the sensory experience (i.e., taste) and the processes of wanting, liking, and learning collectively contribute to DAergic activation, highlighting the importance of behavioral context in modulating neurochemical responses.

Notably, the sensory properties of these beverages may represent an additional factor contributing to DAergic activation. In particular, novel and palatable tastes are known to enhance DA release in both the AcbSh and mPFC, with a particularly pronounced effect in the latter (Bassareo & Di Chiara, 1997). Accordingly, the distinctive taste of AMED, together with the stimulatory action of caffeine on mPFC DAergic transmission (Acquas et al., 2002; De Luca et al., 2007), may have further enhanced the mesocortical response observed under SA conditions.

Moreover, it is important to note that the alcohol intake observed in SA-ALCOHOL cannot be directly compared with that of all the BD groups. In the latter

case, animals consumed a 5% v/v sweetened alcohol solution according to the protocol by Lallai et al. (2016), which was continuously available ad libitum during the 2-hour session. In contrast, SA-ALCOHOL animals were given a 10% unsweetened alcohol solution for only 30 minutes, contingent upon performing the correct operant response. However, BD-AMED was the only group to show an increase in alcohol consumption compared with all other experimental groups, consistent with the pattern observed in SA-AMED, suggesting that this combination may specifically promote higher alcohol intake across different administration paradigms.

Together, these findings clearly indicate in adulthood a dysregulation of both mesocortical and mesolimbic systems induced by AMED exposure in adolescence, disrupting the physiological balance between reward processing and cognitive control circuits and potentially increasing vulnerability to maladaptive alcohol-related behaviors later in life.

These effects can be further understood by considering the pattern and context of substance administration. Specifically, the effects of caffeine and AMED appear to depend critically on the type and schedule of administration, in line with observations for many other psychoactive substances, which produce distinct outcomes under acute versus chronic exposure. Acute caffeine administration, given either prior to or in combination with alcohol, prevented certain alcohol-induced effects, particularly salsolinol formation in the pVTA and mesolimbic DAergic transmission in the AcbSh (Section 7; Bassareo et al., 2024). Similarly, acute AMED administration reduced mesolimbic DAergic transmission in the AcbSh of AC-AMED and decreased the activation of pERK-positive neurons in the same region (Section 8; Puliga et al., in preparation). Intermittent subchronic BD-AMED exposure led to a reduction in the sensitivity of mesocortical DAergic neurons to cue-induced stimuli, accompanied by increased voluntary alcohol intake (Section 9; Dazzi et al., 2024). In contrast, chronic operant SA-AMED resulted in both elevated intake and enhanced mesocortical and mesolimbic DAergic transmission (Section 10). This indicates, in summary, that the effects of caffeine, alcohol, and AMED are determined not only by their pharmacological properties, but also by the interplay between exposure pattern, administration modality, and behavioral context. This

interplay, particularly under subchronic and chronic conditions, leads to the long-term adaptations in the reward circuitry documented in this thesis.

Although the present study provides a detailed characterization of the neurobiological effects of caffeine, RB, alcohol, and their combination, some limitations should be considered. First, as noted earlier, the use of different administration paradigms (acute, intermittent non-contingent BD exposure, and chronic operant SA) limits direct comparisons. However, this approach was necessary to separate purely pharmacological effects from behaviorally relevant ones, allowing us to evaluate how motivational and learning processes interact with drug effects. Moreover, the study focused on the mesocorticolimbic DAergic system, which is central to reward processing, cognitive control, and addiction-related behaviors, but does not represent the only neural circuit or neurotransmitter system involved in AMED effects, which should be investigated in future studies. Another limitation concerns the use of isolated drinking conditions, which, while necessary for accurate intake measurements, do not fully reproduce the social aspects of AMED consumption in human adolescents, which typically occurs in groups and recreational contexts. In addition, the use of only male subjects prevents assessment of possible sex-specific neuroadaptations, which should also be addressed in future studies. Accordingly, from a translational perspective, although animal models allow precise control over developmental timing and experimental variables, direct extrapolation to human behavior must be interpreted with caution due to the complexity of social and cognitive factors influencing AMED use. Nevertheless, the use of developmentally defined exposure PND periods, doses approximating realistic human intake, and contingent administration paradigms increases the translational relevance of the study, modeling key aspects of real-world consumption patterns.
Overall, despite these limitations, the experimental design was specifically developed to capture the complexity of both short- and long-term effects of AMED consumption, which would not have been evident using a single administration protocol.

# CONCLUSIONS

In conclusion, this PhD study confirmed that alcohol administered with RB (AMED) produces differential regulation in the brain reward system across all treatment paradigms (acute, subchronic, and chronic) as well as under both contingent and non-contingent administration.

The main finding of this study is AMED's capacity to alter the mesocorticolimbic DAergic system in a manner dependent on the mode of administration and the behavioral context. Although the modulation of the reward system may vary across the different paradigms, AMED consistently induced significant dysregulation in the balance between mesolimbic and mesocortical Daergic transmission, suggesting that this combination is not safe for adolescent consumption and that repeated exposure may trigger maladaptive mechanisms, potentially increasing vulnerability to AUD in adulthood.

Overall, despite the acknowledged limitations, this study provides novel insights by integrating neurochemical, behavioral, and immunohistochemical analyses across different developmental stages and administration paradigms. These findings emphasize the potential risks of AMED consumption and lay the foundation for future research, which should include female subjects, investigate other neurotransmitter systems relevant for substance-induced reward beyond DA, evaluate the roles of RB components besides caffeine, and assess the reversibility of long-term consequences of AMED exposure.

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