Università degli Studi di Cagliari

# Ph.D. DEGREE

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# TITLE OF THE Ph.D. THESIS

**Phytomedicinal approach toward the treatment of CNS disorders: behavioural and biochemical evaluation of the effects of a methanolic dry root extract of Withania somnifera and its isolated metabolite, Docosanyl Ferulate, in rodent models of anxiety and drug addiction**

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## 1. Abstract

The use of phytomedicinal remedies has increased during the last decades thanks to the relative low cost, low toxicity, and potential synergic effects of plant-derived medications. *Withania somnifera (WS)*, commonly known as Indian Ginseng, is a medical plant belonging to the Indian Traditional Medical System, the Ayurveda. In preclinical studies, *WS* has shown an impressive variety of therapeutic effects in several pathological conditions. Among these effects, the anxiolytic and antiaddictive properties of this plant are the ones discussed in this work of thesis. Both effects have been attributed to a $GABA_A$-mimetic activity of *WS*. This motivated the search for the single isolated compounds responsible for the GABA mimetic component of the plant. Accordingly, our group performed binding stud**ies** on a methanolic dry root extract of *WS* (*WSE*) and recently identified several components of the extract endowed with GABAergic activity. Among these, the Ferulic Acid ester, Docosanyl Ferulate (DF), resulted the most potent $GABA_A$ agonist, being able to enhance the $GABA_A$ receptor inhibitory postsynaptic currents with an IC50 value of 7.9 μM.

This thesis consists of a behavioural and biochemical evaluation of the effects of this newly isolated compound in rodent models of anxiety and substance use disorder. Considering that agonism on the $GABA_A$ receptor is the mechanism of action of benzodiazepines, the most used and prescribed anxiolytic drugs in the world, a behavioural comparison between DF and Diazepam (DZP) as reference compound has been performed in order to assess the eventual anxiolytic activity of DF. The treatment with DF exerted an anxiolytic effect at the Elevated Plus Maze in adult CD1 mice at the doses of 0.25 and 2 mg/kg and the effects of this last dose did not differ statistically from those induced by DZP (2 mg/kg). In addition, the anxiolytic effects of DF were prevented by a pre-treatment with the $GABA_A$ receptor antagonist Flumazenil. Intriguingly, at variance from DZP, DF did not carry motor, mnemonic and addictive side effects and did not potentiate ethanol-induced depressant activity. Moreover, considering the

importance of the $GABA_A$ mimetic component of *WSE* in its antiaddictive effects, we verified whether DF could share with the whole extract the property of preventing the acquisition and expression of ethanol- and morphine-induced Conditioned Place Preference (CPP). Interestingly, DF prevented the acquisition, but not the expression of both substances-induced CPP, probably through a preventive effect on ethanol- and morphine-induced activation of the Extracellular signal-regulated kinase (ERK) in the Shell subregion of the Nucleus Accumbens (AcbSh) in adult CD1 mice. Finally, a very detailed characterization of the role of ethanol's metabolism in ethanol-mediated activation of the mesolimbic pathway was performed. The results pointed out the μ receptors-mediated effect of ethanol-derived salsolinol in the posterior Ventral Tegmental Area (pVTA) as responsible for the ethanol-mediated increases in the dopaminergic traffic in the AcbSh of rats. Hence, considering that *WSE* can prevent ethanol-induced activation of the mesolimbic pathway and increase in the dopaminergic traffic of AcbSh in rats, we verified whether also DF could share this property and if the salsolinol-mediated mechanism of action could be involved in its action. Remarkably, DF prevented alcohol-induced formation of salsolinol in the pVTA and the consequent increase of DA traffic in the AcbSh. In conclusion, these results confirm the efficiency of *WSE* as potential treatment for anxiety and substance use disorder and point out DF as a new potent, safe and interesting molecule to be further characterized in order to better understand its therapeutical potential. Moreover, this thesis suggests, for the first time, a plausible mechanism of action for the ethanol-mediated activation of the mesolimbic pathway *in vivo* which might be of extreme importance to better understand the complex pharmacology of this molecule and to develop novel and specific treatments for alcohol use disorder.

# 2. General introduction

## *2.1 Phytomedicine: definition and brief historical background*

Phytomedicine might be defined as the use of plant-derived medications in the treatment and prevention of pathological conditions (Chikezie, 2015; Bent, 2008). The term phytomedicine was coined by the French physician Henri Leclerc in 1913, but the origin of this science is as old as mankind, as there is consistent evidence supporting that even primitive man used to look for plants-based remedies for therapeutical purposes (Chikezie, 2015; Kaur et al., 2019). Obviously, during pre-historical times, man did not have the means nor knowledge to manage plants-based remedies with a scientific approach; nevertheless, centuries of empirical approaches to the use of this kind of medications lead to what is nowadays considered as modern phytomedicine. In fact, we could say that modern phytomedicine started with the rise of organic chemistry, during the 19$^{th}$ century, when scientists became capable of identifying, isolating and synthetizing the active principles responsible for the pharmacological effects of the plants. The first active substance isolated from a plant was morphine that in 1803 was actually isolated from opium, a secretion of *Papaver somniferum*. Subsequently, the same fate occurred for other medical plants, obtaining more accurate and specific drugs (Miraldi and Baini, 2018). The biologically active compounds present in plants which elicit pharmacological or toxicological effects in humans and animals, are called phytochemicals. These molecules are typically secondary metabolites produced by plants to provide protection against pathogens and diseases and allow adaptation to their environments; there is such a high variety of these molecules that they are part of almost all main classes of organic compounds: aliphatic, aromatic, hydroaromatic and heterocyclic, although they fall into four main classes of compounds: terpenoids, phenolics, nitrogen-containing alkaloids and sulfur-containing compounds. Intriguingly, these molecules exhibit a wide range of therapeutical functions for humans. Some of the most common are:

- Salicylic acid, from *Salix alba*, which exerts anti-inflammatory, analgesic, antipyretic and anticoagulant effects; its ester, acetyl salicylic acid, is the active compound of Aspirin.

- Digoxin and Digitoxin, from *Digitalis purpurea* and other *Digitalis* species, which exert potent cardiotonic effects.

- Caffeine, from *Coffea arabica,* which is one of the most consumed substances worldwide and exerts stimulant effects on the Central Nervous System and on metabolism.

- Taxol, from *Taxus brevifolia* used as antitumoral.

- Vinblastine and Vincristine, from Catharanthus roseus, used especially in leukemia (Kaur et al., 2019).

However, with the possibility of obtaining more specific molecules, the use of natural remedies inevitably suffered the explosion of synthetic drugs and, for decades, took a back seat. Nevertheless, at the end of the 20$^{th}$ century, the interest in phytomedicinal remedies regained the interest of researchers, with a reflexion also in the number of publications. The increasing popularity and demand for herbal drugs can be attributed to the fact that natural plant products are often nontoxic, have low side effects and are available at affordable prices compared to costly chemical drugs. Moreover, the combinations of secondary products present in plants, can sometimes give birth to synergic effects that cannot be reached by isolated compounds (Miraldi and Baini, 2018; Kaur et al., 2019). Accordingly to the raising interest in natural remedies, some years ago, our laboratory started a multi-year research project aimed at the pharmacological and biochemical characterization of a phytomedicinal remedy used for centuries in the Indian Traditional System of Medicine (Ayurveda), *Withania somnifera (WS)* Dunal and started the characterization of its dry methanolic root extract (*WSE).*

### *2.2 Withania somnifera*

*General introduction*

*WS* is a small woody shrub commonly known as "Winter cherry" or "Indian Ginseng" (Figure 1). It belongs to the family of Solanaceae and attains a height of 0.5–2 m. *WS* shows an extreme wide and variegate spectrum of compounds in its different parts: more than 12 alkaloids, 40 withanolides and several sitoindosides have been isolated and reported. The major chemical constituents of *WS are* ***Alkaloids****:* Withanine, withananine, withasomnine, somniferine, tropeltigloate, somniferinine, somninine and nicotine; ***Steroidal lactones****:* Withaferin-A, withanone, withanolide-E, withanolide-F, withanolide-A, withanolide-G, withanolide-H, withanolide-I, withanolide-J, withanolide-K, withanolide-L, withanolide-M; ***Steroids****:* Cholesterol, β-sitosterol, stigmasterol, diosgenin, stigmastadien, sitoinosides VII, sitoinosides VIII, sitoinosides IX, sitoinosides X; ***Salts****:* Cuscohygrine, anahygrine, tropine, pseudotropine, anaferine; ***Flavonoids****:* Kaempferol, quercetin; ***Nitrogen-containing compounds****:* Withanol, somnisol, and somnitol (Dar, 2015). In preclinical studies, *WS* has shown an impressive variety of therapeutic effects, such as: anti-microbial (Bisht and Rawat, 2014;), anti-inflammatory (Minhas et al., 2011; Ku et al., 2014), anti-cancer (Widodo et al., 2008; Mayola et al., 2011), anxiolytic (Kaur and Kaur, 2017; Kaur et al., 2017), neuroprotective (Durg et al., 2015; Singh, 2008) cardioprotective (Ojha and Arya, 2009), and anti-diabetic (Guttam and Kalia, 2013; Andallu and Radhika, 2000). Moreover, *WS* showed, in preclinical studies, very intriguing anti neurodegenerative effects (Kuboyama et al., 2014). Our group provided evidence that *WSE* results efficient in counteracting some effects of Parkinson's Disease and Amyotrophic Lateral Sclerosis in neurodegenerative-models of *Drosophila melanogaster*. In particular, in 2016 De Rose and colleagues demonstrated that the administration of a treatment with *WSE* to a *Drosophila melanogaster* LRRK2 loss-of-function model of Parkinson's disease improved locomotor activity, muscle electrophysiological response to stimuli and also protected against mitochondria degeneration (De Rose et al., 2016). Moreover, they also demonstrated that *WSE* treatment significantly increased lifespan of human Superoxide Dismutase 1 (hSOD1) gain-of-function *Drosophila*

*melanogaster* model of Amyotrophic Lateral Sclerosis (ALS), it significantly rescued climbing impairment, and also latency and other electrophysiological responses; finally it prevented mitochondrial alterations (De Rose et al., 2017). Again, our group studied the same parameters, in addiction to sleep patterns, locomotor activity and response to volatile anaesthetics in another *Drosophila melanogaster* ALS model (TDP43) and demonstrated that *WSE* could partially rescue ALS-altered parameters (Maccioni et al., 2018). Moreover, *WSE* also showed intriguing analgesic features. Orrù and colleagues demonstrated that *WSE* efficiently reduces chemical-induced (formalin and glutamate) nociception in mice, likely through an opioidergic mechanism of action (Orrù et al. 2016). The analgesic properties of *WSE* and its interactions with the opioidergic system have been investigated also in monitoring the effects of this extract in modulating morphine-mediated analgesia. In fact, *WSE* prolongs morphine-induced analgesia and suppresses the development of morphine-induced rebound hyperalgesia through an activity on opioid receptors' gene expression which may likely be mediated by peroxisome proliferator-activated receptors gamma (PPARγ) (Orrù et al., 2014; Caputi et al., 2018; Caputi et al., 2019). Another effect, in particular the anti-addictive property of *WS* has been observed during these last years and it is especially on this effect that our research has focused during the characterization of the pharmacological profile of *WSE*.

*Withania somnifera and drug addiction*

Drug addiction is defined as the progressive loss of control over drug taking caused by repeated exposures to addictive drugs. This is the consequence of a series of neuroadaptations, occurring in selective neural circuits, which leads to the development of a chronic neuroadaptive disorder characterized by behavioural alterations in which compulsive drug-seeking and high risk of relapse play a critical role (Berke and Hyman 2000; Koob 2006; Koob and Le Moal 2001; Nestler 2001, Volkow and Morales, 2015). The scientific interest about the application of phytomedicine and natural remedies for drug addiction treatment has increased in recent years, simultaneously to the number of

publications regarding the effects of crude extracts, active principles, and plant associations on the treatment of addiction and, sadly, accompanied by the failure of classical pharmacological approaches (Lu et al., 2009). The positive effects of *WS* and *WSE* on various aspects of drug addiction have been reported, in preclinical trials, by several studies. In example, *WS* showed activity on reducing some withdrawal-related features: accordingly, Kulkarni and Ninan demonstrated that repeated administration of *WS* (100 mg/kg) for 9 days suppressed morphine-withdrawal jumps, a sign of the development of dependence to opiate as assessed by naloxone (2 mg/kg) precipitated withdrawal on day 10 of testing in mice (Kulkarni and Ninan, 1997); moreover, Kasture and colleagues showed that a treatment with *WSE*, reduced the severity of the morphine-induced withdrawal syndrome when given during chronic morphine but not during withdrawal. In addition, the same treatment with *WSE* during chronic morphine, but again not during withdrawal, fully prevented the reduction of spine density in the nucleus accumbens shell (AcbSh) in spontaneous and pharmacologically precipitated morphine withdrawal (Kasture et al., 2009). Similar positive effects on withdrawal, this time induced by ethanol, were reported by Gupta and Rana who showed that ethanol- mediated withdrawal anxiety was markedly antagonized in dose dependent manner by *WS* at 200 and 500 mg/kg (Gupta and Rana, 2008). In addition, *WSE* resulted able to prevent morphine-induced acquisition and expression of Conditioned Place Preference (CPP) (Ruiu et al., 2013) and also to prevent ethanol-induced acquisition and expression of CPP and Conditioned Place Aversion (CPA) in mice (Spina et al., 2015); in rats, *WSE* reduced the acquisition, maintenance and breakpoint of ethanol self-administration under a FR1 and reduced the deprivation effect and the reinstatement of ethanol-seeking behaviours in adult male Wistar rats (Peana et al., 2014). The ability of *WSE* to preventing the motivational properties of morphine and ethanol has been given a mechanism of action in 2019 when Bassareo and colleagues demonstrated that *WSE* was able to impair morphine- and ethanol-induced stimulation of the mesolimbic pathway, resulting in an impaired neuronal firing of ventral tegmental area (VTA) dopaminergic neurons and of dopamine (DA) transmission in AcbSh, a property that has been pinpointed as a neurochemical feature shared by all substances with addictive

potential (Di Chiara, 1999; Di Chiara et al., 2004). The importance of the mesolimbic pathway in addiction will be further analysed in a dedicated paragraph of this general introduction and in the relative chapters.

*The GABAergic activity of Withania somnifera*

Various studies tried to point out the molecular mechanism behind the neurochemical, electrophysiological and behavioural effects of *WSE* on ethanol and morphine. In the already above-mentioned study, Bassareo and colleagues also performed specific experiments to explain the inhibitory effect of *WSE* on spontaneous firing of VTA DA neurons. They demonstrated that this inhibition was completely abolished by the $GABA_A$ ($GABA_AR$), but not $GABA_B$ ($GABA_BR$), receptor selective antagonist bicuculline, confirming that $GABA_AR$ may be directly involved in this action on VTA DA neurons (Bassareo et al., 2019). This finding totally gets along with other reports showing that $GABA_AR$ are sensitive targets of *WS* in different brain regions (Bhattarai et al., 2010; Yin et al., 2013; Candelario et al., 2015) and with previous receptor binding studies demonstrating that *WSE* shows high to moderate affinities, $k_i$ values being 13 and 130 μg/ml for $GABA_AR$ and $GABA_BR$, respectively (Ruiu et al., 2013; Orrù et al., 2014). An additional support for the involvement of $GABA_AR$ in the pharmacological effects of *WS* came from the observation that *WS* activates $GABA_AR$-mediated $Cl^-$ currents (Bhattarai et al., 2010; Yin et al., 2013; Candelario et al., 2015) and that *WS* (50–200 mg/kg p.o.) makes animals less seizure-prone by exhibiting dose-dependent anticonvulsant effects in mice (Akula et al., 2009). An interaction of *WS* with the $GABA_AR$ is enforced also by the anxiolytic effect of the plant. In fact, both clinical (Pratte et al., 2014) and experimental (Kaur and Kaur, 2017; Kaur et al., 2017) evidence justifies *WS*'s long-standing reputation as an effective anxiolytic treatment. In this regard, modulation of GABA neurotransmission is of fundamental importance from a pharmacotherapeutic perspective (Ngo and Vo, 2019). In keeping with these studies, our group decided to identify and isolate single compounds with affinity to the $GABA_AR$ from *WSE*: nine compounds, including two undescribed withanolides, withasomniferolides A and B, three known withanolides, a ferulic acid dimeric ester, and an inseparable mixture of three long alkyl chain ferulic acid esters, were isolated. The most active compound

resulted Docosanyl Ferulate (DF) (Figure 2), able to enhance the $GABA_AR$ inhibitory postsynaptic currents with an IC50 value of 7.9 μM (Sonar et al., 2019). After this discover, we started exploring the range of actions of this compound and the outcomes of these studies constitutes the most part of this thesis.

### *2.3 The role of mesolimbic dopaminergic transmission in the neurocircuitry of addiction*

Drug addiction is a chronically relapsing disorder that has been characterized by the compulsion to seek and take the drug, the loss of control in limiting intake, and the emergence of a negative emotional state reflecting a motivational withdrawal syndrome when access to the drug is prevented. It might be defined as a neurological disease that includes elements of both impulsivity and compulsivity that contribute to determine a complex cycle composed essentially of three interacting, yet different, stages: "binge/intoxication", "withdrawal/negative affect", and "preoccupation/anticipation" (Koob and Le Moal, 2010). Impulsivity might be defined as "a predisposition toward rapid, unplanned reactions to internal and external stimuli without regard for the negative consequences of these reactions to themselves or others"; compulsivity, on the other hand, could be defined as the manifestation of "perseverative, repetitive actions that are excessive and inappropriate". Thus, if impulsive behaviours can often be accompanied by feelings of pleasure or gratification, compulsions, typical of conditions such as obsessive-compulsive disorder, are conversely performed to reduce tension or anxiety from obsessive thoughts. In drug addiction, the transition from impulsivity to compulsivity is reflected in a shift in the driving force behind the consume of the substance. If during the first phase it is the positive reinforcement that is driving the motivated behaviour, then it is the negative reinforcement and the automaticity which are in charge and induce the motivated behaviour. Contextualized, after this shift, the driving force behind the consume of the substance is not any more the pleasure itself given by the substance (i.e. the main feature of the first stages), but the need to avoid all the negative aspects related difficulties accessing the drug (Koob, 2004, Koob and Volkow, 2016). These three phases are mediated by distinct

circuits. In this introduction I will further describe the first stage, the "binge or intoxication" stage, as it is the one which is more closely linked to the behavioural and biochemical essays performed and described in this thesis. During the binge/intoxication stage, stimuli that were previously neutral, can rapidly start to be associated with drug availability, resulting in gaining incentive salience and promoting habit formation; moreover, this stage also triggers opponent-process responses that reduce reward function and consequently leads to excessive drug seeking (Koob and Volkow, 2016). Although it is not the only mechanism involved, the mesolimbic pathway plays a fundamental role in this early phase. The mesolimbic pathway is a dopaminergic pathway in which the VTA of the midbrain is connected to the ventral striatum, in particular to the basal ganglia of the forebrain, included the AcbSh (Figure 3). Although evidence suggests that also DA-independent reinforcement occurs at the level of the AcbSh, all drugs of abuse activate the mesolimbic DA system resulting in an increase in the DAergic transmission in AcbSh (Koob, 1992; Nestler, 2005; Di Chiara and Imperato, 1988). The drugs showing these properties include central stimulants (e.g., amphetamine and cocaine), opiates (e.g., morphine, methadone, and fentanyl), central depressants (e.g., ethanol), and cholinergic agonists (e.g., nicotine). Activation of the mesolimbic dopaminergic system results in or contributes to reward (Di Chiara and Imperato, 1988). Knowing the role of the mesolimbic pathway is instrumental for the information contained in chapters 2 and 3 of this thesis.

### *2.4 Animal models of addiction and the conditioned place preference paradigm*

During the last decade, animal models of psychiatric disorders have been criticized for their limited translational value to the clinical situation. Failures in clinical trials have often been attributed to the lack of predictive power of preclinical animal models. However, animal models are crucial and informative tools for the identification of pathological mechanisms, target identification, and drug development. These models provide excellent face validity, and the neurochemical and neuroanatomical substrates involved in drug-intake behaviour are similar in laboratory rodents and humans. Consequently, animal models of drug

consumption and addiction provide predictive validity. Some of the best results of this predictive power are well illustrated in alcohol research, in which three approved medications (acamprosate, naltrexone, and nalmefene) were developed by means of animal models and then successfully translated into the clinical situation (Spanagel, 2017). Animal behavioral models of addiction include non-contingent models, in which animals are passively exposed to rewarding substances, usually administered by the experimenter, as well as contingent models such as drug self-administration and relapse. Models based on non-contingent drug exposure are simple and quick to set up. Because of these advantages, many studies have used them to identify key reward-related neurobiological substrates and how drug exposure alters them (Kuhn et al., 2019). The CPP paradigm is still one of the most popular and diffuse non-contingent animal models to perform basic and preliminary studies on rewarding effects of drugs treatments (Tzschentke, 2007). The theoretical aspect behind this animal model is based on the association of a particular environment with the treatment, followed by the association of a different environment with the absence of the treatment (Prus et al., 2009). Normally, the CPP apparatus consists in 2 compartments separated by a guillotine door. At first, spontaneous preference is measured with the door open. Then, animals are conditioned for some days by being treated with the drug and exposed to the drug-associated compartment; conversely, they are exposed to the opposite compartment when they are treated with the vehicle. Normally, compartments differ in visual and tactile stimuli. At the end of this phase, the preference for these compartments is measured and the relative differences are calculated (Figure 4) (see chapter 1 and 2 for more insights on the CPP protocol followed in these experiments). The ability of a stimulus (drug treatment, food, etc.) to produce a preference for the associated environment is governed by Pavlovian conditioning. The effects perceived as a consequence of drug-treatment can be considered as the unconditioned stimulus while the drug-paired compartment as the conditioned stimulus. The rationale behind this motivational paradigm is that pairing the drug to the (drug-associated) compartment results is the transferring of the rewarding properties of the unconditioned stimulus (the drug-effect) directly to the conditioned one (environment), which was somehow neutral/un-preferred before the conditioning. As a

matter of fact, we cannot say that, after conditioning, the conditioned stimulus gains rewarding properties as we intend them, literally, for the drugs and we do not know if being exposed to the drug-associated compartment gives pleasure to the animal; however, we do know that this leads to an increase in DA transmission in the AcbSh (Tzschentke, 2007, Prus et al., 2009). Accordingly drug-elicited place conditioning is regulated through a number of pharmacological approaches targeting the mesolimbic transmission, and this is also valid for both ethanol and morphine, the two substances whose place conditioning effects are discussed in chapter 2 of this thesis. In fact, opiate-induced CPP can be blocked by the DA $D_2$ receptor antagonist haloperidol (Leone et al., 1987; Spyraki et al., 1983) Furthermore, injection of opiates into the VTA or AchSh also produces CPP (Phillips et al., 1980; Van der Kooy et al., 1982; Glimcher et al., 1984; Bozarth 1987). Moreover, ethanol-induced CPP can be attenuated by intra-accumbens administration of $D_2$ or $D_1$ receptor antagonists, such as fluphenazine (Walker and Ettenberg, 2007) or SCH39166 (Spina et al., 2010), respectively.

### *2.5 Extracellular signal-regulated kinase (ERK)*

Extracellular signal-regulated kinase 1 (ERK1) and ERK2, from now on generally called ERKs, are members of a family of structurally related kinases, called mitogen-activated protein kinases (MAPKs). The ERKs are activated by dual phosphorylation on their regulatory Tyr and Thr residues located within the Thr–Xaa–Tyr motif by a large variety of extracellular signals (Shawl and Seger, 2007). Their characterization has revealed how their signalling is involved in essential and precise cellular functions thanks to the wide variety of specific context-expressed substrates. Some of these substrates are localized in the cells' cytoplasm, while others are phosphorylated in the nucleus by ERK molecules that are translocated into this organelle upon stimulation (Figure 5). ERKs signalling controls cell proliferation, survival, growth, metabolism, motility, differentiation, and development (Seger and Krebs, 1995; Lavoie et al., 2020). In order to execute all its functions, sometimes even opposing ones, the ERK cascade is extensively regulated by five distinct mechanisms. 1) Changes in the duration and strength of the signals: regulated via inhibitory signalling

components, such as phosphatases involved in early genes expression. 2) Scaffold proteins: they facilitate the kinetic of activation of the members of the cascade by modulating their proximity. 3) Subcellular localization: it directs the signals into their proper compartments and targets. 4) Crosstalk with other signalling pathway: this mechanism may influence the strength of the signals, and often modulates the activity of downstream targets. 5) The existence of multiple components with distinct functions in each tier of the cascade, which can track the signal into different specific targets for each of these components (Shawl and Seger, 2007). Among the various functions and implications of this cascade, ERKs seem to be heavily involved in signal transduction, neuroplasticity, gene expression, and behavioural changes underlying the reinforcing processes induced by substances of abuse (Valjent et al., 2005). This aspect will be further explained in chapter 2 of this thesis.

### *2.6 Ethanol metabolism and ethanol- dependent synthesis of salsolinol in the pVTA*

As it will be further described later in chapter 3, ethanol metabolism plays an important, if not fundamental, role in its behavioural and neurochemical effects. The main ethanol metabolism pattern takes place in the liver and follows these three steps (Figure 6):

- Ethanol is converted to acetaldehyde with the catalysis of the enzyme alcohol dehydrogenase. This reaction occurs largely in the liver and requires a vitamin-related cofactor called nicotinamide adenine dinucleotide ( NAD). This reaction is reversible.
- The second stage is catalysed by the enzyme aldehyde dehydrogenase. Acetaldehyde is oxidized to acetate; $NAD^+$ as cofactor. This reaction is irreversible, and it consists in the oxidation of acetaldehyde, in the liver, to acetate.
- Finally, great part of the acetate produced by the oxidation of acetaldehyde leaves the liver and circulates to peripheral tissues where it is activated to a key Acetyl CoA.

Among ethanol's metabolites, acetaldehyde seems to play a key role in mediating its effects. Nevertheless, peripheral acetaldehyde produced by ethanol oxidation in the liver does not reach the brain in sufficient amounts (Correa et al., 2012). Conversely, ethanol does, and it is oxidated to acetaldehyde in the brain with a reaction catalysed by CYP2E1 and mostly by the enzyme catalase (Zimatkin and Lindros, 1996), which requires Oxygen Peroxide as cofactor (Figure 7)

$C_2H_5OH + H_2O_2 \rightarrow C_2H_4O + 2H_2O$

As already said, acetaldehyde, the main ethanol's metabolite, was suggested as able to mediate ethanol's reinforcing effects (Chevens, 1953). This hypothesis generated a significant body of research aimed at characterizing the role of ethanol metabolism in its central effects (Karahanian et al., 2011; Correa et al., 2012), in particular with reference to its ability to activate the mesolimbic DA system (Correa et al., 2012; Söderpalm and Ericson, 2013; Israel et al., 2015). In particular, the ability of ethanol to stimulate DA neurons in the posterior region of the VTA (pVTA) (Gessa et al., 1985), activate DA transmission in the AcbSh (Howard et al., 2008; Bassareo et al.,2017), and elicit DA-mediated locomotor activity (Carlsson et al., 1972; Sánchez-Catalán et al., 2009) has been mechanistically related to acetaldehyde. Such role of acetaldehyde has been demonstrated by two independent lines of evidence: first, acetaldehyde administration reproduces, at lower doses than ethanol, DA-dependent central effects (Spina et al., 2010; Correa et al., 2012); second, inhibition of acetaldehyde production, by blockade of peripheral and central ethanol metabolizing enzymes (Spivak et al., 1987; Pastor et al., 2002; Peana et al., 2017a) or by acetaldehyde sequestration (Martí-Prats et al., 2013), prevents both neurochemical and behavioral DA-mediated effects of ethanol. The mechanism(s) by which acetaldehyde exerts such effects is unknown (Peana et al., 2017b). Notably, acetaldehyde has a very short half-life and is highly reactive with molecules such as DA. Indeed, although an enzymatic mechanism has also been claimed (Chen et al., 2018), acetaldehyde and DA can spontaneously generate a condensation product, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline or salsolinol, a molecule undetectable, under control conditions, in the VTA. This led to renewed interest

in ethanol's secondary metabolites (Davis and Walsh, 1970; Davis et al., 1970; Rodd et al., 2008; Hipólito et al., 2012; Deehan et al., 2013; Israel et al., 2015; Quintanilla et al., 2016). Intriguingly, salsolinol, administered either systemically or locally in the pVTA, shares with ethanol and acetaldehyde a spectrum of neurochemical and behavioral DA-mediated effects (Correa et al., 2012; Hipólito et al., 2012; Deehan et al., 2013). Moreover, salsolinol is self-administered by rats in the pVTA (Rodd et al., 2008), and its pVTA administration promotes voluntary binge-like ethanol intake (Quintanilla et al., 2016). The fundamental role of salsolinol and the possibility of DF interfering with its formation will be described in chapter 3.

## 3. Aims of the thesis

1) With the recent identification and isolation of the $GABA_AR$ agonist DF from *WSE*, the first aim of my thesis consisted in executing a behavioural pharmacological characterization of this molecule in order to verify whether DF could be one of the possible effectors of the $GABA_AR$ mimetic effects of the whole extract. Considering that $GABA_AR$ agonism is a feature shared with the most used anxiolytic drugs, benzodiazepines (BDZ), this first behavioural characterization was aimed at verifying whether DF possessed BDZ-like anxiolytic properties which might be blocked by the BDZ site antagonist Flumazenil (FMZ). Moreover, since BDZ have several collateral effects, we sought to verify whether also DF shares these undesired activities. Aim 1 is deeply described in chapter 1 and it is the main topic of the paper entitled "The biologically active compound of Withania somnifera (L.) Dunal, DF, is endowed with potent anxiolytic properties but devoid of typical BDZ-like side effects" by Maccioni et al., 2021 (doi: 10.1177/02698811211008588).

2) Among *WSE* activities justified by a $GABA_AR$-mediated

mechanism of action there are also some effects of the extract on the motivational properties of ethanol and morphine. In particular, *WSE* resulted able to prevent the acquisition and the expression of ethanol- and morphine-induced CPP in mice (Ruiu et al., 2013, Spina et al., 2015). Hence, after having demonstrated that DF might be one of the effectors of the anxiolytic activity of *WSE* (Aim 1), the second aim of the thesis consisted in verifying whether DF might be also responsible of the effects showed by *WSE* on ethanol- and morphine-induced behaviours as determined by CPP. Accordingly, we verified whether DF could prevent the acquisition and the expression of ethanol- and morphine-induced CPP. Moreover, in order to investigate on a plausible molecular mechanism, we also verified whether the activation of Extracellular Regulated Kinase (ERK) in the AcbSh might be involved in these effects. Aim 2 is further described in chapter 2 and it is the main topic of the paper entitled "Differential effects of *WSE* and its $GABA_AR$ agonist constituent, DF, on

acquisition and expression of ethanol- and morphine- CPP and ERK phosphorylation in the AcbSh of CD1 mice" by Maccioni et al. 2022 (doi: 10.1007/s00213-022-06069-w).

3) The third aim of my thesis can be seen as divided into two sub-aims. Aim 3A is focused on the neurochemical and pharmacological investigation of ethanol-induced activation of the mesolimbic pathway. In particular, we elucidated the role of ethanol metabolism and of the formation of salsolinol in the pVTA in the activation of this neuronal circuit. The activation of the mesolimbic pathway is of critical importance during the first stages of alcohol use disorder and it is strongly involved in some of the behavioural effects of ethanol, including the acquisition of ethanol-induced CPP, discussed in Aim 2 of this thesis. Intriguingly, *WSE* is able to prevent, thanks to a $GABA_AR$-mediated mechanism of action, the activation of the mesolimbic pathway and the consequent increase in the DAergic traffic in the AcbSh in rats administered with a single dose of ethanol (i.g. 1 g/kg) (Bassareo et al., 2019). Hence, considering the agonism of DF on the $GABA_AR$ complex we performed, in Aim 3B an initial evaluation of the effects of this molecule on ethanol-dependent salsolinol synthesis and its consequent activation of the mesolimbic dopaminergic pathway. This investigation might help explaining if DF is one of the effectors of *WSE* preventive effects on ethanol-induced activation of mesolimbic pathway and might contribute to justify the effects of this $GABA_AR$ agonist in preventing the acquisition of ethanol-induced CPP (Aim2). Both Aims have been investigated *in vivo* through the application of brain microdialysis in adult male Sprague Dawley rats. Aim 3A is the main topic of the paper entitled "Ethanol-Dependent Synthesis of Salsolinol in the Posterior Ventral Tegmental Area as Key Mechanism of Ethanol's Action on Mesolimbic Dopamine" by Bassareo et al. 2021 (doi:10.3389/fnins.2021.675061) . The data obtained for addressing aim 3B are still unpublished.

# 4. CHAPTER 1 - The biologically active compound of *Withania somnifera* (L.) Dunal, Docosanyl Ferulate, is endowed with potent anxiolytic properties but devoid of typical benzodiazepine-like side effects

## *4.1 Introduction*

As already mentioned in the general introduction, both clinical (Pratte et al., 2014) and experimental (Kaur and Kaur, 2017; Kaur et al., 2017) evidence justifies *WS*'s long-standing reputation as an effective anxiolytic treatment. In this regard, modulation of GABA neurotransmission is of fundamental importance from a pharmacotherapeutic perspective (Ngo and Vo, 2019) and we know that experimental evidence supports the possibility that some constituents of *WS* could target the $GABA_AR$ (Bassareo et al., 2019; Bhattarai et al., 2010; Mehta et al., 1991; Orrù et al., 2014; Ruiu et al., 2013). In keeping with these studies, we recently detected, in *WSE*, some secondary metabolites with affinity for this receptor. In particular, the long alkyl-chain ferulic acid ester, DF (Figure 2), showed the highest modulatory activity on the $GABA_AR$ in rat brain slices (Sonar et al., 2019). Based on this evidence, we designed the present in vivo study aiming to investigate whether this molecule could have anxiolytic effects. We therefore performed a series of assays on mouse behavioural models using diazepam (DZP), a positive allosteric modulator of $GABA_AR$ (Nutt and Blier, 2016), as BDZ reference-compound. The potential DF's anxiolytic effects were investigated in an Elevated Plus Maze (EPM) test (Lister, 1987; Pellow and File, 1986; Rodgers and Johnson, 1995) and, in order to confirm the BDZ-like mechanism of DF effects in the EPM test, we used the BDZ antagonist flumazenil (FMZ) (Razavi et al., 2017). Moreover, although BDZs are among the most prescribed psychiatric medications, they are also classified as addictive drugs (Tan et al., 2010) and furthermore their beneficial effects are restrained by adverse motor and cognitive side effects (Roth et al., 1984; Rowlett et al., 2005). Hence, we also investigated whether DF shares with BDZs their most typical side effects such as motor and mnemonic impairments as well as addictive potential. To this end, we verified whether DF could impair motor abilities in a Static Rods test (Deacon, 2013);

subsequently, we evaluated the performance of DF- and DZP-treated mice in a Novel Object Recognition (NOR) test (Costa et al., 2014) and, in order to evaluate if, similarly to DZP (Acquas et al., 1989; Spyraki et al., 1985), DF presents addictive potential, we also tested whether DF, at its full anxiolytic dose, elicits place conditioning (Tzschentke, 2007). Finally, although BDZs themselves are quite safe medications, an extremely high risk of generating adverse reactions is related to their interaction with other depressant substances including ethanol, the most abundant constituent of alcoholic drinks. Indeed, ethanol and BDZs share the ability to interact with the $GABA_AR$ complex and, for this reason, ethanol increases the misuse of BDZs and BDZs-related cases of overdose (Linnoila, 1990; Votaw et al., 2019). Accordingly, we evaluated whether similarly to DZP, DF is endowed with the property to potentiate ethanol-induced Loss of Righting Reflex (LORR) (Correa et al., 2001; Slater et al., 2016).

### *4.2 Materials and methods*

*Animals*

Adult male CD-1 mice (22–24 g, Charles River, Calco, Italy) (n=336) were housed in groups of eight per cage, under a 12:00/12:00 h light/dark cycle (lights on at 08:00 a.m.) with food (Mucedola Srl, Settimo Milanese (Milan) Italy) and water available ad libitum. All the experiments were carried out during the light phase, between 09:00 and 18:00 h. On the day of the experiment mice, kept in their home cages, were carried in the experimental room where they had 1 h of habituation before the experiments' start. The total numbers of mice were n=116 for the EPM, n=49 for the Static Rods, n=43 for the NOR, n=40 for the Place Conditioning and n=88 for the LORR. All the experimental procedures were performed in accordance with the Principles of laboratory animal care, with the guidelines and protocols approved by the European Union (2010/63/UE L 276 20/10/2010) and with the approval (1177/2016) of the local Committee. Every possible effort was made to minimize animal suffering and discomfort and to reduce the number of experimental subjects.

*Drugs administration*

DF (0.05, 0.25 and 2 mg/kg) (Figure 1), synthetised (purity >98% by HPLC) according to Sonar et al. (2019), DZP (2 mg/ kg) (FIS, Altemantecchio, Vicenza, Italy) and FMZ (10 mg/kg) (gift from Hoffmann-La Roche, Basel, Switzerland), dissolved in Tween 80 (Sigma-Aldrich, Milan, Italy) and suspended in isotonic saline (NaCl 0.9% w/v) were administered at 10 mL/kg of volume injection. Vehicle consisted in the same volume of Tween 80 and isotonic saline used to dissolve the drugs. Ethanol (3 g/kg, 10 mL/kg volume injection) (Sigma-Aldrich, Milan, Italy) was diluted (37% v/v) with isotonic saline. All drugs were administered intraperitoneally (IP). Based on previous literature the doses of DZP, FMZ and ethanol were selected in agreement with, respectively, Löw et al. (2000), Razavi et al. (2017) and Slater et al. (2016).

*Elevated Plus Maze*

The EPM (Figure 8) consisted of a central platform (5 × 5 cm, W × L), two open arms (5 × 25 cm, W × L) aligned perpendicularly to two closed arms (5 × 25 cm, W × L) at a height of 40 cm from the ground. For these experiments two different protocols, 1 and 2, have been followed. In protocol 1 mice were selected randomly and assigned to one of the following groups: VEH (vehicle 10 mL/kg) (n=12), DF 0.05 (DF 0.05 mg/kg) (n=16), DF 0.25 (DF 0.25 mg/kg) (n=16), DF 2 (DF 2 mg/kg) (n=12), DZP (DZP 2 mg/kg) (n=10). After vehicle or drugs were administered, mice were put back in their home cages and 30 min later were tested individually by being placed in the centre of the maze facing an open arm. The spontaneous activity of mice was automatically recorded for 5 min during which the experimenter left the room. After each experiment, the apparatus was cleaned with 10% denatured ethanol allowing some time for evaporation before testing the following mouse. In protocol 2 mice were selected randomly and assigned to one of the following groups: VEH/VEH (vehicle/vehicle) (n=12), FMZ/VEH (flumazenil 10 mg/kg/vehicle) (n=10), VEH/DF 2 (vehicle/DF 2 mg/kg) (n=12), FMZ/DF (flumazenil 10 mg/kg/DF 2 mg/kg) (n=16). After 15 min from pre-treatment (VEH or FMZ), mice were administered VEH or DF 2, and 30 min

later mice were tested following protocol 1. In agreement with Lister (1987) and Pellow and File (1986), data were calculated as the percentage of time spent on the open arms (time on open arms divided by time on open arms + time in closed arms) and, in agreement with Rodgers and Johnson (1995), as the percentage of open arm entries (number of entries into open arms divided by number of entries into open arms + number of entries into closed arms). The analysis was performed by an experimenter blind to treatments on the videos recorded during the tests. Mice that fell from the maze have been discarded from the analysis.

*Motor Coordination*

Motor Coordination has been measured with the Static Rods (Figure 9) test according to Deacon (2013) with minor changes. In particular, we tested motor coordination in four static rods of progressively narrower diameters (25, 20, 15 and 10 mm). Mice were selected randomly and assigned to one of the following groups: VEH (n=11), DF 0.05 (n=9), DF 0.25 (n=10), DF 2 (n=10) and DZP (n=9). Vehicle or drugs were administered and 30 min later each mouse was tested individually following Deacon (2013).

*Novel Object Recognition*

The effects of DF on anterograde memory have been evaluated by the NOR test (Figure 10) according to Costa et al. (2014). Mice were selected randomly and assigned to one of the groups: VEH (n=9), DF 0.05 (n=8), DF 0.25 (n=9), DF 2 (n=9) and DZP (n=8). Mice of VEH, DF or DZP groups were administered 30 min before the acquisition phase. On the test day, the time spent exploring the novel and the familiar object was recorded and subsequently analysed in blind. Data are expressed as time spent exploring the novel object out of total exploring (novel + familiar) time.

*Place Conditioning*

The apparatus consisted of two rectangular Plexiglas boxes (48L × 20W × 30H cm) separated by a guillotine door, placed in a sound-proof room with a constant light of 37.5 Lux (ELD 9010 Luxmeter, Eldes Instruments, Italy) provided by a 40 W lamp placed above each compartment. Different visual and tactile cues distinguished the two compartments: vertically striped black and white walls and white smooth floor for one compartment (A), and horizontally striped black and grey walls and fine grid floor for the other compartment (B). The spontaneous preference was randomly distributed between compartments (55% for compartment A and 45% for compartment B) (one-way ANOVA: $F(3.36)=0.32$). The experiment consisted of three phases. During the first phase (pre-test, day 1), the guillotine door was kept raised and each mouse was placed randomly in one compartment and given access to both compartments of the apparatus for 15 min (900 s). The time spent in one compartment was recorded and taken as indication of spontaneous preference. During the second phase (conditioning, days 2–5), mice of the experimental groups VEH (n=10), DF 0.05 (n=10), DF 0.25 (n=10) and DF 2 (n=10) were administered either vehicle or DF and returned to their home cages for 30 minutes. At the end of this period mice were exposed for 30 min to the given compartment. On the same day, 8 h later, mice of all groups were administered vehicle and, after 30 min, exposed to the opposite compartment. The sequence of administrations of mice of DF groups was alternated in the following days so that on consecutive days mice never received DF and vehicle administrations in the same order. During the third phase (post-conditioning test, day 6), 24 h after the last conditioning session, the guillotine door was kept raised and the time spent by each mouse in the drug-paired compartment out of 15 min was recorded (Figure 4). The conditions of the post-conditioning test were identical to those of the pre-conditioning test. Performances at the pre- and post-conditioning tests were videotaped and subsequently analysed in blind. A statistically significant difference between the time spent during pre- and post-conditioning tests (side preference shift) of the drug group with respect to that of the vehicle group was taken as indication of the development of place conditioning.

*Loss of Righting Reflex*

The interactions between ethanol and DF or DZP were tested through the evaluation of the ethanol-induced LORR (Figure 11) in 88 adult CD1 mice following Correa et al. (2001), with some modifications, and Slater et al. (2016). Mice were casually selected and assigned to the following experimental groups: VEH (n=17), DF 0.05 (n=19), DF 0.25 (n=22), DF 2 (n=18) and DZP (n=12). After habituation in the experimental room, mice were administered DF or vehicle and put back in their home cage for 25 min. At this time, ethanol (3 g/kg) was administered, and mice were placed individually in an empty plexiglass cage in order to be evaluated. The time necessary to lose the righting reflex after ethanol administration was measured and considered as 'latency' (max 20 min). If ethanol succeeded in inducing the LORR, the animal was instantly placed supine on a V-shaped plastic apparatus (with the two faces forming a 45° angle) (4 × 4 × 10 cm, H × W × L). Each mouse was carefully monitored and the length of the LORR was measured (max 300 s). The effect was considered over if the mouse raised its back and touched the V-shaped apparatus with its paws. The percentage of animals in which ethanol succeeded in inducing the LORR was also measured.

*Statistical analyses*

The statistical analyses were performed using StatSoft (v. 8.0, StatSoft Inc., Tulsa, OK, USA). One-way ANOVA, followed by Newman–Keuls post hoc test, was applied in the EPM and the NOR tests to determine significant effects of treatments with DF or DZP and to verify the absence of statistical differences among the spontaneous preferences in the Place Conditioning experiments. Two-way ANOVA, followed by Duncan's post hoc test, was used, in agreement with Gonzalez et al. (1996), to verify the effects of pre-treatment (FLM) and treatment (DF) and their interaction in the EPM tests; repeated measures two-way ANOVA was applied on the Place Conditioning experiments to assess the effects of treatment. Non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparisons,

and Fisher's exact test were applied in the Static Rods and LORR tests. Effects were considered statistically significant when $p<0.05$.

### *4.3 Results*

*DF exerts DZP-like anxiolytic effects blocked by flumazenil*

Figure 12(a) represents the time spent in open arms during the EPM test. One-way ANOVA revealed a significant effect of treatment ($F(4, 61)=12,51$, $p<0.0001$). Post-hoc analysis using Newman–Keuls multiple comparison test revealed that DF, at 0.25 and 2 mg/kg, significantly and dose-dependently increases the time spent in open arms, in comparison to VEH and DF 0.05 groups. Also, as expected, DZP significantly increases the time spent in open arms in comparison to the VEH group. Interestingly, Newman–Keuls test also revealed that DF 2 did not differ significantly from the DZP group on this measure. Figure 12(b) represents the effects of treatments on number of entries in the open arms. One-way ANOVA revealed a significant effect of treatment ($F(4, 61)=6.41$, $p<0.0001$). Post hoc analysis using Newman–Keuls multiple comparison test revealed that DF 0.25 and 2 significantly increases the time spent in open arms, in comparison to VEH and DF 0.05. Intriguingly, both doses did not differ from the DZP group ($p>0.05$). Figure 12(c) represents the number of entries in closed arms. One-way ANOVA failed to reveal any significant effect of treatment. Figures 12(d) and 12(e) show the effects of pre-treatment with FMZ on DF 2-dependent time spent in open arms and number of entries in open arms, respectively. Two-way ANOVA revealed significant effects of pre-treatment ($F_{time}(1,46)=7.14$, $p<0.0001$; $F_{entries}$ $(1,46)=7.41$, $p<0.0001$) and treatment ($F_{time}(1,46)=6.02$, $p<0.0001$; $F_{entries}(1,46)=22.45$, $p<0.0001$) and a significant pre-treatment by treatment interaction ($F_{entries}(1.46)=5.03$, $p<0.0001$); Duncan's post hoc test revealed that pre-treatment with FMZ fully reversed the anxiolytic effect of DF ($p<0.05$ for FMZ/DF vs VEH/DF on both time and entries). Two-way ANOVA, moreover, failed to reveal any significant effect of pre-treatment or treatment on number of closed arms entries (Figure 12(f)).

*Unlike DZP, DF does not impair motor coordination*

Figure 13 shows the effects of treatment with DF and DZP on Orienting (a) and Total Transit (b) Time at the Static Rods test. Non-parametric Kruskal–Wallis test revealed that the treatments have a significant effect on both parameters on the 25, 20 and 15 mm diameter rods ($p<0.05$). Post hoc analysis using Dunn's multiple comparison test revealed that DF at all doses tested is devoid of significant effect on performances on each rod ($p>0.05$) as compared with the VEH group; in contrast, DZP, compared with the VEH group, significantly increases orienting time on the 25, 20 and 10 mm rods ($p<0.05$) and total transit time on the 25 and 20 mm rods ($p<0.05$).

*Unlike DZP, DF does not impair mnemonic performances*

Figure 14 shows the effects of the treatment with DF and DZP on the performance of mice at the NOR test. Two-way ANOVA revealed a significant effect of object ($F(4,77)=137,76$; $p<0.0001$) but not treatment ($F(4,77)=0.001$) and a significant treatment by object interaction ($F(4,77)=13,55$; $p<0.0001$). Post hoc analysis using Newman–Keuls multiple comparison test revealed that VEH- and DF-treated groups spent significantly more time exploring the novel object in comparison to the familiar objects ($p<0.05$). Accordingly, novel object exploration time of DZP treated group was significantly lower in comparison to VEH- and to DF-treated groups ($p<0.05$).

*DF fails to elicit Place Conditioning*

Figure 15 shows the time spent in the drug-associated compartment by mice of the VEH- and DF (0.05, 0.25 and 2 mg/kg)- treated groups during conditioning. Repeated measures two-way ANOVA revealed that treatment with DF, at every dose tested, was devoid of significant effects on place conditioning ($p>0.05$).

*Unlike DZP, DF fails to potentiate ethanol-induced LORR*

Figure 16 shows the effects of treatment with DF and DZP on duration of (a) and latency to (b) ethanol (3 g/kg)-induced LORR. Non-parametric Kruskal–Wallis test revealed that medians are significantly different ($p<0.001$). Post-hoc analysis using Dunn's multiple comparison test indicates that treatment with DF, at every dose tested, has no significant effect with respect to the VEH group ($p>0.05$). In contrast, DZP significantly increased the time of, and decreased latency to, LORR with respect to VEH- and DF 0.05, 0.25 and 2 mg/kg-treated groups ($p<0.05$). Moreover, Figure 16(c) shows that in 100% of DZP-treated mice ethanol succeeded in potentiating the LORR ($p<0.05$), while the percentage of DF-treated mice does not statistically differ from that of VEH ($p>0.05$).

### *4.4 Discussion*

Evidence of anxiolytic properties (Pratte et al., 2014; Kaur and Kaur, 2017; Kaur et al., 2017) and of a GABA mimetic profile (Bassareo et al., 2019; Bhattarai et al., 2010; Mehta et al., 1991; Orrù et al., 2014) of *WS* extracts is consistently present in the literature, although no individual constituents of *WS* have yet been recognised as responsible for these effects. The present study provides the first behavioural pharmacological characterization of DF, a long alkyl-chain ferulic acid ester recently isolated from *WS* and found able to enhance the $GABA_AR$ inhibitory postsynaptic currents in rat hippocampal acute slices with an IC50 value of 7.9 μM (Sonar et al., 2019). The present multilevel behavioural evaluation was conducted in vivo in male CD-1 mice, in order ascertain whether DF is endowed with an anxiolytic profile. The results from the EPM test disclose that DF exerts anxiolytic effects that appear mediated through DF's modulation of the $GABA_AR$ complex activity by interacting with the BDZ binding site since the BDZ competitive antagonist, FMZ (10 mg/kg), completely blocks DF's effects at the EPM (Figure 12). Moreover, since the number of closed arms entries in this model measures the effects of treatments on locomotor activity and exploration (Rodgers and Johnson, 1995), the present results also reveal that DF is devoid of both inhibitory or stimulatory properties on this behavioural component, thus

further pointing out its mere anxiolytic action as revealed in the EPM paradigm. Remarkably, BDZs are known to carry negative side effects on motor coordination, cognition and motivation (Roth et al., 1984; Tan et al., 2011). Hence, we also extended our investigations on DF's pharmacological profile to assess whether, at anxiolytic-exerting doses, it could also carry the typical BDZ's adverse effects. Strikingly, we found that DF, unlike DZP, lacks the property of impairing motor coordination (Figure 13) and anterograde memory (Figure 14). Moreover, also in contrast with DZP's ability to exert reinforcing properties in the place conditioning procedure (Acquas et al., 1989; Spyraki et al., 1895; Tzschentke, 1998) and to potentiate the ethanol-induced LORR (Figure 6), DF at full anxiolytic doses fails to elicit place conditioning (Figure 15) and to enhance ethanol's depressant properties in this assay (Figure 16). In the present study, in application of the 3R principle and based on previous literature (Acquas et al., 1989; Spyraki et al., 1895; Tzschentke, 1998), we did not repeat the DZP groups in the place conditioning experiments. A possible explanation to interpret the differences between DF and DZP could be that in spite of their common site of action at the BDZ site of the $GABA_AR$, DF interacts with $GABA_AR$ whose subunits have a different stoichiometry than those responsible for mediating the adverse effects of DZP. In this regard, previous pharmacological and behavioural studies found a correlation between BDZs' effects and $GABA_AR$ $\alpha$ subunit isoforms (Tan et al., 2011), with the outcome of the anxiolytic effect seemingly being mediated mostly by $\alpha_2$-containing $GABA_ARs$ (Löw et al., 2000). Accordingly, we speculate that DF may exert its keen anxiolytic properties by selectively binding to $\alpha_2$-containing $GABA_ARs$, thus avoiding undesired side effects mediated by other $\alpha$ GABAARs subunits (Biggio et al., 2001). Further studies with the model of Fear Conditioning (Curzon et al., 2009) and other models of anxiety (Bailey et al., 2009) will validate this hypothesis. Moreover, functional and electrophysiological studies will also have to be performed in order to explain why DF, unlike DZP, fails to potentiate ethanol's depressant activity (LORR). In conclusion, this study points out, for the first time, a single possible $GABA_AR$/BDZ-acting effector of the anxiolytic properties of WS and, also, suggests the possibility that ferulic acid esters efficiently interact with the $GABA_AR$ to induce BDZs'- like

anxiolytic effects. Overall, DF shows a promising pharmacological profile worth of future studies that suggests it is a safe, selective and anxiolytic compound devoid of critical side effects that could reduce its compliance and manageability.

# 5. CHAPTER 2 - Effects of Docosanyl Ferulate, a constituent of *Withania somnifera* Dunal, on ethanol- and morphine-elicited conditioned place preference and ERK phosphorylation in the accumbens shell of CD1 mice

## *5.1 Introduction*

Drug addiction is defined as the progressive loss of control over drug taking caused by repeated exposures to addictive drugs. This is the consequence of a series of neuroadaptations, occurring in selective neural circuits, which leads to the development of a chronic neuroadaptive disorder characterized by behavioural alterations in which compulsive drug-seeking and high risk of relapse play a critical role (Berke and Hyman 2000; Koob 2006; Koob and Le Moal 2001; Nestler 2001; Volkow and Morales 2015). Ethanol and morphine are two substances that are well-characterized to induce such neuroadaptations. Accordingly, ethanol is one of the most used and abused psychoactive substances worldwide, is a high-risk factor for several multi organ diseases (Axley et al. 2019; Rehm et al. 2017) and is responsible for the potential of alcoholic drinks to trigger their pathological consumption (Abrahao et al. 2017). Morphine is, instead, the lead compound prescribed for the treatment of multiple and diverse chronic painful conditions and although developing addiction is not an issue in this case, a high rate of dependence in those who take morphine chronically has been reported with debilitating side-effects such as constipation and respiratory depression (Benyamin et al. 2008). Notably, although the mechanism of action by which ethanol and morphine may elicit addiction is different, though not fully understood, these drugs share the ability to increase mesolimbic dopamine (DA) transmission (Di Chiara et al. 2004; Bassareo et al. 2019; Bassareo et al. 2021) and induce the phosphorylation and subsequent activation of the ERKs (Ibba et al. 2009; Porru et al. 2020; Rosas et al. 2016; Spina et al. 2015; Valjent et al. 2004), two biochemical indexes critical for addiction-related behavior in laboratory studies (Di Chiara 1999; Di Chiara et al. 2004).

The CPP paradigm (Figure 4), widely used to study the rewarding properties of unconditioned stimuli (drugs, food, sex, etc.), is endowed with great translational impact

due to its face, construct and predictive validity (Tzschentke 2007). The acquisition and the expression of CPP are two critical phases of this paradigm. In particular, the acquisition of place conditioning is grounded on associative learning (Di Chiara et al. 2004) and represents the phase in which the reinforcing properties of an unconditioned stimulus are transferred to the conditioned, otherwise neutral, stimulus; on the other hand, the expression of place conditioning represents the phase in which the reinforcing properties of the unconditioned stimulus, that have been transferred to the conditioned one, are recognized (recalling) by the animals that may hence emit a response toward that stimulus (positive side-preference shift: animals spend, in the environment associated with the unconditioned stimulus, longer time than before conditioning).Thus, in translational perspective, acquisition and expression of CPP, by modelling two distinct critical conditions of the clinical, naturalistic, setting of drug addiction allow to investigate, respectively, the phase in which subjects attribute drug's reinforcing properties to the context (acquisition and/or maintenance of drug-taking) and that in which the conditioned stimulus becomes eventually capable of triggering relapse into drug-taking (expression, reinstatement). This, in turn, makes treatments that may prevent any of these critical phases of drug addiction highly desirable. ERKs are part of the MAPK signalling cascade (Figure 5) and play a central role in signal transduction, neuroplasticity and gene expression (Sweatt 2004) as well as in the behavioral changes underlying the reinforcing processes induced by drugs of abuse (Valjent et al. 2005). ERKs are highly expressed also in reward-related brain areas and their activation through phosphorylation (pERK) occurs after both acute (Acquas et al. 2007; Brami-Cherrier et al. 2005; Ibba et al. 2009; Rosas et al. 2016) or chronic (Berhow et al. 1996; Muller and Unterwald 2004) administration of drugs of abuse. Notably, both acquisition and expression of place conditioning have been strictly associated with the increased activation, by phosphorylation, of ERKs. In particular, ERKs phosphorylation has been shown to be increased, in key brain regions such as those of the mesolimbic system and the extended amygdala, during the acquisition and expression of place conditioning (Gerdjikov et al. 2004; Mazzucchelli et al. 2002; Porru et al. 2020; Salzmann et al. 2003; Valjent et al. 2000; Valjent et al. 2001). Accordingly, several studies have demonstrated that the

pharmacological inhibition of the Mitogen activating ERK Kinase (MEK), the kinase responsible for ERKs phosphorylation (Sweatt 2004), prevents pERK expression and CPP acquisition elicited by several drugs of abuse (Gerdjikov et al. 2004; Lu et al. 2006; Porru et al. 2020; Salzmann et al. 2003; Spina et al. 2010; Valjent et al. 2000; Valjent et al. 2001), including ethanol (Rosas et al. 2017), its metabolite, acetaldehyde (Correa et al. 2012; Spina et al. 2010), and morphine (Lin et al. 2010; Spina et al. 2010). On a similar vein, place conditioning and self-administration studies have shown that increased pERK is strictly associated with the emission of the acquired response (CPP expression) (Mazzucchelli et al. 2002; Porru et al. 2020). Consequently, prevention of ERKs phosphorylation during the post-conditioning test prevents the behavioral outcome (CPP expression) (Rosas et al. 2017; Porru et al. 2020). Thus, increased ERKs phosphorylation appears involved, although with some exceptions (Rosas et al. 2018), not only in the attribution of motivational value to neutral stimuli when paired with the primary effects of addictive substances (acquisition of conditioned responses) (Gerdjikov et al. 2004; Rosas et al. 2018; Valjent et al. 2001) but also in the recognition and recall of drug-conditioned stimuli (expression, i.e. behavioral response to presentation of conditioned stimuli) (Mazzucchelli et al. 2000; Porru et al. 2020). *WS* Dunal (Figure 1) is a medicinal plant originally included in the Ayurveda, the Indian traditional system of medicine, whose central properties extend from anxiolytic and neuroprotective to anti-inflammatory and anti-neurodegenerative (Dar et al. 2015; Maccioni et al. 2018, Singh et al. 2011). Interestingly, *WS's* standardized methanolic root extract, *WSE*, has also been reported, in preclinical rodent models of drug addiction, to prevent the acquisition and the expression of ethanol (Spina et al. 2015)- and morphine (Ruiu et al., 2013)-elicited CPP. Moreover, recent evidence demonstrated that *WSE*, through a $GABA_AR$-mediated mechanism, also prevents the ethanol- and morphine-dependent increases of DA transmission in the rat AcbSh (Bassareo et al. 2019). This evidence, given the role of mesolimbic DA in drug-elicited place conditioning (Acquas and Di Chiara, 1994; Di Chiara et al. 2004; Fenu et al. 2006), suggests a plausible mechanism for *WSE*'s positive effects on motivated behaviours. Based on these premises, given i) the profile of DF (Figure 2) as most active constituent of *WSE* on $GABA_AR$, with anxiolytic properties without sedative,

amnesic, motivational and motor coordination-impairing effects (Sonar et al., 2019), and ii) the importance of the proposed $GABA_AR$-mediated mechanism for *WSE's* actions on mesolimbic DA function (Bassareo et al. 2019), the present study aimed at verifying, whether DF could affect the acquisition and/or the expression of ethanol- and morphine-induced CPP. Moreover, based on the critical connection between CPP and ERKs activation (Gerdjikov et al. 2004; Lu et al. 2006; Mazzucchelli et al. 2000; Porru et al. 2020; Salzmann et al. 2003; Spina et al. 2010; Valjent et al. 2000; Valjent et al. 2001), the study also aimed at verifying whether *WSE*, at a dose (50 mg/kg) at which it prevents acquisition and expression of ethanol (Spina et al. 2015)- and morphine (Ruiu et al. 2013)-elicited CPP, and DF, at a dose (2 mg/kg), at which it shows robust anxiolytic properties with no undesired side-effects (Maccioni et al. 2021), could prevent pERK expression elicited by ethanol (Porru et al. 2020; Rosas et al. 2017) or morphine (Rosas et al. 2016) in the AcbSh.

### *5.2 Materials and Methods*

*Animals*

Adult male CD1 mice (22–24 g, Charles River, Calco, Italy) (n=305) were housed in groups of eight per cage for at least 6 days before the experiments began, under a 12:00/12:00 h light/dark cycle (lights on at 08:00 a.m.) with food (Mucedola Srl, Settimo Milanese, Milan, Italy) and water available *ad libitum*. All the experiments were carried out during the light phase, between 09:00 and 18:00 h. The total number of mice used for CPP and immunohistochemistry experiments was 179 and 126, respectively. All the experimental procedures were performed in accordance with the principles of laboratory animal care, with the guidelines and protocols approved by the European Union (2010/63/UE L 276 20/10/2010) and with the approval of the local committee (authorization number 371/2020-PR). Every possible effort was made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

*Drugs*

Ethanol (EtOH) (Sigma-Aldrich, Milan, Italy) was diluted in saline (NaCl 0.9% w/v) to 20% (v/v) and administered at the dose of 2 g/kg (12.5 ml/kg volume injection). Morphine hydrochloride (MOR) (Franchini Prodotti Chimici Srl, Mozzate, Como, Italy) was dissolved in saline (10 ml/kg volume injection) and administered at the dose of 5 mg/kg. *WSE* (Natural Remedies Pvt Ltd, Bangalore, India) was dissolved in saline and administered at the dose of 50 mg/kg (10 ml/kg volume injection). DF, synthesised (purity >98% by HPLC) according to Sonar et al. (2019), dissolved in Tween 80 (Sigma-Aldrich, Milan, Italy) and suspended in saline, was administered at the dose of 2 mg/kg (10 ml/kg volume injection). Sodium pentobarbital (Pentothal Sodium, MSD Animal Health S.r.l, Italy) was dissolved in saline and administered at the dose of 50 mg/kg. All drugs were administered intraperitoneally (i.p.) at doses in accordance with previous experiments (Ibba et al. 2009; Maccioni et al. 2021; Porru et al. 2020; Rosas et al. 2016; Ruiu et al. 2013; Spina et al. 2015).

*Conditioned Place Preference (CPP)*

The apparatus consisted of two rectangular Plexiglas boxes (48L × 20W × 30H cm) (Figure 4) separated by a guillotine door, placed in a sound-proof room with a constant light of 37.5 Lux (ELD 9010 Luxmeter, Eldes Instruments, Italy) provided by a 40 W lamp placed above each compartment. Different visual and tactile cues distinguished the two compartments: vertically striped black and white walls and white smooth floor for one compartment (A), and horizontally striped black and grey walls and fine grid floor for the other compartment (B). The spontaneous preference was randomly distributed between compartments (49% for compartment A and 51% for compartment B) and did not differ statistically among the experimental groups (table 1).

Table 1

| Experimental group | Spontaneous preference (seconds±SEM/900) | N | One-way ANOVA |
|---|---|---|---|
| Acquisition of ethanol-induced CPP | | | [$F_{(3,52)}$=0.61, p>0.05] |
| Veh/Veh | 379±15 | 12 | |
| DF/Veh | 348±28 | 10 | |
| Veh/EtOH | 379±24 | 16 | |
| DF/EtOH | 389±16 | 18 | |
| Acquisition of morphine-induced CPP | | | [$F_{(3,40)}$=0.08, p>0.05] |
| Veh/Veh | 394±16 | 12 | |
| DF/Veh | 386±20 | 10 | |
| Veh/MOR | 382±20 | 12 | |
| DF/MOR | 385±18 | 10 | |
| Expression of ethanol-induced CPP | | | [$F_{(3,35)}$=0.16, p>0.05] |
| Veh/Veh+Veh | 394±20 | 10 | |
| Veh/Veh+DF | 390±18 | 8 | |
| Veh/EtOH+Veh | 376±27 | 8 | |
| Veh/EtOH+DF | 387±10 | 13 | |
| Expression of morphine-induced CPP | | | [$F_{(3,36)}$=0.03, p>0.05] |
| Veh/Veh+Veh | 394±20 | 10 | |
| Veh/Veh+DF | 391±18 | 8 | |
| Veh/MOR+Veh | 392±16 | 9 | |
| Veh/MOR+DF | 387±17 | 13 | |

Table 1

Average pre-conditioning test time (sec/900±SEM) (spontaneous preference) of the experimental groups of the acquisition and expression experiments.

*CPP Acquisition experiments*

The experiment consisted of three phases. During the first phase (pre-conditioning test, day 1), the guillotine door was kept raised and each mouse was placed randomly in one compartment and given access to both compartments of the apparatus for 15 minutes (900 seconds). The time spent in one compartment was recorded and taken as indication of spontaneous preference. During the second phase (conditioning, days 2-5), mice of the experimental groups (as indicated above) were administered (pre-treatment) either vehicle (Veh) or DF and returned to their home cages for 30 minutes. At the end of this period mice were administered (treatment) either vehicle (Veh) or ethanol (EtOH) or morphine (MOR) and exposed for 30 minutes to the given compartment. On the same day, 8 hours later, mice of all groups were administered veh or DF (pre-treatment) and, after 30 min, immediately after being administered the drug (EtOH or MOR) or Veh (treatment) were exposed to the opposite compartment for 30 minutes. The sequence of the administrations of Veh or DF, as pre-treatment, and of Veh or drug (EtOH or MOR), as treatment, was alternated in the following days so that on consecutive days mice never received Veh or DF (pre-treatment) and Veh or EtOH or MOR (treatment) administrations in the same order. During the third phase (post-conditioning test, day 6), 24 hours after the last conditioning session, the guillotine door was kept raised and the time spent, out of 15 minutes, by each mouse in the drug-paired compartment was recorded. The conditions of the post-conditioning test were identical to those of the pre-conditioning test. Performances at the pre- and post-conditioning tests were videotaped and subsequently analysed in blind. A statistically significant difference between the time spent during pre- and post-conditioning tests (side preference shift) of the drug group with respect to that of the vehicle group was taken as indication of the development of place conditioning.

*CPP Expression experiments*

The general protocol was the same of the one used for the acquisition experiments with two differences: **i)** during conditioning (phase 2) mice were administered only Veh (pre-treatment) and either Veh or EtOH or MOR (treatment) (groups: Veh/Veh and Veh/EtOh or

Veh/MOR) and **ii)** 30 minutes before performing the post-conditioning test (phase 3) mice were administered either Veh or DF (groups: Veh/Veh+Veh, Veh/Veh+DF, Veh/EtOh or Veh/MOR+Veh and Veh/EtOh or Veh/MOR+DF). As for the post-conditioning test of the acquisition experiments, a statistically significant difference between the time spent during pre- and post-conditioning tests (side preference shift) of the drug group with respect to that of the vehicle group was taken as indication of the expression of place conditioning.

*Immunohistochemistry*

Drug-elicited ERKs phosphorylation in the AcbSh is critical for the acquisition of drug-elicited place conditioning (Gerdjikov et al., 2004; Salzmann et al., 2003). The immunohistochemistry experiments of this study have been planned in order to investigate whether *WSE* and DF may prevent the ability of ethanol and morphine to elicit ERKs phosphorylation in the AcbSh in order to allow us to indirectly infer that *WSE* (Spina et al., 2015; Ruiu et al., 2013) and DF's (present study) property to prevent CPP acquisition may be attributed to its ability to affect ethanol- or morphine-elicited ERKs phosphorylation. Thus, since for this technique, animals have to be sacrificed in order to allow processing their brains, distinct cohorts of animals were utilized for these experiments. Mice of different experimental groups were carried in the experimental room and given 1 hour of habituation time. Subsequently, they were administered Veh or *WSE* (50 mg/kg) or DF (2 mg/kg) (pre-treatment). After 30 minutes, mice were administered Veh or EtOH (2 g/kg) or MOR (5 mg/kg) (treatment). Experimental groups consisted, accordingly, in Veh/Veh (n=8), *WSE*/Veh (n=8), Veh/EtOH (n=6) and *WSE*/EtOH (n=6) and Veh/Veh (n=8), *WSE*/Veh (n=8), Veh/MOR (n=8), *WSE*/MOR (n=10) for the experiments performed with vehicle and *WSE* as pre-treatment; Veh/Veh (n=8), DF/Veh (n=7), Veh/EtOH (n=7), DF/EtOH (n=9) and Veh/Veh (n=8), DF/Veh (n=7), Veh/MOR (n=8), DF/MOR (n=10) for the experiments performed with vehicle or DF as pre-treatment. Mice of the ethanol-related experiments were anesthetized, with sodium pentobarbital (50 mg/kg), 15 minutes after the treatment (Ibba et al. 2009; Rosas et al. 2017), whilst subjects of the morphine-related experiments were anesthetized, with sodium pentobarbital (50 mg/kg), 20 minutes after the treatment (Rosas et al. 2016). Under

deep anaesthesia, animals were subjected to transcardial perfusion with 0.9% NaCl followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4). After perfusion, brains were removed and post-fixed for 2 hours in 4% PFA (4°C). Two coronal brain slices (40 μm) of the region of interest were cut on ice-cold PBS with a vibratome (Leica VT1000, Leica, Germany) according to plates 21-23 (approximately from antero-posterior (AP) 1.18 to AP 0.98 mm from bregma) of the Paxinos and Franklin (2001) mouse brain atlas. Sections were then processed under the diaminobenzidine (DAB) technique to quantify neurons positive to the phospho-(44/42) ERK as a marker of neuronal activation. After three rinses in PBS, sections were first incubated for 30 minutes in 1% $H_2O_2$, and then for 1 hour in 3% Bovine Serum Albumin (BSA) (Sigma-Aldrich, Milan, Italy). The incubation with the primary anti pERK antibody (Cell Signaling Technology, Beverly, MA, USA (1:350)) was conducted overnight at 4°C. The following day, after rinsing in PBS, slices were incubated for 1 hour with the biotinylated secondary antibody (1:800). After three rinses, slices were incubated in an avidin biotin peroxidase complex prepared according to the manufacturer's suggestions (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) and a 3-3'-diaminobenzidine solution (10 mg/ml) was added until development of brown staining. Finally, sections were mounted onto glass slides coated with gelatine in Eukitt mounting medium for microscope visualization. Standard control experiments were performed by omission of the primary or secondary antibody and yielded no cellular labeling (data not shown). Images were obtained with an epifluorescence microscope (Axio Scope A1, Zeiss, Germany) connected to a digital camera (1.4 MPixels, Infinity 3-1, Lumenera, Canada). Brain sections immune-stained for pERK were evaluated using a 10X objective lens to acquire two images representing the whole left and right AcbSh. Then, the total number of pERK positive neurons was counted by using the manual particle counting option of ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Analysis was performed in a blinded manner. Since no significant differences in the counts of pERK-positive neurons were found among the two coronal sections of the AcbSh from the same mouse, values obtained from these sections were averaged.

*Statistical analysis*

To determine statistically significant differences between pre-conditioning values of the experimental groups, one-way Analysis of Variance (ANOVA) was applied. To determine the effects of pre-treatment (vehicle or DF) and treatment (vehicle or ethanol or morphine) as well as of their interaction on acquisition of CPP, data were analysed by three-way ANOVAs with pre-treatment and treatment as independent factors (between subjects), and with pre-conditioning and post-conditioning values as a within-subjects factor (repeated measures). All statistical analyses were carried out (StatSoft, v. 8.0, StatSoft Inc., Tulsa (OK), USA) using data from the experimental groups depicted in each figure. Newman-Keuls post hoc analyses also between pre- and post-conditioning times within each conditioning group were undertaken if significant effects were found ($p<0.05$).

pERK-positive neurons/area following each treatment were expressed as the average number of pERK-positive neurons/area of each experimental group and indicated as pERK-positive neurons/area (pERK expression). These values were used for statistical analyses by two-way ANOVAs with pERK-positive neurons/area as dependent variables and with pre-treatment (vehicle or *WSE* or DF) and treatment (vehicle or ethanol or morphine) as independent variables. All statistical analyses were carried out using data from the experimental groups depicted in each figure. Newman-Keuls post hoc analyses were undertaken if significant effects were found ($p<0.05$).

### *5.3 Results*

*Effects of DF on acquisition and expression of ethanol-induced CPP*

Figure 17A shows the effects of pre-treatment with Vehicle (Veh) or DF (2 mg/kg) 30 minutes before the administration of vehicle (Veh) or ethanol (EtOH) and exposure to the associated compartment for 30 minutes. One-way ANOVA revealed that pre-conditioning preference times did not significantly differ between experimental groups ($p>0.05$). Repeated measures three-way ANOVA with preference times (pre- and post-conditioning) as dependent factors, and with pre-treatment (Veh or DF) and treatment (Veh or EtOH) as independent factors, revealed significant effects of time [$F_{(1,52)}=9.40$, $p<0.005$], pre-treatment [$F_{(1,52)}=4.39$,

p<0.05] and treatment [$F_{(1,52)}$=6.58, p<0.05]=confirming=that EtOH stimulates a significant preference shift=(Porru et al. 2020; Spina et al. 2015) and indicating that DF on its own is devoid of conditioning properties (Maccioni et al. 2021) and significantly prevents the acquisition of CPP induced by EtOH (p<0.05). Figure 17B shows the effects of treatment with vehicle (Veh) or DF (2 mg/kg) 30 minutes before the exposure to the two compartments for the post-conditioning test of mice conditioned with (pre-treatment/treatment) Veh/Veh and Veh/EtOH. One-way ANOVA revealed that pre-conditioning preference times did not significantly differ between experimental groups (p>0.05). Repeated measures three-way ANOVA with preference times (pre- and post-conditioning) as dependent factors, and with conditioning treatment (Veh or EtOH) and post-conditioning test treatment (Veh or DF) as independent factors, revealed significant effects of time [$F_{(1,35)}$=9.59, p<0.005] and conditioning-treatment [$F_{(1, 35)}$=7.96, p<0.05], and a significant time by conditioning-treatment interaction [F(1,35)=11.69, p<0.005] but not a significant effect of post-conditioning test treatment [F(1,35)=0.71, p>0.05 confirming that EtOH stimulates a significant preference shift (p<0.05) and that DF fails to prevent this effect: in fact, EtOH-conditioned and DF-treated (30 minutes before the post-conditioning test) mice had a significant shift from 387±10 to 524±47 seconds/900 (p<0.05), underlying that DF fails to affect the expression of EtOH-induced CPP.

*Effects of DF on acquisition and expression of morphine-induced CPP*

Figure 18A shows the effects of pre-treatment with vehicle (Veh) or DF (2 mg/kg), 30 minutes before the administration of vehicle (Veh) or morphine (MOR) and exposure to the associated compartment for 30 minutes. One-way ANOVA revealed that pre-conditioning preference times did not significantly differ between experimental groups (p>0.05). Repeated measures three-way ANOVA with preference times (pre- and post-conditioning) as dependent factors, and with pre-treatment (Veh or DF) and treatment (Veh or MOR) as independent factors, revealed significant effects of time [$F_{(1,40)}$=14.02, p<0.005] and pre-treatment [$F_{(1,40)}$=7.05, p<0.05] and significant time by pre-treatment [$F_{(1,40)}$=5.34, p<0.05] and time by pre-treatment by treatment [$F_{(1, 40)}$=4.85, p<0.05] interactions. Post-hoc analysis using

the Newman-Keuls test confirmed that morphine stimulates a significant preference shift from 382±20 to 547±26 seconds/900 ($p<0.05$) (Ruiu et al. 2013) and that DF is devoid of conditioning properties (Maccioni et al. 2021) and showed that DF significantly prevents the acquisition of CPP induced by morphine ($p<0.05$). Figure 18B shows the effects of treatment with vehicle (Veh) or DF 30 minutes before exposure to the two compartments for the post-conditioning test of mice conditioned with (pre-treatment/treatment) Veh/Veh and Veh/MOR. One-way ANOVA revealed that pre-conditioning preference times did not significantly differ between experimental groups ($p>0.05$). Repeated measures three-way ANOVA with preference times (pre- and post-conditioning) as dependent factors, and with conditioning (Veh or MOR) and post-conditioning test treatments (Veh or DF) as independent factors, revealed significant effects of time [$F_{(1,36)}=11.24$, $p<0.005$] and conditioning-treatment [$F_{(1, 36)}=15.73$, $p<0.005$] and a significant time by conditioning-treatment interaction [$F_{(1,36)}=14.01$, $p<0.001$] but not a significant effect of post-conditioning test treatment [$F_{(1,36)}=1.10$, $p>0.05$ confirming that morphine stimulates a significant preference shift from 392±16 to 519±35 sec/900 ($p<0.05$) and that the post-conditioning test treatment with DF fails to prevent this effect: in fact, morphine-conditioned and DF-treated (30 minutes before the post-conditioning test) mice had a significant shift from 391±17 to 525±28 seconds/900 ($p<0.05$), underlying that DF fails to affect the expression of morphine-induced CPP.

*Effects of WSE on ethanol- and morphine-induced ERK phosphorylation in AcbSh*

Figure 19A shows the effects of pre-treatment with vehicle (Veh) or *WSE* (50 mg/kg) 30 minutes before the administration of vehicle (Veh) or ethanol (EtOH, 2 g/kg) (treatment) on the number of pERK-positive neurons (pERK expression) in the AcbSh. Two-way ANOVA with pre-treatment and treatment as categorical variables and pERK-positive cells counts as dependent variable revealed significant effects of pre-treatment [$F_{(1, 24)}=7.86$; $p<0.05$) and treatment [$F_{(1, 24)}=5.03$; $p<0.05$). Post-hoc analysis using the Newman-Keuls test confirming that ethanol increases the number of pERK-positive neurons (Ibba et al. 2009; Porru et al. 2020) and showing that pre-treatment with *WSE* significantly prevents ethanol-induced

ERK phosphorylation ($p<0.05$). Figure 19B shows the effects of pre-treatment with vehicle (Veh) or *WSE* (50 mg/kg) 30 min before the administration of vehicle (Veh) or morphine (MOR, 5 mg/kg) (treatment) on the number of pERK-positive neurons in the AcbSh. Two-way ANOVA with pre-treatment and treatment as categorical variables and pERK-positive cells counts as dependent variable revealed significant effects of pre-treatment [$F_{(1,30)}=15.04$; $p<0.005$) and treatment [$F_{(1,30)}=7.16$; $p<0.05$] and a significant pre-treatment by treatment interaction [$F_{(1,30)}=10.40$; $p<0.005$]. Post-hoc analysis using the Newman-Keuls test confirmed that morphine increases the number of pERK-positive neurons in the AcbSh (Rosas et al. 2016) and showed that pre-treatment with *WSE* significantly prevents this effect ($p<0.05$). Representative images of these effects are shown in Figure 21.

*Effects of DF on ethanol- and morphine-induced ERK phosphorylation in AcbSh*

Figure 20A shows the effects of pre-treatment with Veh or DF (2 mg/kg) 30 minutes before the administration of vehicle (Veh) or ethanol (EtOH, 2 g/kg) (treatment) on the number of pERK-positive neurons (pERK expression) in the AcbSh. Two-way ANOVA with pre-treatment and treatment as categorical variables and pERK-positive cells counts as dependent variable revealed significant effects of treatment [$F_{(1,27)}=4,95$; $p<0.05$] and a significant pre-treatment by treatment [$F_{(1,27)}=9,58$; $p<0.005$] interaction. Post-hoc analysis using Newman-Keuls test confirmed that EtOH increases the number of AcbSh pERK-positive neurons (Ibba et al. 2009; Porru et al. 2020) and showed that pre-treatment with DF prevents this effect ($p<0.05$). Figure 20B shows the effects of pre-treatment with vehicle (Veh) or DF (2 mg/kg) 30 minutes before the administration of vehicle (Veh) or morphine (MOR, 5 mg/kg) on the number of pERK-positive neurons in the AcbSh. Two-way ANOVA with pre-treatment and treatment as categorical variables and positive cells counts as dependent variable revealed significant effects of treatment [$F_{(1,28)}=7.97$; $p<0.05$] and a significant pre-treatment by treatment [$F_{(1,28)}=6.62$; $p<0.05$] interaction. Post-hoc analysis using Newman-Keuls test confirmed that morphine increases the number of AcbSh pERK-positive neurons (Rosas et al. 2016) and showed that pre-treatment with DF significantly prevents this effect ($p<0.05$). Representative images of these effects are shown in Figure 21.

### *5.4 Discussion*

Previous studies have shown the property of *WSE* to prevent both ethanol (Spina et al. 2015)- and morphine (Ruiu et al. 2013)-elicited acquisition of CPP as well as both ethanol- and morphine-elicited CPP expression, i.e. the ability of environmental stimuli, conditioned to both ethanol (Spina et al. 2015) and morphine (Ruiu et al. 2013), to determine a positive side-preference shift (CPP expression) at the post-conditioning test. In fact, due to the failure of classical pharmacological approaches (Lu et al. 2009) to treat drug addiction in humans, the scientific interest on the application of phytomedicine and natural remedies for the treatment of drug addiction has increased. In particular, the efficacy of *WSE* in pre-clinical models of drug addiction is strongly supported by over a decade of research (Gupta and Rana 2008; Kasture et al. 2009; Kulkarni et al. 1997; Peana et al. 2014; Ruiu et al. 2013; Spina et al. 2015; Bassareo et al. 2019). Thus, as a follow-up of previous studies from our laboratory, the present investigation was aimed at i) characterizing further the potentially beneficial application of *WSE* to counteract the ability of acutely administered ethanol (Ibba et al. 2009; Porru et al. 2020; Porru et al. 2021; Rosas et al. 2017) and morphine (Rosas et al. 2016) to increase ERKs phosphorylation in the AcbSh and ii) establishing whether *WSE*'s active compound, DF (Maccioni et al. 2021; Sonar et al. 2019), may be responsible for such effects as well as for *WSE*'s effects on acquisition and expression of ethanol- and morphine-elicited CPP. The results of the present behavioral experiments confirm that ethanol (Spina et al. 2015) and morphine (Ruiu et al. 2013) elicit a significant CPP. The present results also reveal that DF, at the dose of 2 mg/kg, at which was found fully effective in exerting anxiolytic properties, without showing sedative, amnesic and motivational effects (Maccioni et al. 2021), significantly prevents the acquisition (Figures 17A and 18A) but not the expression (figures 17B and 18B) of ethanol- and morphine-elicited CPP. Moreover, these data appear in partial agreement with our previous reports on the effects of *WSE* on acquisition and expression of ethanol- and morphine elicited CPP, suggesting that the $GABA_AR$-mimetic component, represented by DF, is critical for *WSE*'s ability to affect the acquisition but not the expression of CPP elicited by ethanol and morphine. This interpretation is supported by the observation that AcbSh DA has been reported to be

critical for the acquisition, but not the expression, of morphine-elicited CPP (Fenu et al. 2006) and appears overall in agreement with the role played by mesolimbic DA in the associative learning (Di Chiara 1998; Di Chiara and Bassareo 2007) at the basis of CPP acquisition (Di Chiara et al. 2004). Accordingly, *via* a $GABA_AR$-mediated mechanism, *WSE* was reported to significantly suppress the stimulatory actions of both ethanol and morphine on the neuronal firing of VTA DA neurons (Bassareo et al. 2019) and to prevent ethanol- and morphine-mediated increases of AcbSh DA release (Bassareo et al. 2019). The complex relationship between $GABA_AR$ modulators and the reinforcing properties of both ethanol and morphine has been addressed, although with no conclusive results, in the literature. In particular, studies investigating the interactions between $GABA_AR$ modulators and the reinforcing properties of ethanol showed that $GABA_AR$ ligands, both agonists (Hodge et al. 1995) and antagonists (Hodge et al. 1995; June et al. 1998), reduce ethanol self-administration and that $GABA_AR$ antagonists increase ethanol-induced CPP and conditioned taste aversion in mice (Chester and Cunningham 1999).Moreover, in addition to such uncertainty on the role of $GABA_AR$ on the reinforcing properties of ethanol and morphine, these results also suggest that rather than being related to the actions of ethanol and morphine, the involvement of $GABA_AR$ may be related to their critical role in the learning process at the basis of the acquisition of the conditioned response. Furthermore, the present behavioral findings also indicate that other components of *WSE*, besides DF, may be responsible for *WSE*'s ability to affect the expression of drug-induced CPP. This conclusion is fully compatible, in a complementary perspective, with the observation that distinct neural processes and anatomical structures may differentially underlie distinct phases of drug-elicited place conditioning (Bardo 1998; Tzschentke 2007). The results of the present study also confirm that both ethanol (Porru et al. 2020; Porru et al. 2021; Rosas et al. 2017) and morphine (Rosas et al. 2016; Valjent et al. 2004) activate ERKs phosphorylation in the AcbSh of CD1 mice and show for the first time that both *WSE* (50 mg/kg) and DF (2 mg/kg) are able to prevent these increases. The ability of WSE and DF to prevent ERKs phosphorylation in the AcbSh upon ethanol or morphine administration was assessed, in distinct cohorts of animals, in order to allow to infer that WSE and DF may be able to prevent the acquisition of ethanol- or

morphine-elicited CPP as a consequence of their ability to prevent ethanol- or morphine-elicited ERK phosphorylation upon their acute administration. Thus, while we acknowledge that this evidence is indirect since obtained from mice that had not conditioning, our suggestion that WSE and DF may be preventing the acquisition of ethanol- or morphine-elicited CPP by preventing ERKs phosphorylation in the AcbSh is also supported by the critical role of pERKs in associative learning (Gerdjikov et al., 2004; Marotta et al., 2014; Salzman et al., 2003). Moreover, these findings appear overall in agreement with the observation that activated ERKs play a critical role in the conditioned approach properties of drugs as assessed in the place conditioning paradigm (Gerdjikov et al. 2004; Lu et al. 2006; Rosas et al. 2018; Salzmann et al. 2003; Spina et al. 2010; Valjent et al. 2000; Valjent et al. 2001) but also with the ability of *WSE*, *via* a $GABA_AR$-mediated mechanism, to control ethanol- and morphine-stimulated AcbSh DA transmission (Bassareo et al. 2019) as well as with the role of DA in ethanol (Ibba et al. 2009)- and morphine (Rosas et al. 2016)-elicited ERK phosphorylation in the AcbSh. However, while all this reasoning applies coherently to the recognition of the mesolimbic dopaminergic system in place conditioning (Di Chiara et al., 2004; Tzschentke, 2007), we also acknowledge that other brain areas, such as the hippocampus (Bagherpasand et al. 2019; Zhang et al. 2016) as well as pERK expression therein (Bagherpasand et al. 2019; Zhang et al. 2016), may be critically responsible for the acquisition of place conditioning. The relationship between $GABA_AR$ and ERKs phosphorylation can also be interpreted in light of the observation that a putative phosphorylation site for ERKs was found in almost all known alpha subunits of the $GABA_AR$, including the ubiquitously expressed alpha1 subunit (Bell-Horner et al. 2006). Interestingly, this study demonstrated that this site is functional and that pERK acts as a negative $GABA_AR$ modulator as its inhibition, through pharmacological inhibition of MEK, results in an amplification of $GABA_AR$ currents in HEK293 cells (Bell-Horner et al. 2006). Hence, the relationship between pERK and $GABA_AR$ might be bidirectional, as $GABA_AR$ agonists lead to a decrease in pERK expression and reduction of pERK expression by MEK inhibition, in turn, induces an increase of $GABA_AR$-mediated currents. Overall, the previous (Ruiu et al. 2013; Spina et al. 2015) and present data on *WSE* support the view of selective

products of phytomedicine and natural remedies as useful strategies for the treatment of brain disorders as well as for further understanding the underpinning subcellular mechanisms. The relevance of these findings comes not only from this observation, but also because DF's data contribute significantly to characterize the relationship between *WSE*, pERK, two distinct phases of the place conditioning paradigm and $GABA_AR$ pointing to DF as a potential pharmacological agent for the management of drug addiction. In fact, DF already resulted a promising molecule in its first behavioural characterization, in which showed an anxiolytic activity comparable to the $GABA_AR$ positive modulator, DZP, (Maccioni et al. 2021; Nutt and Blier 2016), however combined with the lack of undesired motor and mnemonic effects, of addictive potential as well as of the DZP's property to potentiate ethanol's depressant central effects (Maccioni et al. 2021). In conclusion, these results may support the suggestion of the suitability of both *WSE* and DF as strategies for the management of distinct phases of drug addiction, the establishment of associative memories (modelled by the CPP acquisition) and the triggering of drug-seeking by contextual conditioned stimuli (modelled by CPP expression). This suggestion is further supported on one hand by the observation that both *WS* (Dar et al. 2015) and DF (Maccioni et al. 2021) present a robust anxiolytic profile and, on the other hand, by the observation that anxiety disorders and drug addiction may co-occur at high rates (Smith and Book 2008). Hence, the anxiolytic profile and the ability to prevent ethanol- and morphine-elicited CPP may truly be useful in the development of an efficient therapeutical strategy, especially considering the lack of undesired effects. Additional behavioural and biochemical studies will have to be performed to characterize further their suitability for the treatment of drug addiction.

## 6. CHAPTER 3. DF interferes with the formation of salsolinol in the pVTA and prevents ethanol-dependent effects on mesolimbic dopaminergic transmission

### *6.1 Introduction*

Ethanol, key ingredient of alcoholic drinks, is one of the most used and abused psychoactive substances worldwide and underlies the potential of alcoholic drinks to trigger their abnormal/heavy consumption (Abrahao et al., 2017). In addition, ethanol is a risk factor for other serious illnesses including cancer and liver and cardiovascular diseases (Rehm et al., 2017; Axley et al., 2019). The molecular mechanism by which ethanol may trigger its excessive ingestion has been the subject of intense research (Koob, 2004; Belmer et al., 2016; Koob and Volkow, 2016). Several studies demonstrated that ethanol's reinforcing properties are mediated by actions on the mesolimbic DA system (Figure 3) (Di Chiara et al., 2004; Howard et al., 2008; Bassareo et al., 2017, 2019; Volkow et al., 2017; Wise and Robble, 2020), but the underlying mechanism and molecular target have remained not fully understood. However, a pivotal role is recognized to DA neurons in the posterior, but not anterior, VTA (pVTA) (Rodd et al., 2004; Hauser et al., 2011; Ding et al., 2012) and their projections (Ungerstedt, 1971) to the AcbSh Di Chiara et al., 2004). These neurons are a key component of a brain motivation circuit activated by addictive drugs (Di Chiara et al., 2004; Volkow et al., 2017; Wise and Robble, 2020). Ethanol is no exception to this general framework, and the role of ethanol metabolism in the pVTA, in this regard, has been emphasized. Accordingly, already in the early 1950s, acetaldehyde, the main ethanol's metabolite, was suggested as able to mediate ethanol's reinforcing effects (Chevens, 1953). This hypothesis generated a significant body of research aimed at characterizing the role of ethanol metabolism in its central effects (Karahanian et al., 2011; Correa et al., 2012), in particular with reference to its ability to activate the mesolimbic DA system (Correa et al., 2012; Söderpalm and Ericson, 2013; Israel et al., 2015). Likewise, this evidence suffers a lack of general consensus (Söderpalm and Ericson, 2013) and of a specific molecular mechanism (Israel et al., 2015; Peana et al., 2017b, 2019). This notwithstanding, the ability of ethanol to stimulate DA

neurons in the pVTA (Gessa et al., 1985), activate DA transmission in the AcbSh (Howard et al., 2008; Bassareo et al., 2017), and elicit DA-mediated locomotor activity (Carlsson et al., 1972; Sánchez-Catalán et al., 2009) has been mechanistically related to ethanol's main metabolite, acetaldehyde. Such role of acetaldehyde has been demonstrated by two independent lines of evidence: first, acetaldehyde administration reproduces, at lower doses than ethanol, DA-dependent central effects (Spina et al., 2010; Correa et al., 2012); second, inhibition of acetaldehyde production, by blockade of peripheral and central ethanol metabolizing enzymes (Spivak et al., 1987; Pastor et al., 2002; Peana et al., 2017a) or by acetaldehyde sequestration (Martí-Prats et al., 2013), prevents both neurochemical and behavioral DA-mediated effects of ethanol. The mechanism(s) by which acetaldehyde exerts such effects is unknown (Peana et al., 2017b). Notably, acetaldehyde has a very short half-life and is highly reactive with molecules such as DA. Indeed, although an enzymatic mechanism has also been claimed (Chen et al., 2018), acetaldehyde and DA can spontaneously generate a condensation product, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline or salsolinol (Figure 22), a molecule undetectable, under control conditions, in the VTA. This led to renewed interest in ethanol's secondary metabolites (Davis and Walsh, 1970; Davis et al., 1970; Rodd et al., 2008; Hipólito et al., 2012; Deehan et al., 2013; Israel et al., 2015; Quintanilla et al., 2016). Intriguingly, salsolinol, administered either systemically or locally in the pVTA, shares with ethanol and acetaldehyde a spectrum of neurochemical and behavioral DA-mediated effects (Correa et al., 2012; Hipólito et al., 2012; Deehan et al., 2013). Moreover, salsolinol is self-administered by rats in the pVTA (Rodd et al., 2008), and its pVTA administration promotes voluntary binge-like ethanol intake (Quintanilla et al., 2016). Notwithstanding, to date, the hypothesis of ethanol as the prodrug of salsolinol for the activation of the mesolimbic DA system has not been directly addressed in vivo (Polache and Granero, 2013; Peana et al., 2016). The present study aimed at challenging this hypothesis. To this end, we applied in vivo brain microdialysis to freely behaving rats implanted two microdialysis probes, one in the pVTA and another in the AcbSh of either the same side (ipsilateral) or of the opposite side of the brain (contralateral). We hypothesized that the systemic administration of a dose of ethanol known to result in

pharmacologically and clinically relevant blood ethanol concentrations (BEC) (Gill et al., 1986; Majchrowicz, 1975; Nurmi et al., 1994) could result in pharmacologically meaningful salsolinol concentrations in the pVTA and in salsolinol-dependent increases of DA release in the AcbSh. In fact, since the prevailing VTA–AcbSh projections are ipsilateral (Ikemoto, 2007; Breton et al., 2019) with only approximately 5% of midbrain neurons projecting contralaterally (Jaeger et al., 1983; Geisler and Zahm, 2005), we planned to explore the impact of preventing the conversion of ethanol into salsolinol only in the pVTA of one side (either left or right). We reasoned that applying by reverse dialysis in the pVTA of one side either an acetaldehyde-sequestering agent (thus reducing acetaldehyde's availability) (Martí-Prats et al., 2013) or a catalase inhibitor (thus preventing catalase-mediated oxidation of ethanol into acetaldehyde (Pastor et al., 2002) or a DA D2/D3 receptor agonist (thus reducing synaptic availability of DA) (Kohl et al., 1998) would prevent salsolinol formation and hence ethanol's effects on DA release in the ipsilateral, but not in the contralateral, AcbSh. Finally, based on the evidence that both the neurochemical and the behavioral pVTA-dependent effects of ethanol and salsolinol (Hipólito et al., 2011; Xie et al., 2012) are mediated by μ opioid receptors, we tested the hypothesis that ethanol-derived salsolinol in the pVTA increases DA release in the AcbSh via a mOR-mediated mechanism. After having investigated this complex mechanism, in line with the main focus of this thesis, we verified whether DF could also be active on the formation of salsolinol and, in this way, prevent the increase in the DA traffic of the AcbSh. In fact, the most likely candidate mechanism of action by which *WSE* impairs ethanol-induced acquisition of CPP (Spina et al., 2015) is represented by its ability to affect ethanol-elicited increase of the spontaneous firing rate of VTA DA neurons as well as of phasic DA transmission in the AcbSh. This hypothesis was confirmed in 2019 by Bassareo and colleagues who demonstrated that *WSE* was indeed capable of the above-mentioned neurochemical and electrophysiological effect via a $GABA_AR$-mediated mechanism of action (Bassareo et al., 2019). Hence, considering that DF resulted the most active, amongst the *WSE*-isolated compounds, on the $GABA_AR$ complex (Sonar et al., 2019), it was reasonable to hypothesize an activity of this molecule on ethanol-induced DA transmission in the AcbSh. Moreover, in light of the demonstration that

ethanol elicits AcbSh DA transmission through a newly generated salsolinol-dependent mechanism (Bassareo et al., 2021) it was worth verifying whether DF may also affect the ability of ethanol to elicit DA transmission in the AcbSh by impairing salsolinol formation in the VTA.

### *6.2 Materials and methods*

*Animals*

Male Sprague–Dawley rats (275–325 g) (Envigo, Desio, Italy) were used for the experiments. Animals had access to water and standard food (Stefano Morini, S. Polo D'Enza, RE, Italy) ad libitum and were handled throughout the experimental procedures in accordance with the guidelines for the care and use of experimental animals of the European Community Council (2010/63/UE L 276 20/10/2010) and with Italian law (DL 04.03.2014, N 26; Authorization n 1177/2016). Every effort was made to minimize suffering and reduce the number of animals used.

*Surgery*

Beginning 3 days before surgery, rats were handled once daily for 5 min to habituate them to the experimental procedures [intraperitoneal (i.p.) and intragastric (i.g.) administrations]. Under deep anesthesia by equitesin (0.97 g pentobarbital, 4.25 g chloral hydrate, 2.1 g MgSO4, 42.8 ml propylene glycol, 11.5 ml 90% ethanol/100 ml; 5 ml/kg i.p.) (Bassareo et al., 2019), vertical microdialysis probes were stereotaxically implanted in the pVTA and in the AcbSh using the following coordinates according to Paxinos and Watson (1998): AP: –5.8 mm and ML: +/- 0.5 mm from bregma and DV: –8.0 mm from dura, for the pVTA; AP: 1.8 mm and ML: +/- 1 mm from bregma and DV: –7.6 mm from dura, for the AcbSh. After surgery, rats were housed in individual hemispheric cages under the same standard conditions, left undisturbed for at least 24 h, and fed with 20 g of standard chow. Water was available ad libitum throughout, except during the microdialysis experiments.

*Probe Preparation*

Vertical dialysis probes (Figure 23) (dialyzing portion: 1.5 mm; outer diameter: 300 mm) were prepared with AN69 fibers (Bassareo et al., 2019) (Hospal-Dasco, Bologna, Italy) for both the pVTA and the AcbSh. In the case of pVTA, however, we acknowledge that such length of the dialyzing portion may have extended dorsally outside the anatomical border of the targeted area.

*Drugs and Treatments*

Ethanol, diluted at the concentration of 20% (v/v) in tap water (vehicle), was administered intragastrically (i.g.) at the dose of 1 g/kg/10 ml. (+/-)-Salsolinol (Santa Cruz Biotechnology Inc., Dallas, TX, United States) was dissolved in normal Ringer (see below) to 10 nM and used in place of normal Ringer to perfuse the microdialysis probe in the pVTA. D-penicillamine (DP), an acetaldehyde-sequestering agent; 3-amino-1,2,4-triazole (3AT), a non-competitive catalase inhibitor; quinpirole, a dopamine D2/D3 receptor agonist; and naltrexone, a m opioid receptor antagonist, were dissolved in normal Ringer at 75 mM (DP), 1 nM (3AT and naltrexone), and 2.5 mM (quinpirole) and were used in place of normal Ringer to perfuse the microdialysis probe in the pVTA of one side (either left or right) starting 30 min before the administration of vehicle or ethanol. pVTA perfusion with salsolinol, DP, 3AT, quinpirole, or naltrexone was maintained until the end of the microdialysis experiment. Ethanol, DP, 3AT, quinpirole, and naltrexone were purchased from Sigma-Aldrich, Milan, Italy. The dose of ethanol (Howard et al., 2008; Bassareo et al., 2019) and the concentrations of salsolinol (Hipólito et al., 2009, 2011), DP (Orrico et al., 2017), 3AT (Rodd et al., 2005; Melis et al., 2015), quinpirole (Rodd et al., 2004; Hauser et al., 2011), and naltrexone (Latagliata et al., 2014) for pVTA reverse dialysis were selected based on previous literature. DF, synthesised (purity >98% by HPLC) according to Sonar et al., (2019), dissolved in Tween 80 (Sigma-Aldrich, Milan, Italy) and suspended in saline, was administered i.p. at the dose of 2 mg/kg (5 ml/kg volume injection) 30’ before the oral-treatment with ethanol or vehicle.

*Microdialysis Experiments*

On the experiment day, in awake and freely moving animals, pVTA and AcbSh probes were connected to an infusion pump and perfused with normal Ringer solution (in mM: 147 NaCl, 4 KCl, 2.2 CaCl2) at a constant flow rate of 1 ml/min, and when for technical reasons this could not be guaranteed, the experiment was dropped. Dialysate samples (10 ml) were injected without purification into a high-performance liquid chromatograph (HPLC) equipped with a reverse-phase column (LC-18 DB, 15 cm, 5 mm particle size, Supelco-Waters, Milford, MA, United States) and a coulometric detector (ESA-Coulochem II, Bedford, MA, United States) to simultaneously quantify salsolinol and DA (Figure 24). The first electrode of the detector was set at C125 mV (oxidation) and the second at –175 mV (reduction). The composition of the mobile phase was as follows (in mM: 50 NaH2PO4, 0.1 Na2-EDTA, 0.5 n-octyl sodium sulfate, and 15% (v/v) methanol, pH 5.5 obtained adding Na2HPO4). Under these conditions, the sensitivity of the assay for salsolinol and DA in the pVTA and DA in the AcbSh was 5 femtomoles (fmol)/sample). DA concentration in pVTA dialysates was often erratic due to its value often below the detection limit of our analytical systems, and for this reason, it was not assayed. Salsolinol was undetectable in AcbSh dialysates, both under basal conditions and after administration of ethanol, in agreement with the lack of catalase's expression in AcbSh (Zimatkin and Lindros, 1996). Every subject was utilized for only one microdialysis experiment. Salsolinol and DA data were calculated and plotted as the average SEM of the same time points of subjects from each experimental group, before and after changes of perfusion fluid and/or systemic administrations.

*Histology*

At the end of the microdialysis experiment, rats were anesthetized as previously reported (Bassareo et al., 2019), probes were removed, and brains were kept in a 4% (w/v) formaldehyde solution for at least 1 week and successively cut with a vibratome in serial coronal slices oriented according to the rat brain atlas of Paxinos and Watson (1998). The

location of the probes was reconstructed and referred to the rat brain atlas plates (Paxinos and Watson, 1998; Figure 25). Data from three rats were excluded for probe misplacement.

*Blood Ethanol Concentration*

One hour after vehicle or ethanol administration to rats of separate experimental groups, rats were deeply anesthetized and sacrificed for trunk blood sample (2 ml) collection into heparinized (2,000 UI/2 ml) centrifuge tubes. pVTA-salsolinol and AcbSh-DA data from these animals are not shown (N = 8). Quantitative analysis of ethanol was performed by GC-FID according to a previously published method (Penton, 1985) and data were expressed as average +/- SEM g/L.

*Statistics*

The statistical analysis was carried out by Statistica 8.0 (StatsSoft Inc., Tulsa, OK, United States) for Windows. Basal dialysate salsolinol and DA were calculated as the average +/- SEM of the last three consecutive samples differing by no more than 10%, collected during the time period preceding each treatment. Changes in dialysate salsolinol and DA were expressed as fmol/10 ml of dialysate and were analyzed by two- or three-way analysis of variance (ANOVA) with repeated measures over time. Salsolinol data, in addition, in order to take into account the fact that data were under a non-normal distribution, were also analyzed under the Friedman's ANOVA and Kendall's coefficient of concordance non-parametric test (general linear model). BEC data were analyzed by one-way ANOVA. Results from treatments showing significant overall changes were subjected to Tukey's post hoc tests with statistical significance set at $p < 0.05$.

### *6.3 Results*

*Effects of Ethanol on pVTA Salsolinol, AcbSh DA, and BEC*

Figure 26 shows that the intragastric administration of vehicle (10 ml/kg) does not affect salsolinol concentration in pVTA dialysates (Figure 26A) and basal DA release in the AcbSh (Figure 26B). In contrast, the intragastric administration of ethanol (1 g/kg) determines the appearance, in the pVTA, of salsolinol (Figure 26C), a molecule therein undetectable under control conditions, and affects AcbSh DA release (Figure 26D). Two-way ANOVA of salsolinol concentrations revealed a significant effect of treatment (EtOH vs. vehicle) [$F_{(1, 13)}$ = 95.98; p<0.001] and time [$F_{(18, 234)}$ = 4.85; p < 0.001] [$F_{(18, 234)}$=4.85; p<0.001 and a significant treatment  time interaction [$F_{(18, 234)}$ = 4.72; p<0.001]. Moreover, Friedman's ANOVA and Kendall's coefficient of concordance analysis resulted in $\chi 2$ .N D 17, df D 18 = 96.91, p<0.00001; coeff. of concordance = 0.32; average rank r = 0.27. Three-way ANOVA of DA data disclosed a significant effect of treatment (EtOH vs. vehicle) [$F_{(1, 16)}$ = 19.9; p<0.001] and time [$F_{(18, 288)}$ = 3.26; p<0.001] and a significant treatment  time interaction [$F_{(18, 288)}$= 2.76; p<0.001]. Tukey's post hoc analysis revealed that ethanol significantly increases salsolinol and both ipsilateral and contralateral DA with respect to basal values (p<0.05). Moreover, ethanol administration also results in a BEC of 0.61  0.08 g/L. One-way ANOVA of BEC revealed a significant difference between average BEC from vehicle- and ethanol-treated rats [$F_{(1, 6)}$= 748.31; p<0.001].

*Effects of pVTA Perfusion with Salsolinol on pVTA Salsolinol and Ipsilateral and Contralateral AcbSh DA*

To verify the prevailing ipsilateral pVTA–AcbSh projections, we unilaterally perfused with salsolinol, by reverse dialysis, the pVTA (Figures 26G,H). This affects both salsolinol in pVTA (Figure 26G) and DA concentrations in ipsilateral AcbSh (Figure 26H) dialysates. Two-way ANOVA of salsolinol concentrations showed a significant effect of pVTA perfusion (normal Ringer vs. salsolinol) [$F_{(1, 11)}$ = 121.63; p<0.001] and time [$F_{(9, 99)}$ = 28.46;

p<0.001] and a significant treatment  time interaction [$F_{(9, 99)}$ = 28.37; p<0.001]. Three-way ANOVA of DA data showed a significant effect of pVTA perfusion (normal Ringer vs. salsolinol) [$F_{(1, 12)}$ = 12.47; p<0.005], probe placement (ipsilateral vs. contralateral) [$F_{(1,12)}$ = 21.88; p<0.0005], and time [$F_{9, 108}$ = 4.28; p<0.001] and significant treatment  probe placement [$F_{(1,12)}$ = 15.63; p<0.002], treatment  time [$F_{(9, 108)}$ = 2.65; p<0.008], and probe placement  time [$F_{(9, 108)}$ = 3.59; p<0.001] interactions. Tukey's post hoc analysis revealed that perfusion with salsolinol in normal Ringer caused the appearance of a significant concentration of salsolinol in the pVTA (Figure 26G) and a significant increase of ipsilateral AcbSh DA release with respect to basal values (p<0.05) (Figure 26H). In contrast, unilateral application of salsolinol in the pVTA does not affect DA release in the contralateral AcbSh (Figure 26H), confirming the ipsilateral specificity of the pVTA–AcbSh projection.

*Effects of Prevention of pVTA Salsolinol formation on Ipsilateral and Contralateral AcbSh DA*

To assess whether the unilateral prevention of salsolinol production in the pVTA could affect ethanol-mediated stimulation of DA release in the ipsilateral, but not contralateral, AcbSh, we exploited sequestering newly formed acetaldehyde in the pVTA or blocking catalase-mediated production of acetaldehyde. To this end, the acetaldehyde-sequestering agent, DP (75 mM), or the non-competitive catalase-H2O2 inhibitor, 3AT (1 nM), was unilaterally applied through the pVTA probe. As shown in Figure 27, pVTA perfusion with DP fails, also after vehicle administration, to affect the concentration of salsolinol in pVTA dialysates (Figure 27A) as well as those of DA in the ipsilateral and contralateral AcbSh (Figure 27B). However, pVTA perfusion with DP affects both ethanol-induced formation of salsolinol in the pVTA (Figure 27C) and stimulation of DA release in the ipsilateral, but not in the contralateral, AcbSh (Figure 27D). Two-way ANOVA of salsolinol concentrations in the presence of DP perfusion of the pVTA failed to reveal significant effects of treatment (EtOH vs. vehicle) [$F_{(1, 15)}$ = 1.09; p = 0.3] and time [$F_{(12, 180)}$ = 0.72; p = 0.7] and a significant treatment  time interaction [$F_{(12, 180)}$ = 0.72; p = 0.73]. Moreover Friedman's ANOVA and Kendall's coefficient of concordance analysis resulted in $\chi$2 N D 17, df D 12/ = 24.76, p<0.02;

coeff. Of concordance = 0.12; average rank r = 0.66. Three-way ANOVA of DA data showed a significant effect of treatment (EtOH vs. vehicle) [$F_{(1, 12)}$= 11.4; p<0.005] and time [$F_{(12, 144)}$= 2.98; p<0.001] and the following significant interactions: probe placement × time [$F_{(12, 144)}$ = 2.75; p<0.005], treatment × time [$F_{(12, 144)}$ = 4.42; p<0.001], and probe placement × treatment × time [$F_{(12, 144)}$ = 2.35; p<0.008]. Tukey's post hoc analysis revealed that DP significantly prevents both pVTA salsolinol formation and ipsilateral AcbSh DA increases with respect to basal values (p<0.05). Similarly, pVTA perfusion with 3AT fails, also after vehicle administration, to affect the concentration of salsolinol in pVTA dialysates (Figure 27E) as well as those of DA in the ipsilateral and contralateral AcbSh (Figure 27F). However, pVTA perfusion with 3AT affects both ethanol-induced formation of salsolinol in the pVTA (Figure 27G) and stimulation of DA release in the ipsilateral, but not contralateral, AcbSh (Figure 27H). Two-way ANOVA of salsolinol concentrations in the presence of 3AT perfusion of the pVTA failed to reveal significant effects of treatment (EtOH vs. vehicle) [$F_{(1, 15)}$ = 1.15; p = 0.3] and time [$F_{(12, 180)}$ = 0.67; p = 0.77] and also a significant treatment × time interaction [$F_{(12, 180)}$ = 0.71; p = 0.78]. Moreover, Friedman's ANOVA and Kendall's coefficient of concordance analysis resulted in $\chi 2$ .N D 17, df = 12/ = 15.91, p < 0.19; coeff. of concordance = 0.078; average rank r = 0.02. Three-way ANOVA of DA data revealed a significant effect of treatment (EtOH vs. vehicle) [$F_{(1, 13)}$ = 7.52; p<0.02] and time [$F_{(12, 156)}$ = 3.37; p<0.001] and the following significant interactions: probe placement × time [$F_{(12, 156)}$ = 2.50; p<0.005], treatment × time [$F_{12, 156}$ = 4.88; p < 0.001], and probe placement × treatment × time [$F_{(12, 156)}$= 3.14; p<0.001]. Tukey's post hoc analysis revealed that 3AT significantly prevents both ethanol-induced increases of pVTA salsolinol and stimulation of ipsilateral AcbSh DA release with respect to basal values (p<0.05). In addition, two-way ANOVA of contralateral AcbSh DA data (shown, respectively, in Figures 26D, 27D,H) disclosed a significant effect of time [$F_{(12, 168)}$ = 17.68; p < 0.0001] but not of treatment [$F_{(2, 14)}$= 0.81; p>0.005] nor a significant treatment × time interaction [$F_{(24, 168)}$ = 1.23; p>0.005]. Moreover, as shown in Figure 28, by perfusing the pVTA with the DA D2/D3 receptor agonist, quinpirole (2.5 mM), we also exploited the ability of $D_2/D_3$ autoreceptor stimulation to prevent DA availability for condensation with acetaldehyde to generate salsolinol. Quinpirole does not affect the concentration of salsolinol in pVTA

dialysates (Figure 28A) as well as that of DA in the ipsilateral AcbSh (Figure 28B). However, after ethanol administration, pVTA perfusion with quinpirole affects salsolinol formation in the pVTA (Figure 28C) and DA release in the ipsilateral AcbSh (Figure 28D). Two-way ANOVA of salsolinol concentrations showed significant effects of treatment (EtOH vs. vehicle) [$F_{(1, 6)}$ = 11.01; p<0.02] and time [$F_{18, 108}$ = 3.06; p<0.001] and a significant treatment time interaction [$F_{(18, 108)}$ = 3.06; p<0.001]. Moreover, Friedman's ANOVA and Kendall's coefficient of concordance analysis resulted in χ2 .N D 10, df D 18/ = 44.16, p<0.00055; coeff. of concordance = 0.24; average rank r = 0.16. Two-way ANOVA of DA data failed to reveal significant effects of treatment (EtOH vs. vehicle) [$F_{(1, 6)}$ = 0.07; p = 0.8] and time [$F_{(12, 72)}$ = 1.08; p = 0.4] and a significant treatment time interaction [$F_{(12, 72)}$ = 1.69; p=0.09]. Tukey's post hoc analysis revealed that quinpirole significantly prevents both ethanol induced increase of salsolinol in the pVTA and DA release in the ipsilateral AcbSh with respect to basal values (p<0.05).

*Effects of pVTA μ Receptors Blockade on pVTA*

Salsolinol and Ipsilateral and Contralateral AcbSh DA Finally, to test the hypothesis that ethanol's effects on AcbSh DA release are mediated by the ability of ethanol-derived salsolinol to stimulate pVTA μ Receptors, naltrexone (1 nM) was applied by reverse dialysis to the pVTA. Figure 29 shows that naltrexone, also after vehicle administration, does not affect the concentration of salsolinol in pVTA dialysates (Figure 29A) as well as those of DA in the ipsilateral and contralateral AcbSh (Figure 29B). Moreover, after ethanol administration, naltrexone does not affect the generation of salsolinol in the pVTA (Figure 29C) but affects the ability of ethanol to stimulate DA release in the ipsilateral, but not contralateral, AcbSh (Figure 29D). Two-way ANOVA of salsolinol concentrations revealed a significant effect of treatment (EtOH vs. vehicle) [$F_{(1, 17)}$ = 595.18; p < 0.001] and time [$F_{15, 255}$ = 45.14; p<0.001] and a significant treatment time interaction [$F_{(15, 255)}$ = 45.06; p < 0.001]. Moreover, Friedman's ANOVA and Kendall's coefficient of concordance analysis resulted in χ2 .N D 19, df D 18 = 55.45, p<0.00001; coeff. of concordance = 0.16; average rank r = 0.12.

Three-way ANOVA of DA data failed to reveal significant effects of treatment (EtOH vs. vehicle) [$F_{(1, 9)} = 0.35$; $p = 0.57$], probe placement [$F_{1, 9} = 0.58$; $p = 0.46$], and time [$F_{12, 108} = 0.94$; $p = 0.51$] and significant treatment probe placement [$F_{1, 9} = 2.75$; $p = 0.13$], treatment time [$F_{12, 108} = 1.48$; $p = 0.14$], probe placement time [$F_{(12, 108)} = 1.34$; $p = 0.2$], and treatment probe placement time [$F_{(12, 108)} = 0.96$; $p = 0.5$] interactions. Tukey's post hoc analysis revealed that naltrexone significantly prevents ethanol-induced increase of ipsilateral AcbSh DA release with respect to basal values ($p<0.05$) while leaving unaffected the formation of salsolinol in the pVTA

*Effects of DF pre-treatment on ethanol-induced pVTA Salsolinol and AcbSh DA*

As shown in Figure 30, systemic DF administration fails, also after vehicle administration, to affect the concentration of salsolinol in pVTA dialysates (Figure 30A) as well as that of DA in the ipsilateral AcbSh (Figure 30B). However, DF affects both ethanol-induced formation of salsolinol in the pVTA (Figure 30A) and stimulation of DA release in the ipsilateral AcbSh (Figure 30B). Three-way ANOVA of salsolinol concentrations after DF administration revealed significant effects of pretreatment (DF vs saline) [$F_{(1, 15)} = 42.36$; $p<0.05$] treatment (EtOH vs. vehicle) [$F_{(1, 15)} = 35.98$; $p<0.05$] and time [$F_{(18, 270)} = 4.85$; $p<0.01$] and a significant pretreatment x treatment × time interaction [$F_{(18, 270)} = 4.72$; $p<0.01$]. (EtOH vs. vehicle) [$F_{(1, 10)} = 89.6$; $p<0.3$] and time [$F_{(12, 180)} = 0.72$; $p = 0.7$] and a significant treatment × time interaction [$F_{(12, 180)} = 0.72$; $p = 0.73$]. Three-way ANOVA of DA data showed a significant effect of pretreatment [DF vs vehicle] [$F_{(1, 10)} = 107.6$; $p<0.005$], treatment (EtOH vs. vehicle) [$F_{(1, 10)} = 89.6$; $p<0.005$] and time [$F_{(12, 180)} = 5.15$; $p<0.001$] and the following significant interactions: pretreatment × treatment [$F_{(1, 10)} = 99.5$; $p<0.005$], pretreatment × time [$F_{(12, 180)} = 4.8$; $p<0.001$], treatment × time [$F_{(12, 180)} = 2.8$; $p<0.001$], and pretreatment × treatment × time [$F_{(12, 180)} = 2.7$; $p<0.001$]. Tukey's post hoc analysis revealed that DF significantly prevents both pVTA salsolinol formation and ipsilateral AcbSh DA increases with respect to basal values ($p<0.05$).

### *6.4 Discussion*

The present study aimed at challenging, in vivo, the hypothesis that ethanol activates the mesolimbic DA system acting as the prodrug of salsolinol. To directly test this hypothesis (Polache and Granero, 2013; Peana et al., 2016), we envisioned to detect salsolinol and DA through microdialysis probes implanted in the pVTA and AcbSh, respectively, of the same side (ipsilateral) or of the opposite side (contralateral) of the rat brain, taking advantage of the mostly ipsilateral VTA–AcbSh projections (Geisler and Zahm, 2005; Ikemoto, 2007; Breton et al., 2019). In this regard, two anatomical details enforce some attention: the first one refers to the anteroposterior heterogeneity of the VTA. This has been recently in-depth reviewed (Sanchez- Catalan et al., 2014) disclosing that the ability of ethanol (but also of acetaldehyde and salsolinol) to sustain its intracranial self-administration and elicit locomotor activation resides in the posterior, but not anterior, portion of this brain structure (Rodd-Henricks et al., 2000, 2002; Rodd et al., 2004, 2005, 2008; Ding et al., 2009, 2012; Sánchez-Catalán et al., 2009; Hipólito et al., 2010, 2011; Marti-Prats et al., 2010, Martí-Prats et al., 2013). For this reason, in the present experiments, the microdialysis probes were implanted in the pVTA at brain stereotaxic coordinates in agreement with the above studies. Also, it is indeed critical, in this context, to point out that since over 95% of pVTA projections to AcbSh are ipsilateral, second anatomical detail that enforces attention (Figure 29A), our study exploited this anatomical peculiarity to reach the demonstration that reveals something that had long been suggested by indirect evidence but never proven: the until now unknown mechanism by which ethanol stimulates DA release in the AcbSh and exerts the potential to trigger its addictive liability. The present results confirmed that the oral administration of a dose of ethanol responsible of a BEC that produces mild euphoriant effects in humans (Majchrowicz, 1975; Gill et al., 1986; Nurmi et al., 1994) increases DA release in the AcbSh (Howard et al., 2008; Bassareo et al., 2019). In addition, the results disclose for the first time that this systemic administration of ethanol also determines the appearance of salsolinol, a molecule undetectable in pVTA dialysates under control conditions, no matter whether in dialysates of the same or of the opposite side with respect to that where we could simultaneously detect the increase of AcbSh DA release. However

this ethanol dependent appearance of salsolinol in pVTA dialysates, although representing on its own the first in vivo demonstration of the long-sought evidence (Polache and Granero, 2013) of the ethanol-dependent formation of salsolinol in the pVTA, still necessitated to be causally linked to the ethanol-dependent increase of AcbSh DA release (Polache and Granero, 2013; Peana et al., 2016). Hence, we firstly sought to determine whether, in agreement with the mostly ipsilateral VTA–AcbSh projections (Jaeger et al., 1983; Geisler and Zahm, 2005; Ikemoto, 2007; Breton et al., 2019), the application of salsolinol in the pVTA of one side would result in increased DA release only in the ipsilateral AcbSh. In agreement with previous studies (Hipólito et al., 2009, 2011), salsolinol, applied by reverse dialysis in the pVTA, significantly increases DA release in the ipsilateral AcbSh but does not affect it in the AcbSh of the opposite side. Notably, given the small percentage of pVTA neurons that project to the AcbSh of the opposite side (Jaeger et al., 1983; Geisler and Zahm, 2005), a detection system such as fast-scan cyclic voltammetry, endowed with greater analytical and temporal resolution, might have allowed to detect similar effects also contralaterally. However, the analytical sensitivity (5 fmol/10 ml) and the relatively low temporal resolution of sample collection of our assay prevented to find similar effects also in the contralateral AcbSh. In this regard, this apparent analytical limitation provided us, on the contrary, the optimal experimental conditions to test our working hypothesis. Thus, to temporally and mechanistically lock ethanol-dependent pVTA salsolinol to ethanol-dependent increases of AcbSh DA release, we sought to interfere with the formation of salsolinol. To this end, according to a common and validated approach of the studies aimed at assessing the role of acetaldehyde in ethanol's central effects (Spivak et al., 1987; Pastor et al., 2002; Correa et al., 2012; Martí-Prats et al., 2013; Peana et al., 2017a), we prevented acetaldehyde's bioavailability by blocking catalase-mediated ethanol metabolism with 3AT or by sequestering acetaldehyde with DP (Quertemont et al., 2003; Font et al., 2006a,b; Correa et al., 2008; Peana et al., 2015). The results of these experiments demonstrate that without acetaldehyde formation and/or availability in the pVTA, there is no detectable salsolinol in pVTA dialysates nor increased DA release in the ipsilateral AcbSh after ethanol's systemic administration. In contrast, in the AcbSh of the side opposite to that

where pVTA was reverse applied with 3AT or DP, systemic ethanol significantly increases DA release. Thus, based on previous observations in the ipsilateral pVTA and AcbSh, we can conclude that the increases of DA release in the contralateral AcbSh are further indirect proof of the newly formed salsolinoldependent increase of AcbSh DA release. In this regard, it is relevant to observe that salsolinol was detected at a concentration in the low nM range (15 fmol/10 ml, i.e., 1.5 nM), very similar to that (10 nM) used in the experiments in which salsolinol, reverse applied in the pVTA (Figure 26), increases DA release in the ipsilateral AcbSh. Notably, in agreement with the observation that no catalase staining could be found in the nucleus accumbens (Zimatkin and Lindros, 1996), we could not detect salsolinol in AcbSh dialysates (Figure 25). In summary, the present in vivo results (Figure 29) point to the catalase-mediated metabolic conversion of ethanol into acetaldehyde in the pVTA and to the subsequent condensation of acetaldehyde with DA to generate salsolinol, as key mechanisms for the ability of ethanol to excite DA neurons and, consequently, to stimulate DA release in the AcbSh (Di Chiara et al., 2004; Howard et al., 2008; Bassareo et al., 2017, 2019; Volkow et al., 2017;Wise and Robble, 2020). Previous extensive literature indicates that the reduced activity of DA cells in the posterior, but not anterior, VTA (Rodd et al., 2004, 2008) by local injections of quinpirole can reduce ethanol intake (Nowak et al., 2000) and both ethanol (Hodge et al., 1993; Hauser et al., 2011) and salsolinol (Rodd et al., 2008) self-injections in the pVTA. Hence, since salsolinol is formed by DA and acetaldehyde (Davis and Walsh, 1970; Davis et al., 1970) no matter, in this context, whether spontaneously (by Pictet– Spengler condensation) or enzymatically (Chen et al., 2018), we also addressed the possibility of exploiting the reduction of synaptic availability of DA in the pVTA, by reverse application of quinpirole, to further challenge our working hypothesis. The results of this experiment (Figure 28) disclose that, after ethanol's systemic administration, the stimulation of pVTA DA $D_2/D_3$ auto receptors at a concentration in the range that prevents ethanol (Rodd et al., 2004; Hauser et al., 2011) and salsolinol (Rodd et al., 2008) self-administration does not affect basal AcbSh DA release but significantly reduces the formation of salsolinol in the pVTA and prevents the ethanol-elicited increase of DA release in the ipsilateral AcbSh. These results rule out the possibility that the effects of ethanol in the pVTA could be

directly mediated by acetaldehyde instead of salsolinol and are in agreement with our recent report that in the absence of newly synthesized DA, unlike salsolinol, neither ethanol nor acetaldehyde can stimulate pVTA DA neurons recorded in mesencephalic, pVTA containing, slices (Melis et al., 2015). Moreover, while the effect of ethanol on the firing of pVTA DA neurons in slices from a-methyl-p-tyrosine-treated mice could be restored by the application of exogenous DA, this was blocked by catalase inhibition (Melis et al., 2015). Notably, although after ethanol administration in the presence of pVTA perfusion with quinpirole we could still detect a significant formation of salsolinol (Figure 28C), this was significantly lower in comparison with that determined in the presence of pVTA perfusion with normal Ringer. Moreover, although detectable (Figure 28C), such salsolinol's concentration was insufficient to trigger AcbSh DA release (Figure 28D). A possible interpretation of this finding might also be that pVTA perfusion with quinpirole, at the concentration that on its own does not affect basal AcbSh DA release, may have exerted a further preventive action (Rodd et al., 2004, 2008; Hauser et al., 2011) on the stimulatory effect of ethanol-derived salsolinol in the pVTA (Hipólito et al., 2009, 2011; Melis et al., 2015). The present data also point to μ Receptors as those through which the ethanol-derived, newly formed, salsolinol stimulates DA neurons in the pVTA (Figure 29). This is consistent with ex vivo and in vivo evidence that μ Receptors blockade prevents salsolinol-induced excitation of DA neurons (Xie et al., 2012), DA release in the Acb (Hipólito et al., 2009, 2011), locomotor activation (Hipólito et al., 2010), and CPP (Matsuzawa et al., 2000; Hipólito et al., 2011; Campos- Jurado et al., 2020). In this regard, the present study provides a mechanistic framework for naltrexone as FDA-approved treatment for alcohol use disorder (AUD) (American Psychiatric Association, 2013). However, apparently at variance with both the present and previous data (Melis et al., 2015), it has recently been proposed that KCNK13 potassium channels may be the molecular target for the stimulatory action of ethanol on pVTA DA neurons and for ethanol-dependent behaviors (You et al., 2019). Nevertheless, this does not preclude the possibility that these effects are mediated through stimulation of μ Receptors by ethanol-derived salsolinol. In this regard, it would be critical to test, in brain slices from a-methyl-p-tyrosine-treated animals, the suggestion put forward by You et al.

(2019). This part of the present study demonstrates that the intragastric administration of ethanol results, in the pVTA, in pharmacologically effective concentrations of salsolinol that, via local stimulation of μ Receptors, increases DA release in the AcbSh, the neurochemical mechanism potentially responsible for triggering ethanol's abnormal consumption (Di Chiara et al., 2004; Volkow et al., 2017; Wise and Robble, 2020). However, we acknowledge that these findings would be of greater impact if also investigated on female rats. Moreover, while we also acknowledge that this is still far from conclusively linking newly formed salsolinol in the pVTA to AUD, we envision that the significance and impact of the present discovery would be greatly amplified if confirmed also under voluntary consumption by ethanol-preferring rats. Ethanol, in fact, being a molecule endowed with addictive potential, may become responsible for AUD and for its negative consequences on health (Rehm et al., 2017; Axley et al., 2019) when encounters genetic vulnerability to its excessive consumption. Hence, the disclosure of ethanol's precise site and mechanism of action on both genders, together with the discovery of the genetic bases of proclivity to excessive ethanol consumption, will represent significant progresses not only for the scientific community but also for the organizations that at different levels, are in charge of individual and societal healthcare policies. Moreover, the data regarding the efficiency of DF in preventing the formation of salsolinol in the pVTA (Figure 30A ) and, hence, in line with the above-discussed data, the increase of DA in the AcbSh (Figure 30B), gives a plausible explanation to the positive effects of this molecule on ethanol-induced acquisition of place conditioning (Chapter 2) and strengthen its potential application for the treatment of AUD. However, the precise mechanism by which salsolinol's formation is prevented by DF administration remains unknown. It could be speculated that DF, being a derivative of ferulic acid, which is known to be endowed of potent antioxidant properties (Zduńska K et al., 2018; Srinivasan M, 2007), might be oxidated by $H_2O_2$, thus reducing the availability of the cofactor needed for catalase activity and, consequently, reducing the availability of ethanol-derived acetaldehyde which is necessary for the formation of salsolinol. However, future studies are required to better understand this mechanism of action. In conclusion, all these limitations notwithstanding, the present demonstration validates and sustains the development of

future studies devoted to further investigate the disclosed mechanism after voluntary consumption and to define preventive and therapeutic strategies based on acetaldehyde sequestration (Orrico et al., 2017) or on the use of appropriate antioxidant agents (Aragon et al., 1991).

## 7. General discussion and conclusions

The results obtained in this study consist in a heterogeneous, yet strictly connected and coherent, pool of data which underlies the therapeutic potential of the *WSE*-isolated $GABA_AR$ agonist, DF, in the treatment of anxiety and addiction, providing also candidate mechanisms of action for these activities. Moreover, this thesis digs deep in the complex relationship between ethanol metabolism and its addiction-related neurochemical features (Correa et al., 2012; Hipolito et al., 2012), revealing that DF, and perhaps *WSE*, could exert its effects on ethanol at this stage too. The outcomes from chapter 1 point out DF as a single possible $GABA_AR$/BDZ-acting effector of the anxiolytic properties of *WS* surprisingly and intriguingly devoid of any typical collateral effect. Moreover, in chapter 2 we demonstrated how *WSE* and DF differentially interfere with the property of ethanol and morphine to acquire and express CPP through preventing ERKs phosphorylation in the AcbSh. As already discussed above, this paradigm is endowed with great translational impact due to its face, construct, and predictive validity (Tzschentke, 2007). In particular, in fact, in contrast to *WSE* (Ruiu 2013, Spina 2015), DF resulted able to prevent ethanol- and morphine-induced acquisition but not expression of CPP, suggesting that the $GABA_AR$-mimetic activity of DF might be critical for *WSE*'s ability to affect the acquisition but not the expression of CPP elicited by ethanol and morphine. This interpretation is supported by the observation that AcbSh DA has been reported to be critical for the acquisition, but not the expression, of morphine-elicited CPP (Fenu et al., 2006) and appears overall in agreement with the role played by mesolimbic DA in the associative learning at the basis of CPP acquisition (Di Chiara et al., 2004). In the last part of this study, described in chapter 3, our group performed an accurate and complex study on how the acute administration of ethanol can activate the mesolimbic pathway , with a focus on its metabolism. The results obtained have a great scientific relevance, we believe, because they represent the first successful attempt of identifying a key mechanism for ethanol-mediated activation of mesolimbic pathway in vivo. In fact, this study demonstrated that ethanol's metabolism in the brain plays a crucial role in the activation of this important reward circuit. In particular, the reaction between ethanol-derived acetaldehyde and DA in the pVTA, resulting in the

formation of the otherwise undetectable salsolinol, appears of dramatic importance for the ability of ethanol to activate DA firing (Melis et al., 2015) and hence increase the DA traffic in the AcbSh (Bassareo et al., 2021). Through various and complex pharmacological approaches, our group managed to demonstrate that i) preventing the formation of salsolinol also prevents the increase of DA in the AcbSh; ii) salsolinol itself can activate pVTA DA neurons and induce an increase of DA traffic in the AcbSh; iii) salsolinol effects in the pVTA are mediated by μ Receptors. Accordingly to the results of chapter 2, DF resulted able to prevent ethanol-mediated increase of the DA traffic in the AcbSh and, surprisingly, this effect appeared related to a preventive action on the formation of salsolinol (chapter 3). Taken together, the results of this thesis may support the suggestion of the suitability of both *WSE* and DF as strategies for the management of anxiety and of distinct phases of drug addiction . In fact, anxiety disorders and drug addiction may co-occur at high rates (Smith and Book, 2008) hence, the anxiolytic profile and the anti-addictive properties of DF may truly be useful in the development of an efficient therapeutical strategy. Moreover, since the most prescribed anxiolytic drug, BDZs, are known to carry negative side effects on motor coordination, cognition, and motivation (Roth et al., 1984; Tan et al., 2011), the fact that DF lacks the property of impairing motor coordination and anterograde memory, together with its failure in potentiating ethanol-induced LORR and to induce CPP, makes this molecule worthy of future studies, suggesting it is a safe, selective, anxiolytic and anti-addictive compound devoid of critical side effects that could reduce its compliance and manageability. In conclusion, this thesis represents a valuable example of how the application of a phytomedicinal approach to the investigation on CNS disorders, might result, in pre-clinical studies, in the individuation of suitable and safe candidates for the treatment of the above-cited disorders. In particular, this study strengthens the already acknowledged therapeutic potential of *WSE*, and discloses a new potential use for its metabolite, DF, in the treatment of anxiety and drug addiction. Indeed, after the behavioural and biochemical investigations conducted during my PhD course, we added new pieces in the puzzle of how *WSE* might be able to exert part of its neurological effects and, by characterizing its isolated metabolite, DF, we could disclose that it can be

one of the main actors in the whole extract with the great positive aspect of being potent but, to the best of our knowledge, so far, surprisingly safe. In fact, as already discussed, the reduction of collateral effects is one of the main reasons that induced researchers to renew their interest in phytomedicinal approaches, even for the most complicated disorders. Moreover, the important outcomes deriving from the application of brain microdialysis *in vivo* gives a great scientific relevance to this thesis, providing new fundamental knowledges which helps to better understand the extremely complicated pharmacology of ethanol and might suggest new approaches to counteract AUD.

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## 9. Figures

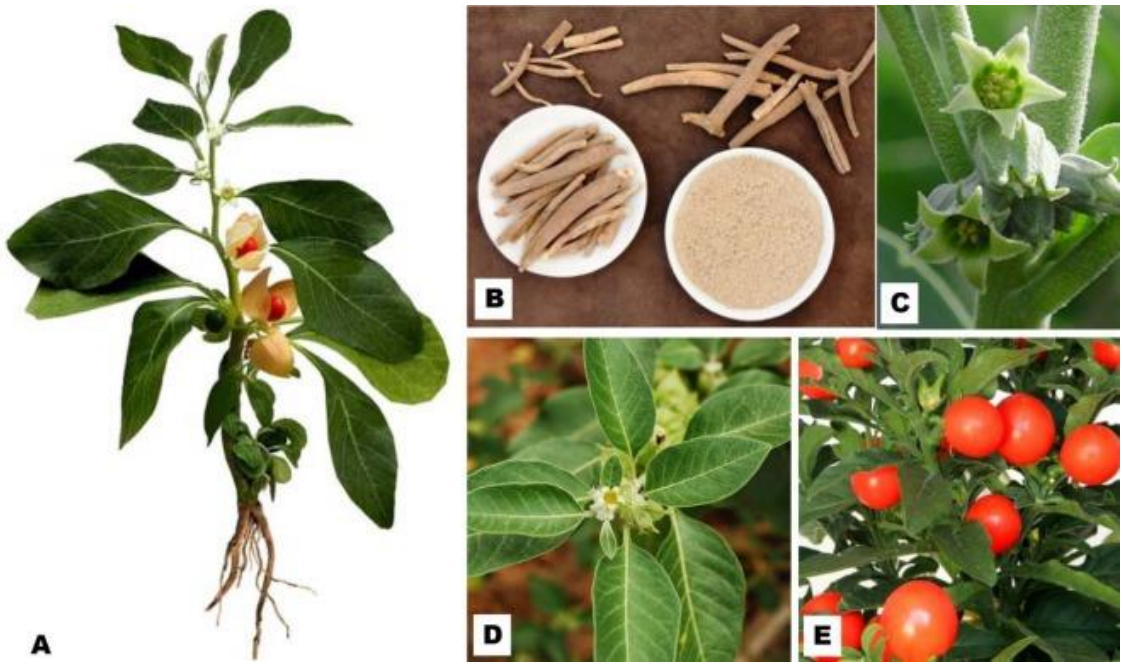


**Figure 1.** *WS* plant: (A) plant; (B) roots and root powder; (C) flowers; (D) leaves; and (E) and fruits (Sengupta et al., 2017).

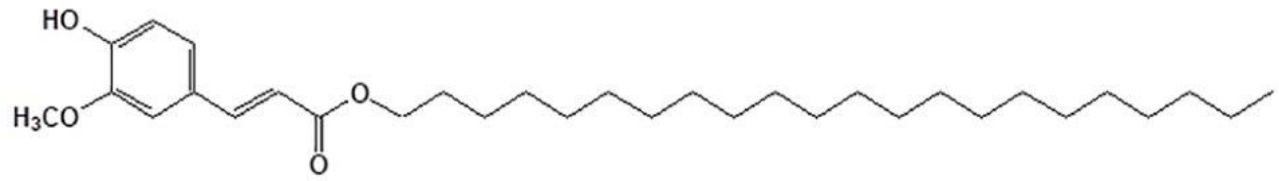


**Figure 2.** Chemical structure of DF, bioactive secondary metabolite isolated from methanolic extract of the roots of *WS* (Sonar et al., 2019).

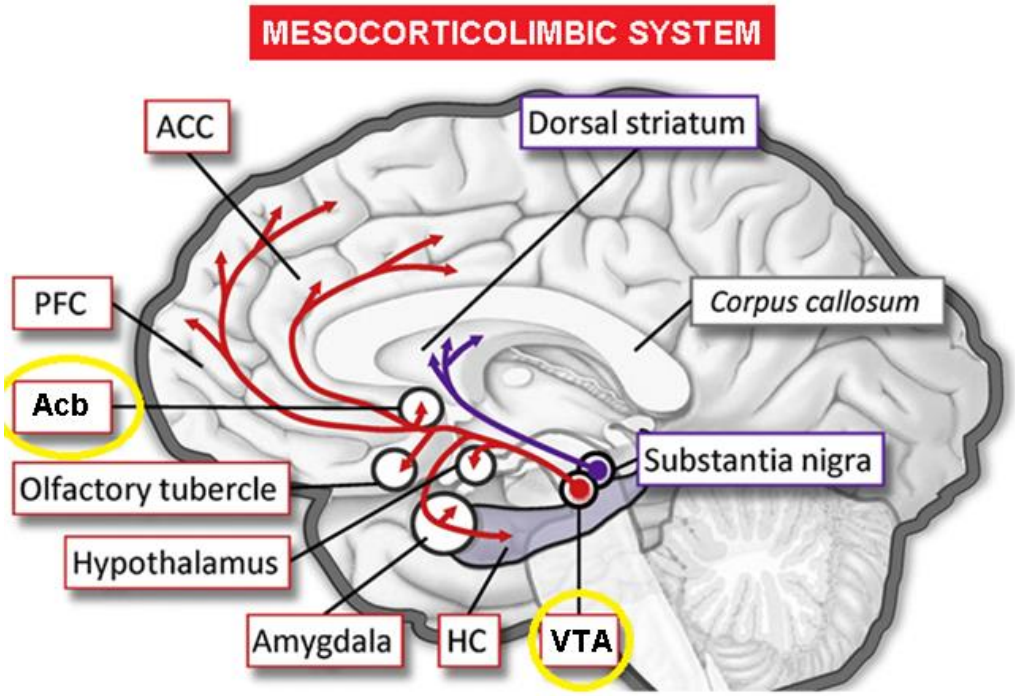


**Figure 3.** Main ascending mesocorticolimbic and nigrostriatal dopaminergic pathways. Projections from VTA to Acb forms the mesolimbic system. Abbreviations: ACC: anterior cingulate cortex; HC: hippocampal complex; Acb: nucleus accumbens; PFC: prefrontal cortex; VTA: ventral tegmental area (Perogamvros L and Schwartz S).

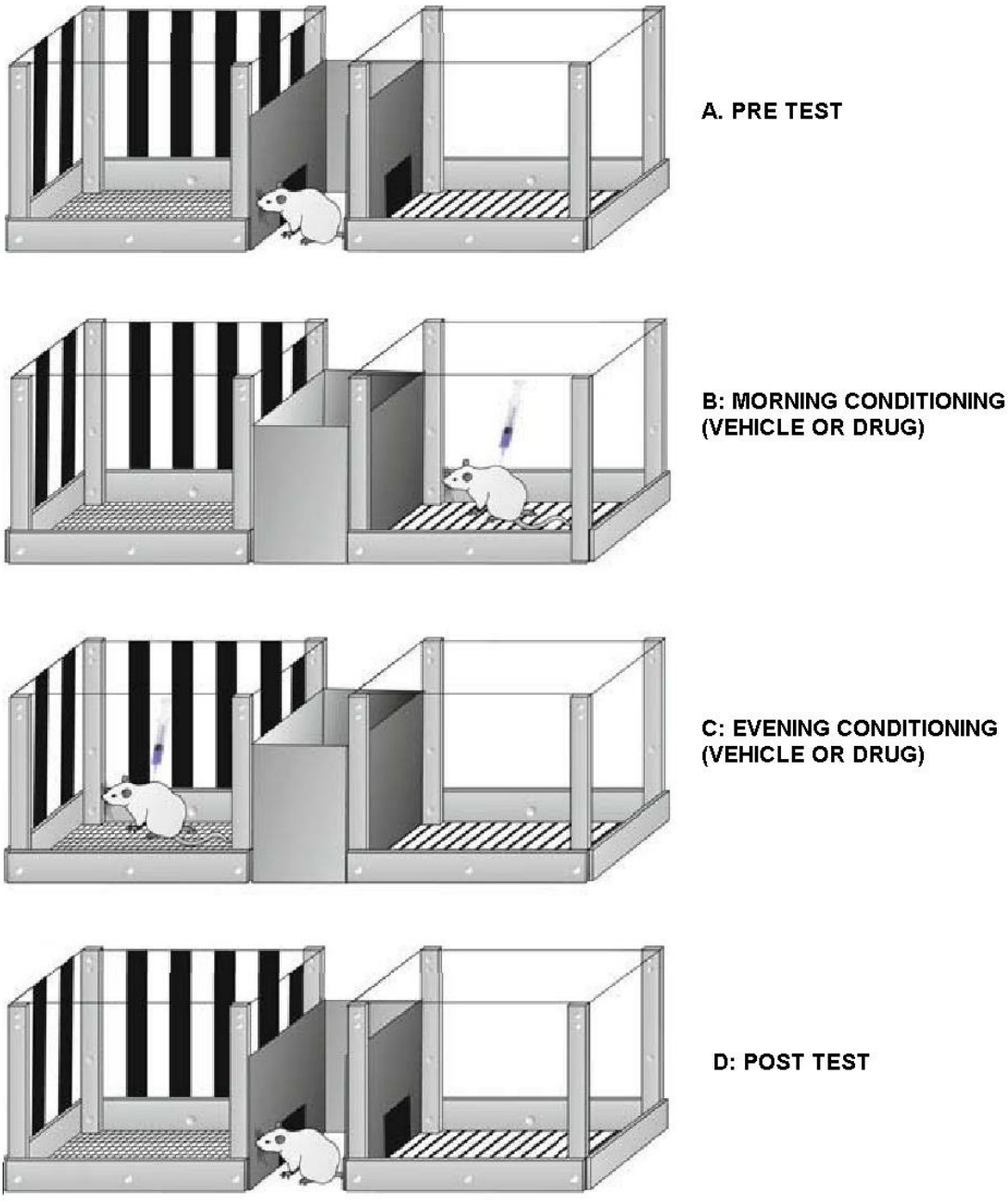


**Figure 4.** CPP paradigm. 4A represents the pre-test phase in which subjects' spontaneous preference is recorded. 4B represents the conditioning phase: if the animal is injected with vehicle and exposed to its favourite compartment in the morning, it will be injected with drug and exposed to the opposite compartment in the evening and vice versa. 4D represents the post-test phase in which subjects' shifts in spontaneous preference are recorded (Olive MF and Kalivas PW, 2010)

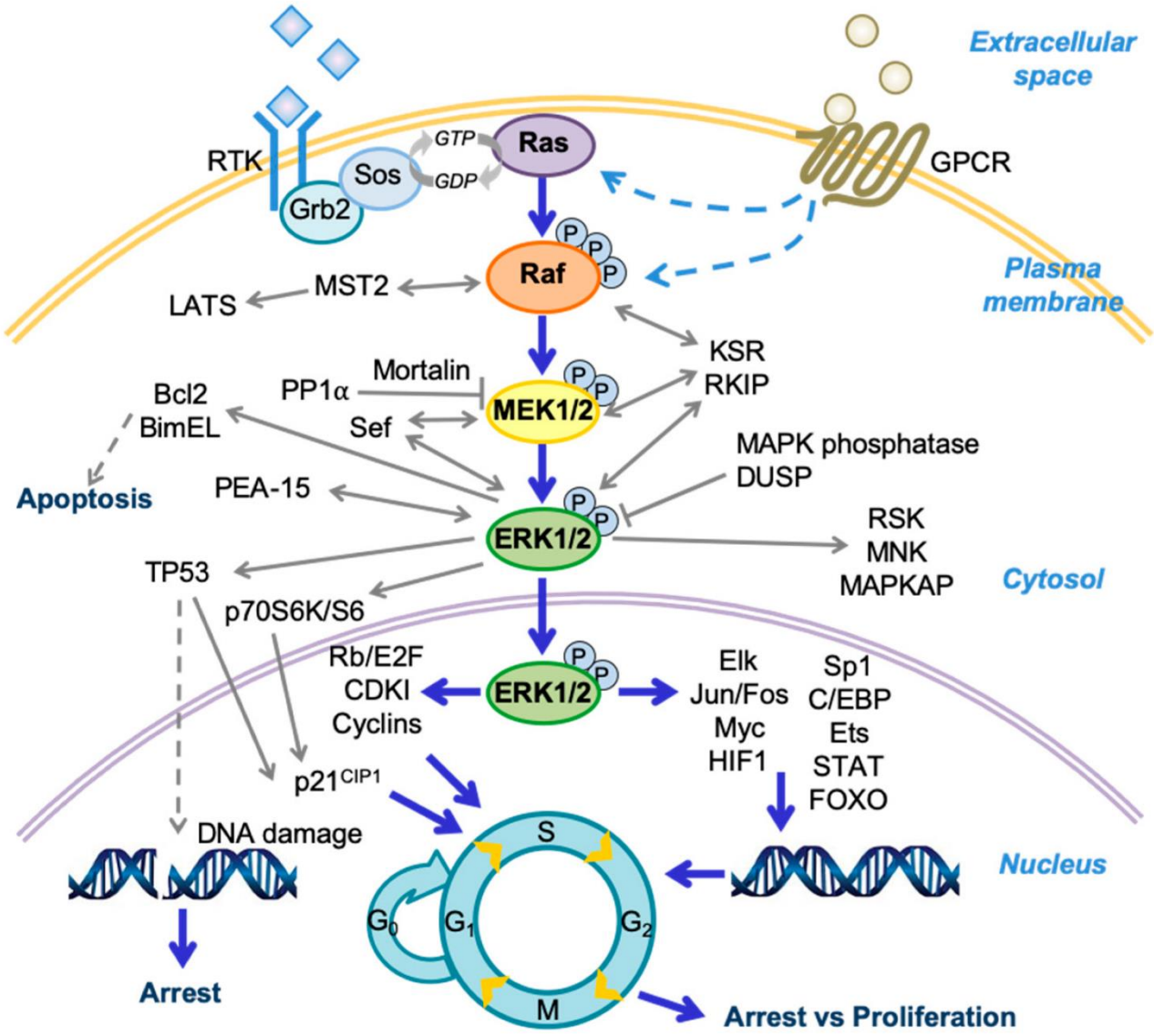


**Figure 5.** The extreme complexity of the ERK pathway (Wu PK et al., 2020).

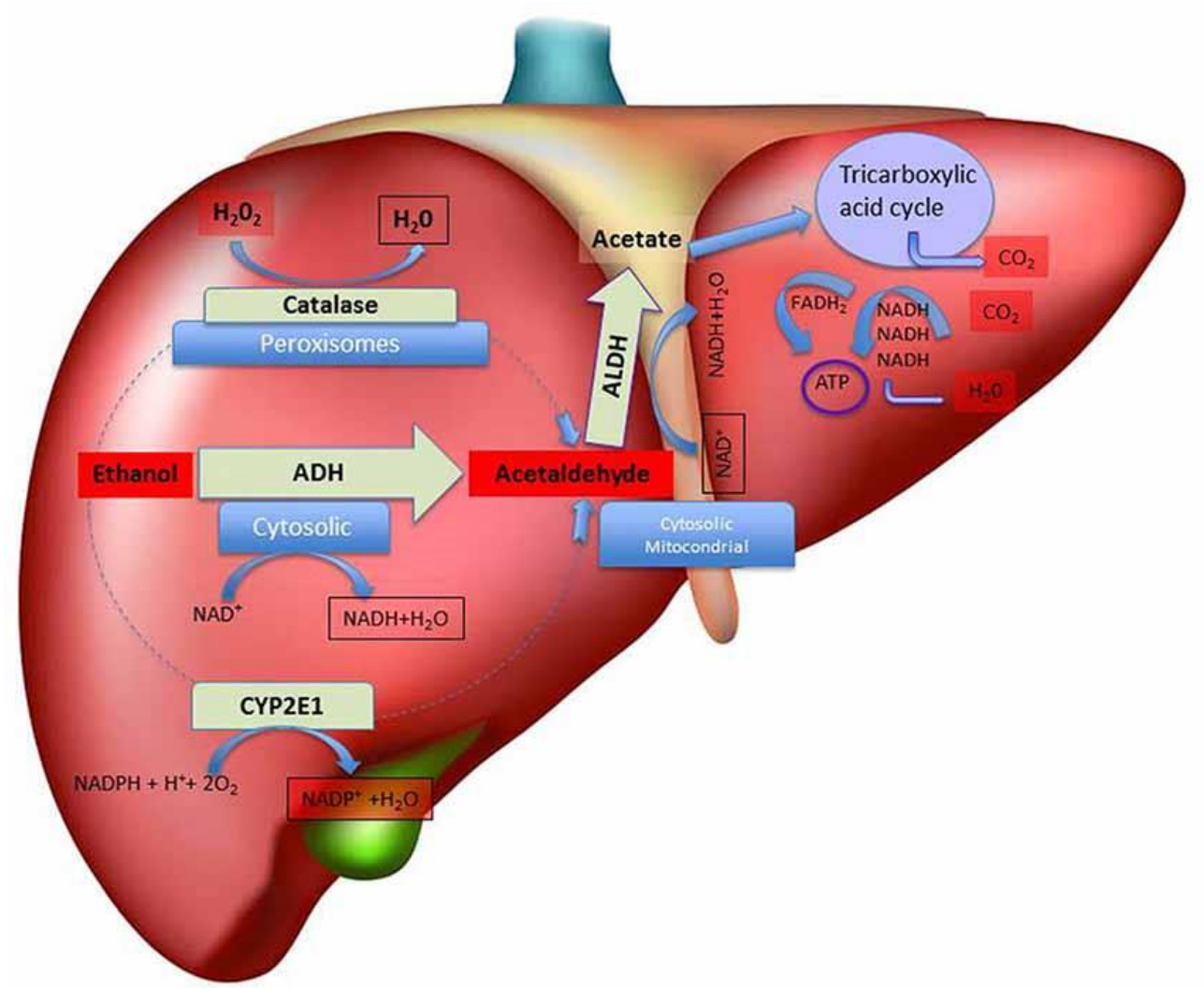


**Figure 6.** The figure depicts, in the liver, the sub-cellular (cytosolic, peroxisomal and mitochondrial) localization of the main pathways of ethanol oxidative metabolism to acetaldehyde and of the main pathways of ethanol by-products (acetaldehyde and acetate) disposal, with indication of the relative co-factors involved. Abbreviations: ADH, Alcohol dehydrogenase; ALDH, Aldehyde dehydrogenase; ATP, adenosine triphosphate; CYP2E1, isoform 2E1 of cytochrome P450; FADH2, flavin-adenine dinucleotide coenzyme in its reduced form; NAD+, nicotinamide adenine dinucleotide coenzyme; NADPH, Nicotinamide Adenine Dinucleotide Phosphate coenzyme in its reduced form. (Peana et al., 2017)

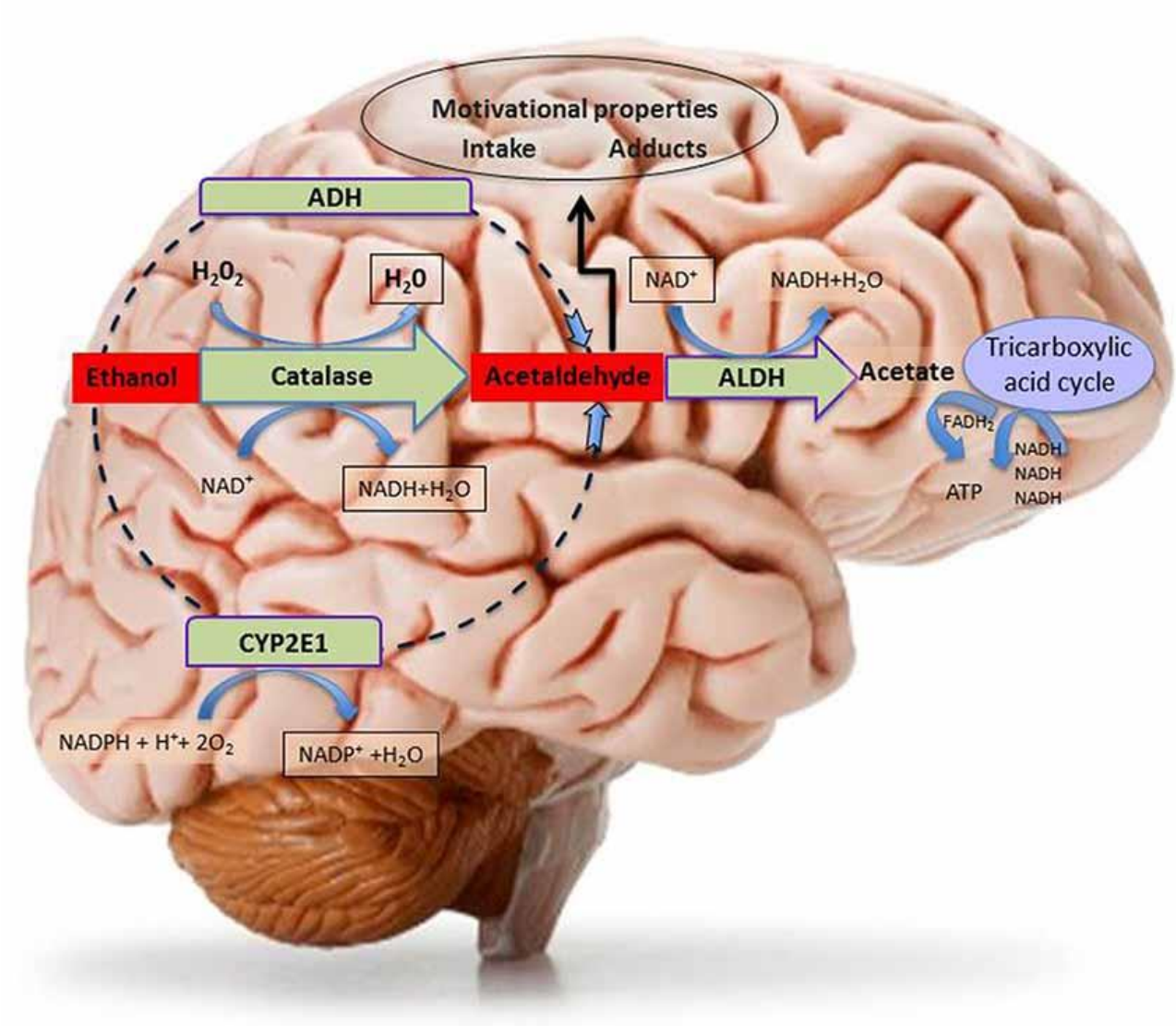


**Figure 7.** Schematic representation of central metabolism of ethanol. The figure depicts the three main central metabolic pathways of ethanol oxidative metabolism to acetaldehyde and the main metabolic pathways of ethanol by-product (acetaldehyde and acetate) disposal, with indication of the relative co-factors involved. Abbreviations: ADH, Alcohol dehydrogenase; ALDH, Aldehyde dehydrogenase; ATP, adenosine triphosphate; CYP2E1, isoform 2E1 of cytochrome P450; FADH2, flavin-adenine dinucleotide in its reduced form; H2O2, Hydrogen peroxide; NAD+, nicotinamide adenine dinucleotide coenzyme; NADPH, Nicotinamide Adenine Dinucleotide Phosphate coenzyme in its reduced form. (Peana et al., 2017)

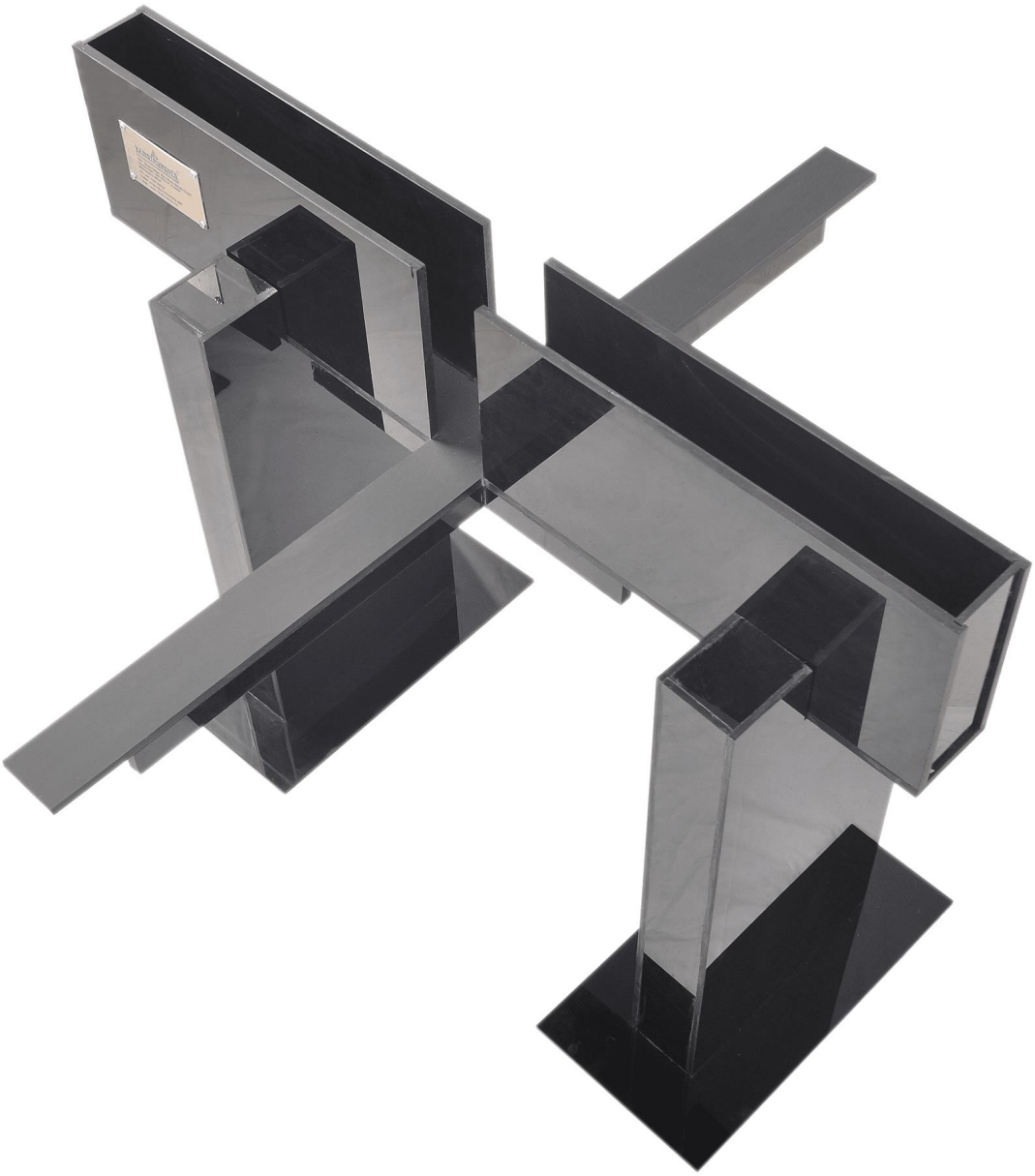

**Figure. 8.** Elevated Plus Maze (EPM)

**Figure 9.** Horizontal Static Rods

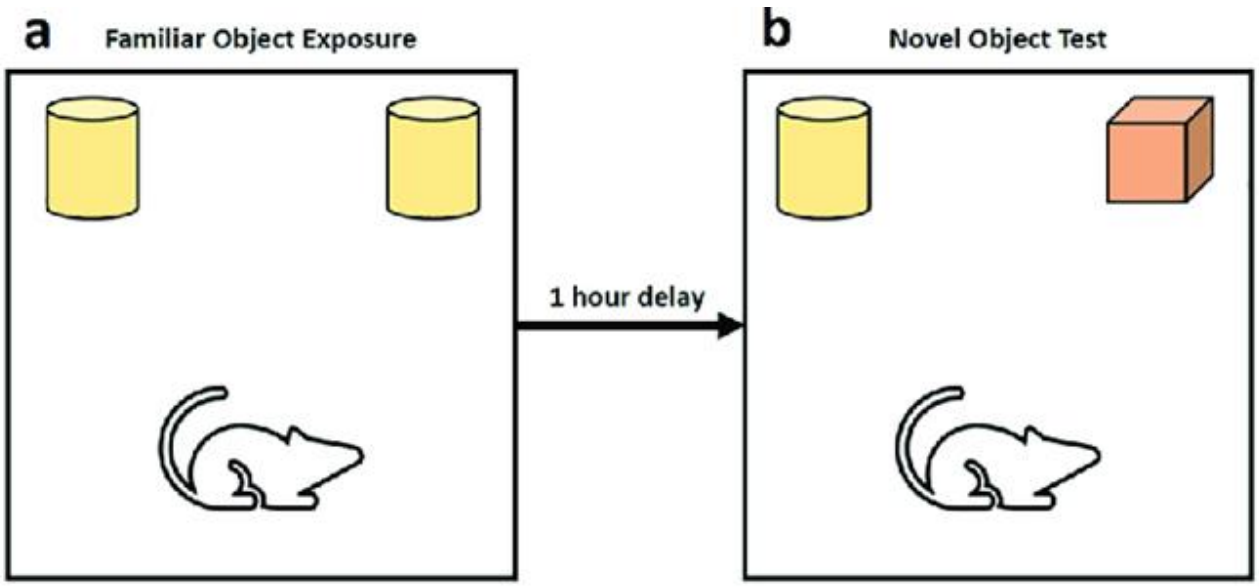


**Figure 10.** Schematic representation of the Novel Object Recognition (NOR) test.

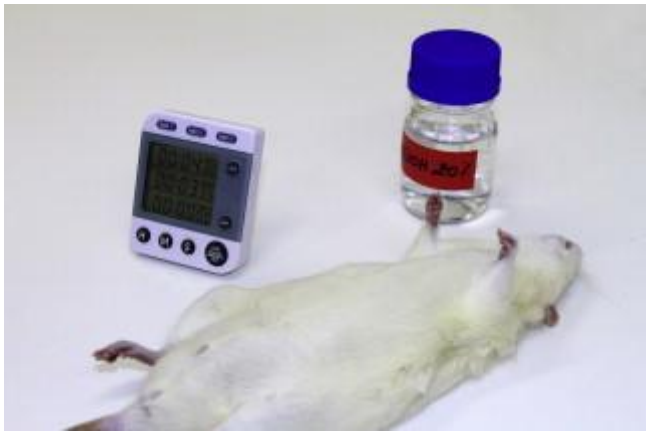

**Figure 11.** Example of Loss of Righting Reflex (LORR) evaluation in rodents.

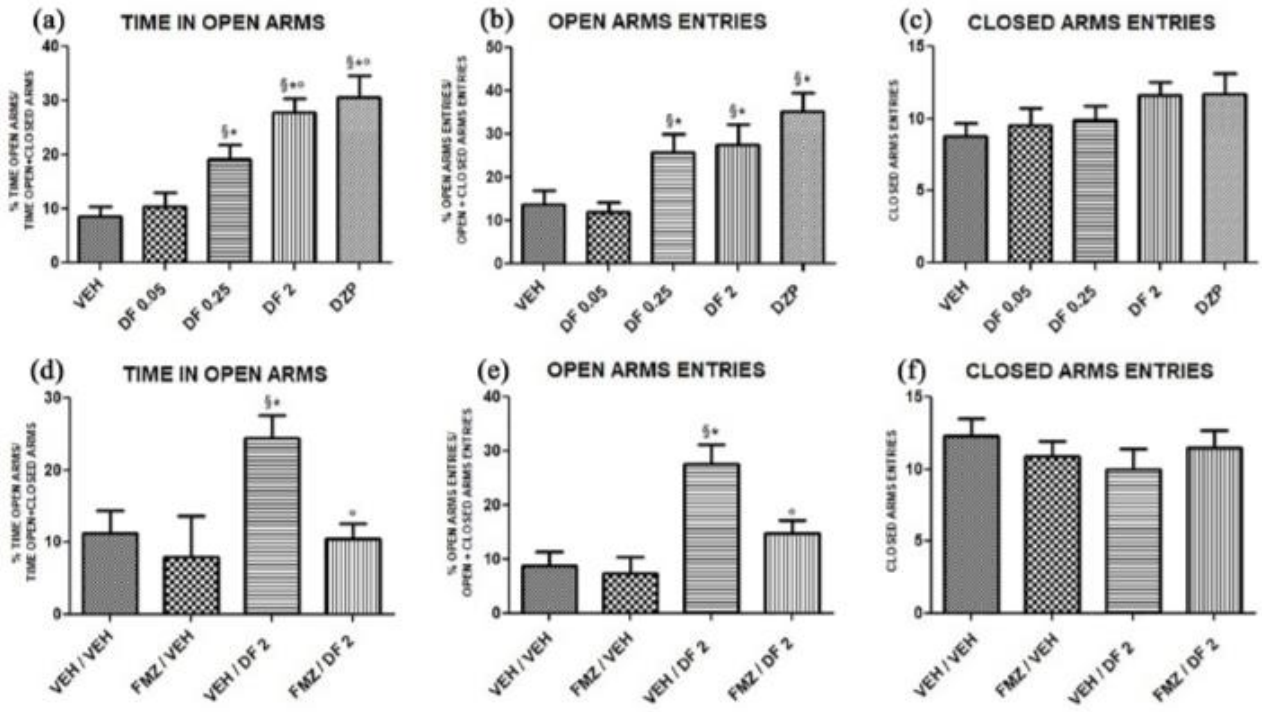


**Figure 12.** Effects of treatment with DF and DZP (a–c) and effects of pre-treatment with FMZ on the effects of DF 2 mg/kg (d–f) on the EPM; (a) and (d) % time in open arms (time in open arms divided by time in open arms + time in closed arms), expressed as % of VEH group (100%); (b) and (e) % number of entries in open arms (entries in open arms divided by entries in open arms + entries in closed arms), expressed as % of VEH group; (c) and (f) number of entries in closed arms. Values are expressed as mean + SEM. (a, b): §p < 0.05 vs VEH; *p < 0.05 vs DF 0.05; °p < 0.05 vs DF 0.25; (d, e): §p < 0.05 vs VEH/VEH; *p < 0.05 vs FMZ/VEH; °p < 0.05 vs DF 2 (Maccioni et al., 2021).

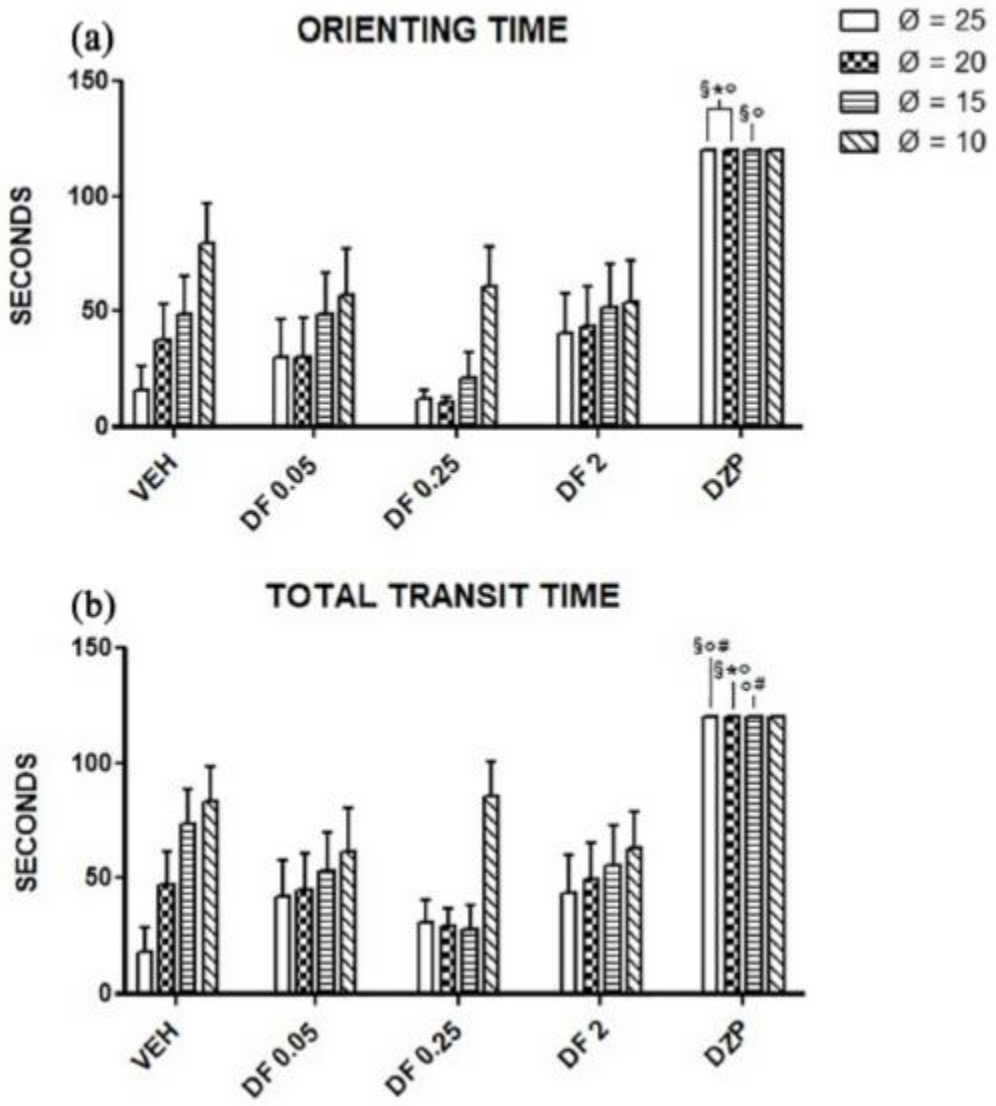


**Figure 13.** Effects of DF on motor coordination evaluated on four progressively narrower horizontal static rods (diameter = 25, 20, 15 and 10 mm). (a) Time (s) spent by mice orienting themselves 180° from initial position. (b) Time (s) of orientation plus time spent to cross the rod. Values are expressed as mean + SEM. §$p < 0.05$ vs VEH. *$p < 0.05$ vs DF 0.05. °$p < 0.05$ vs DF 0.25. #$p < 0.05$ vs DF 2 (Maccioni et al., 2021).

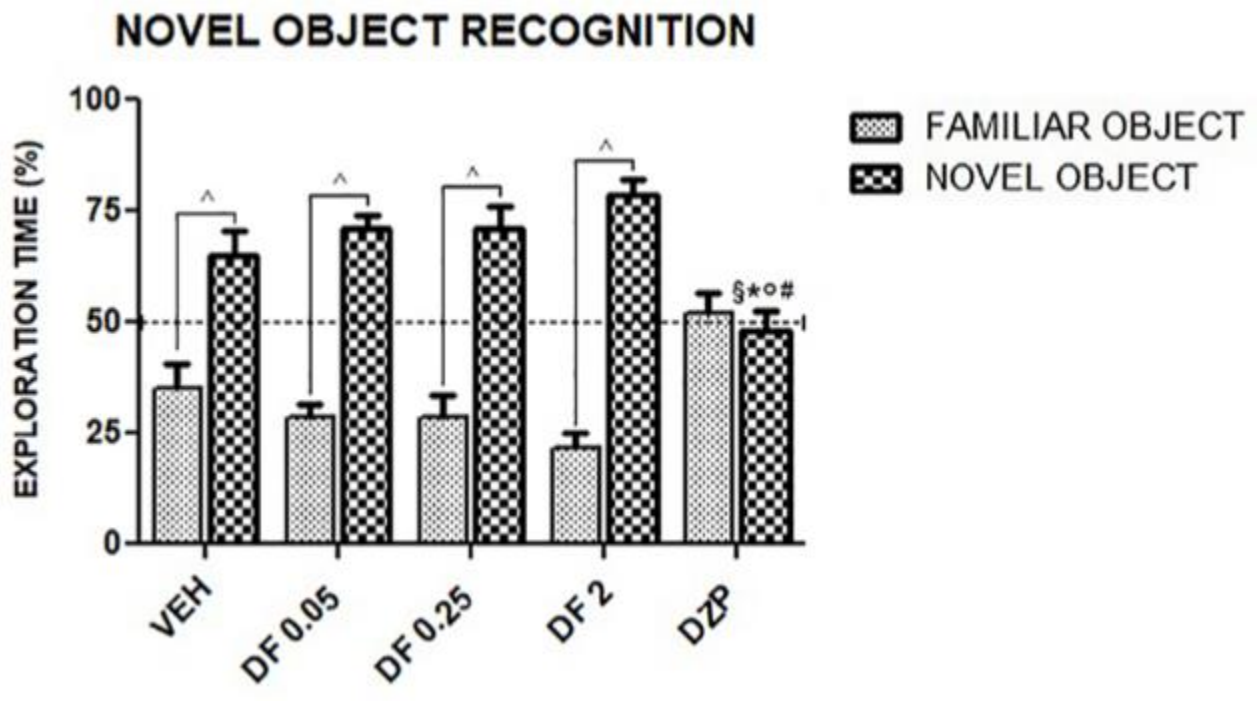


**Figure 14.** Effects of DF and DZP at the NOR test. The histograms represent the time (expressed as % of total exploration time) exploring the familiar and the novel object. Values are expressed as mean + SEM. ^p < 0.05 vs familiar object; §p < 0.05 vs VEH; *p < 0.05 vs DF 0.05; °p < 0.05 vs DF 0.25; #p < 0.05 vs DF 2 (Maccioni et al., 2021).

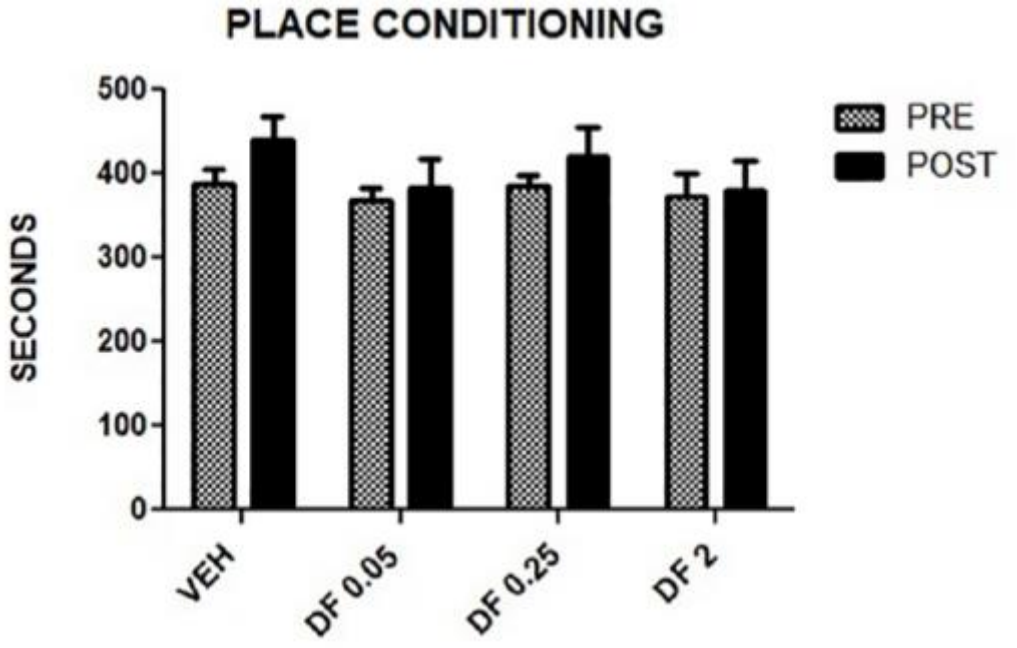


**Figure 15.** Effects of DF on place conditioning. Histograms represent the time (s) in the drug-paired compartment, before (pre) and after (post) conditioning. Values are expressed as mean + SEM (Maccioni et al., 2021).

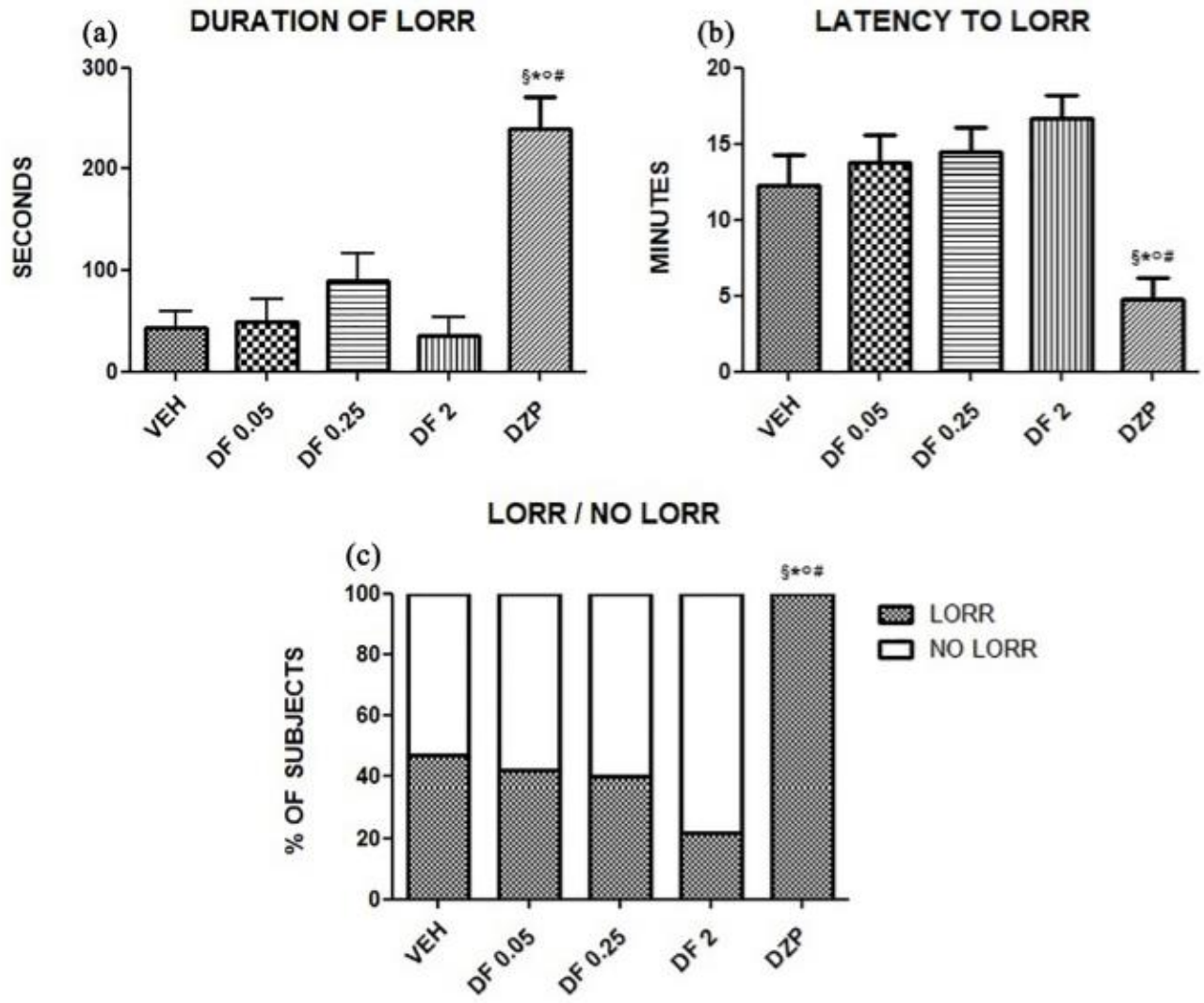


**Figure 16.** Effects of DF and DZP on ethanol-induced LORR. (a) Duration (s) of LORR. (b) Time (min) to LORR (latency). (c) Proportion of subjects in which ethanol induced/did not induce LORR, expressed as % of the total number of mice. Values are expressed as mean + SEM. §$p < 0.05$ vs VEH; *$p < 0.05$ vs DF 0.05; °$p < 0.05$ vs DF 0.25; #$p < 0.05$ vs DF 2 (Maccioni et al., 2021).

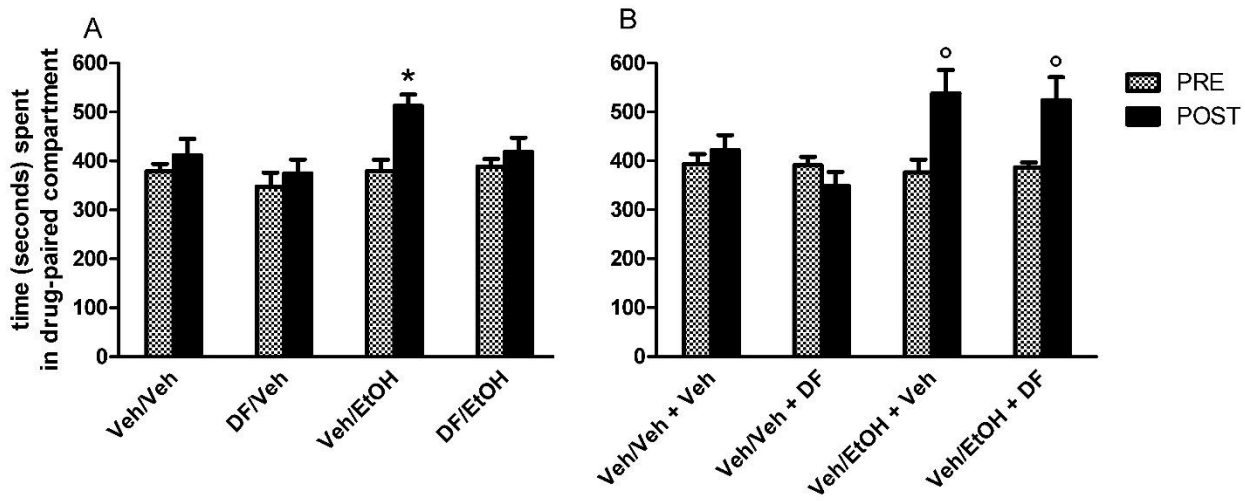


**Figure 17.** Effects of DF (2 mg/kg i.p.) on ethanol-induced CPP acquisition (A) and expression (B). Histograms represent the time (seconds/900) spent in the drug-paired compartment before and after conditioning expressed as mean + SEM. *indicates a significant difference ($p<0.05$) vs pre conditioning values (same group) in figure 17A; °indicates a significant difference ($p<0.05$) vs conditioning values (same groups) in figure 17B.

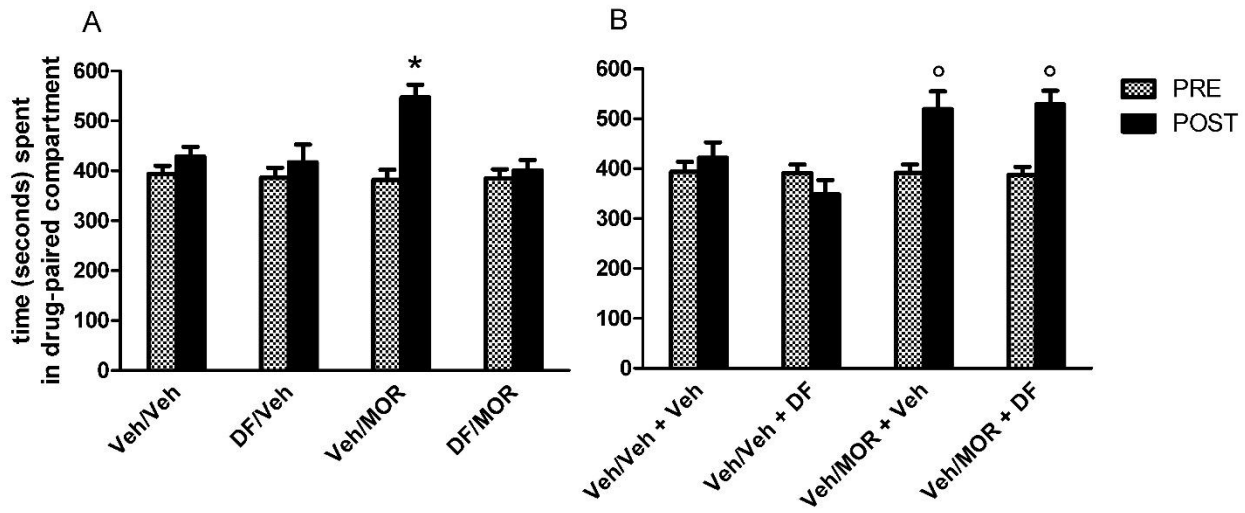


**Figure 18.** Effects of DF (2 mg/kg i.p.) on morphine-induced CPP acquisition (A) and expression (B). Histograms represent the time (seconds/900) spent in the drug-paired compartment before and after conditioning expressed as mean + SEM. *indicates a significant difference ($p<0.05$) vs all groups in figure 18A; °indicates a significant difference ($p<0.05$) vs the Veh/Veh+Veh and Veh/Veh+DF groups in figure 18B.

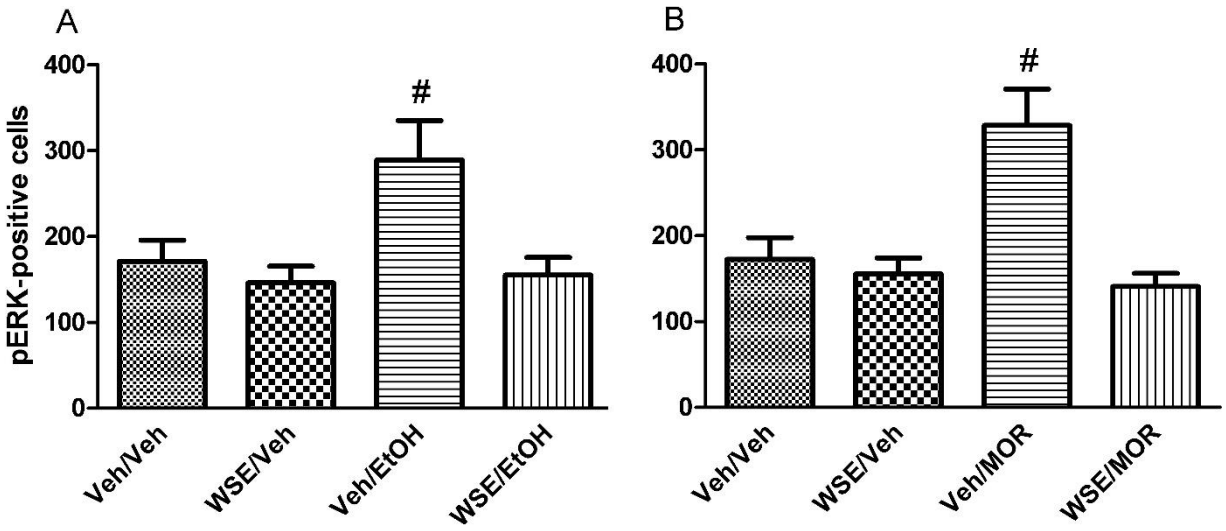


**Figure 19.** Effects of acute administration of *WSE* (50 mg/kg i.p.) on the expression of ethanol (2 g/kg i.p.)- (A) and morphine (5 mg/kg i.p.)- (B) elicited pERK-positive neurons in the AcbSh of adult male CD1 mice. Data are shown as mean + SEM of pERK-positive neurons/area. #indicates a significant difference ($p<0.05$) vs all groups.

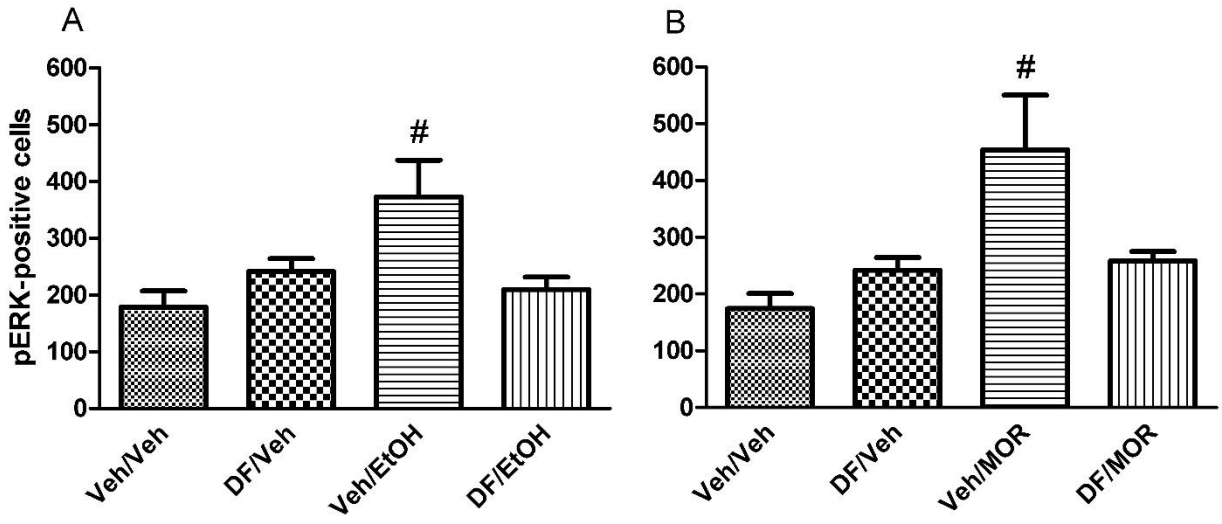


**Figure 20.** Effects of acute administration of DF (2 mg/kg i.p.) on the expression of of ethanol (2 g/kg i.p.)- (A) and morphine (5 mg/kg i.p.)- (B) elicited pERK-positive neurons in the AcbSh of adult male CD1 mice.. Data are shown as mean + SEM of pERK-positive neurons/area. # indicates a significant difference ($p<0.05$) vs all groups.

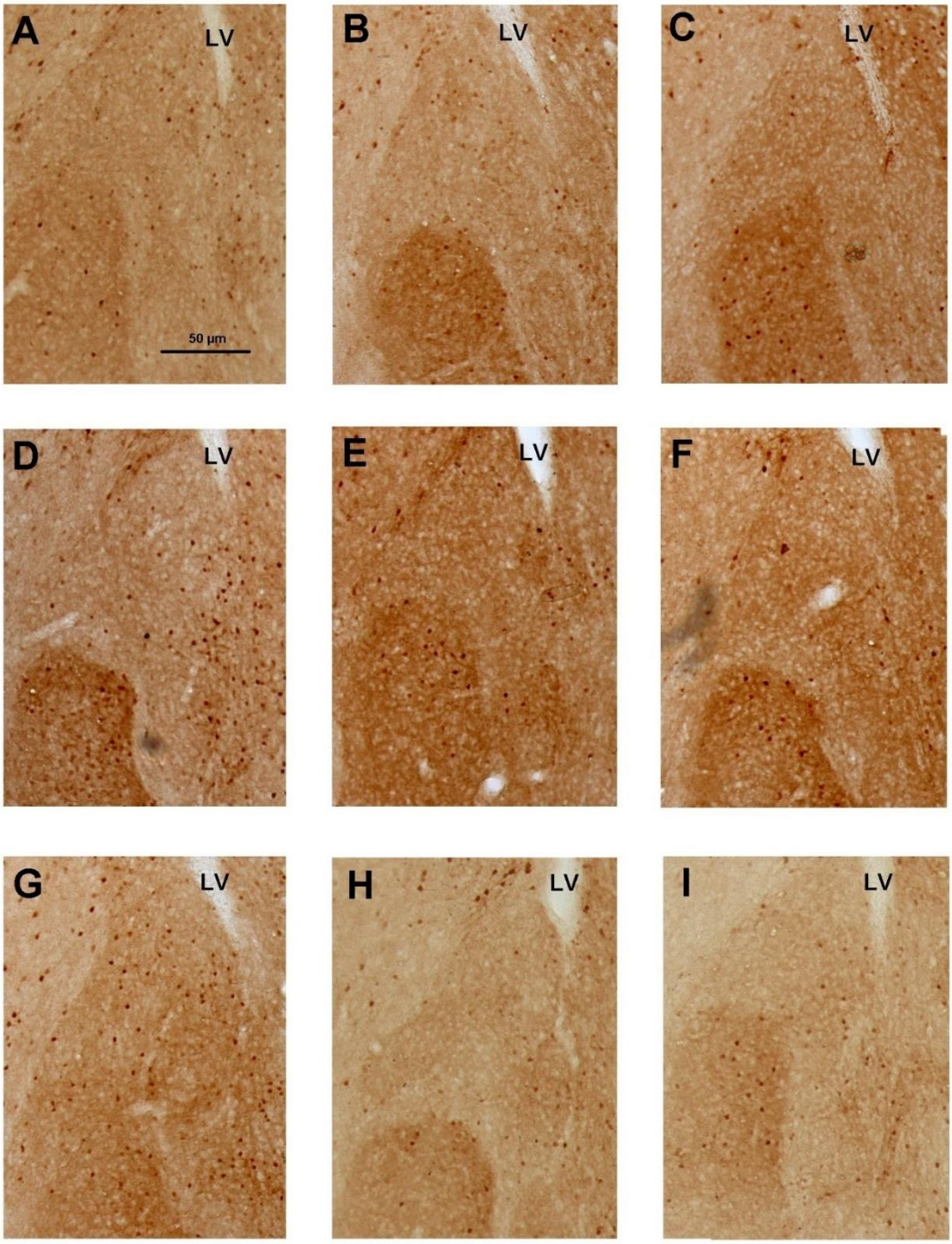


**Figure 21.** Low (10X) magnification representative images of the effects of the acute administration of *WSE* and DF on ethanol- and morphine-elicited pERK-immunostaining in the AcbSh from mice representative of treatment groups: A) Veh/Veh, B) *WSE*/Veh, C) DF/Veh, D) Veh/EtOH, E) *WSE*/EtOH, F) DF/EtOH, G) Veh/MOR, H) *WSE*/MOR, I) DF/MOR. Abbreviations: LV - lateral ventricle.

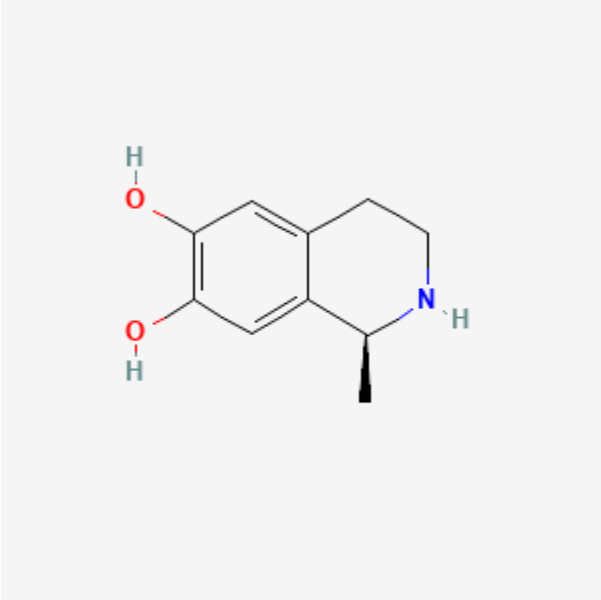


**Figure 22.** Chemical structure of Salsolinol

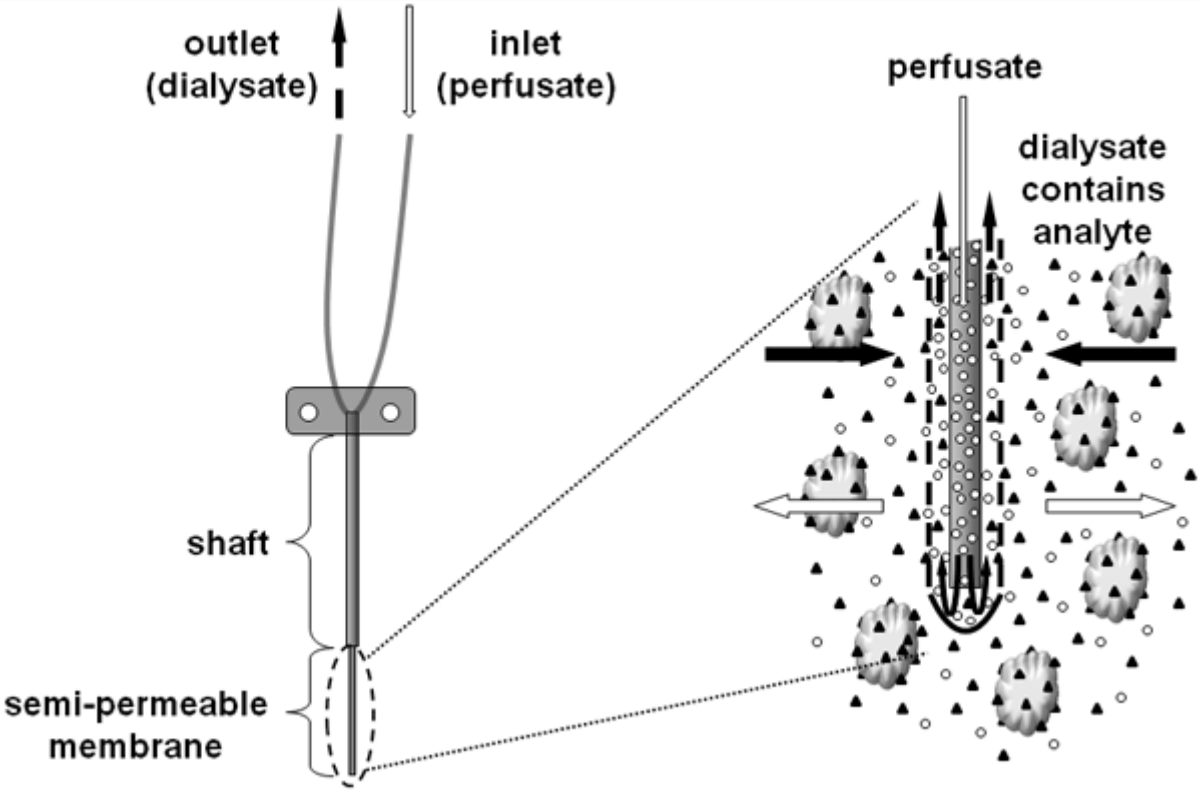


**Figure 23.** Schematic representation of a microdialysis probe. Isotonic fluid (perfusate) is perfused in the brain through the inlet fiber; the semi-permeable membrane, inserted in the area of interest, allows osmotic exchanges of molecules between the perfusate and the brain. The dialysate will be collected from the outlet fiber and will be enriched of brain-derived molecules. The analysis of the dialysate will be performed through HPLC.

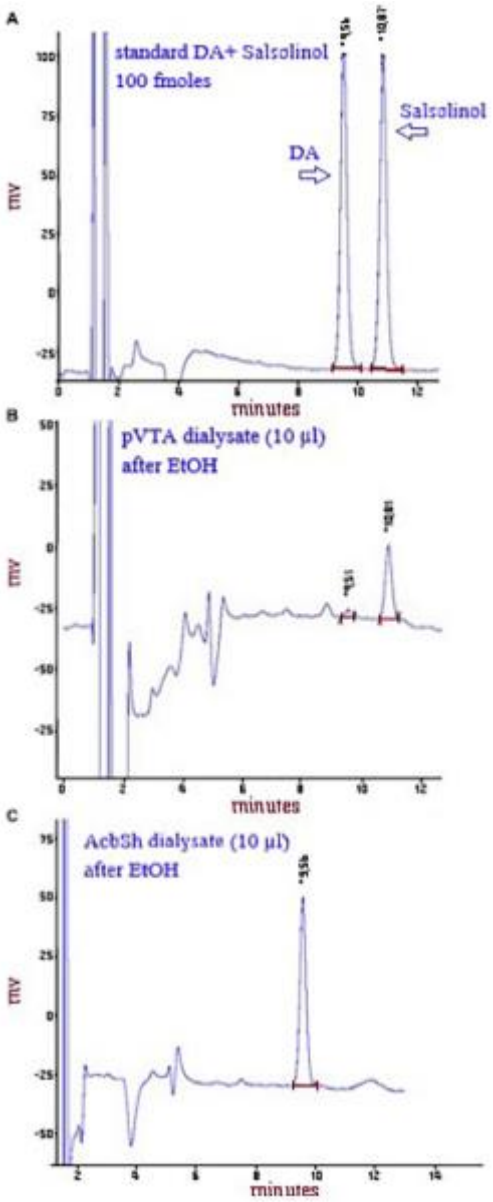


**Figure 24.** Representative chromatograms showing the peaks of DA and salsolinol after the HPLC injection of a standard solution of 100 fmol of both DA and salsolinol/10 ml (A) or of DA and salsolinol after the HPLC injection of a pVTA dialysate (10 ml) from a rat administered ethanol (B) or of DA, but not salsolinol after the HPLC injection of an AcbSh dialysate

(10 ml) from a rat administered ethanol (C) (Bassareo et al., 2021).

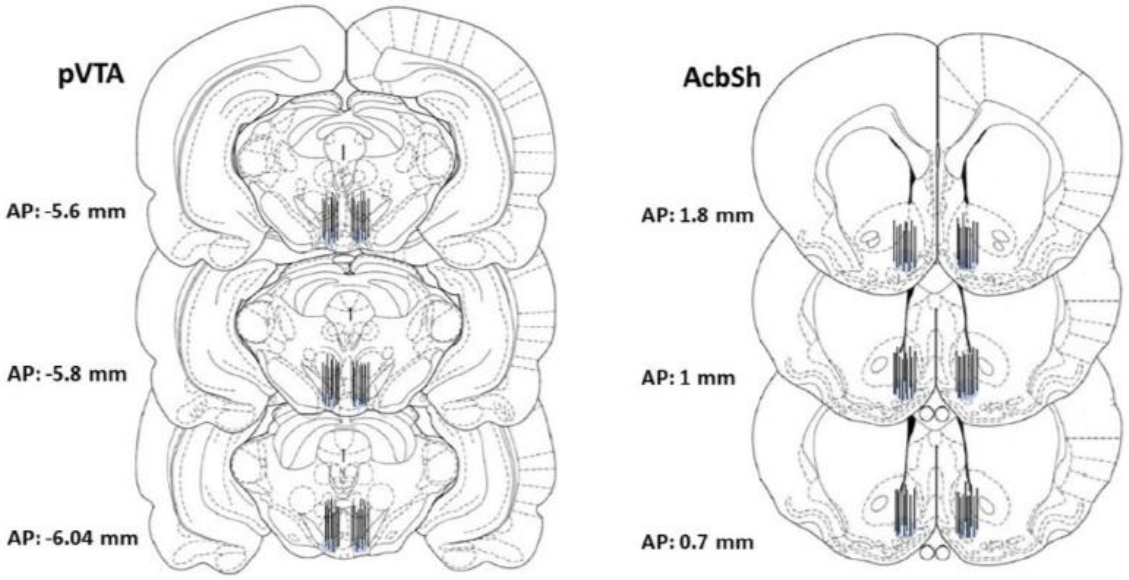


**Figure 25.** Representative images of the localization, within the pVTA and the AcbSh, of the dialyzing portion of dialysis probes drawn, after histological examination, in the brain atlas plates showing the pVTA and the AcbSh at different AP distances from bregma according to the rat brain atlas of Paxinos and Watson (1998) (Bassareo et al., 2021).

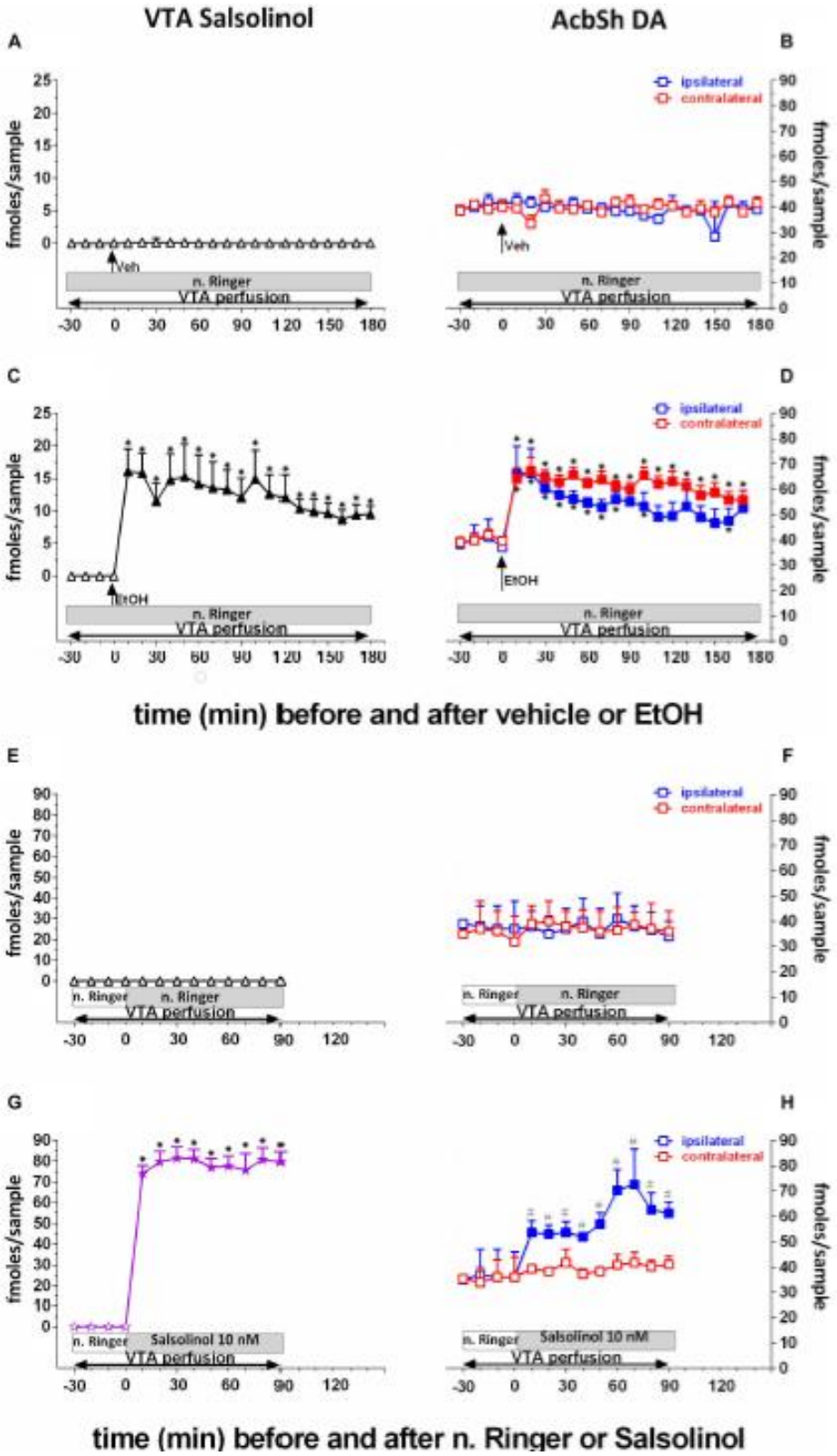
VTA Salsolinol
AcbSh DA
A
B
C
D
E
F
G
H
fmoles/sample
ipsilateral
contralateral
Veh
EtOH
n. Ringer
VTA perfusion
Salsolinol 10 nM
time (min) before and after vehicle or EtOH
time (min) before and after n. Ringer or Salsolinol

**Figure 26.** Effects of intragastric administration of vehicle (tap water, 10 ml/kg) (A, B) or ethanol (EtOH) (1 g/kg, 20% v/v) (C, D) or of reverse dialysis application in the pVTA of normal Ringer (n. Ringer) (E, F) or salsolinol (10 nM) (G,H), on pVTA salsolinol (A,C,E,G) and on ipsilateral and contralateral AcbSh DA (B,D,F,H) dialysates. The purple color was used here to highlight the fact that indeed these concentrations of salsolinol were from the reverse dialysis application of salsolinol (10 nM)-enriched n. Ringer. Horizontal bars depict the contents of the pVTA perfusion fluid along the experiments. Vertical arrows indicate the last pVTA or AcbSh sample before vehicle or EtOH administration. Filled symbols indicate samples representing $p < 0.001$ vs. basal; $p < 0.01$ vs. vehicle administration; #$p < 0.01$ vs. contralateral area (Bassareo et al., 2021).

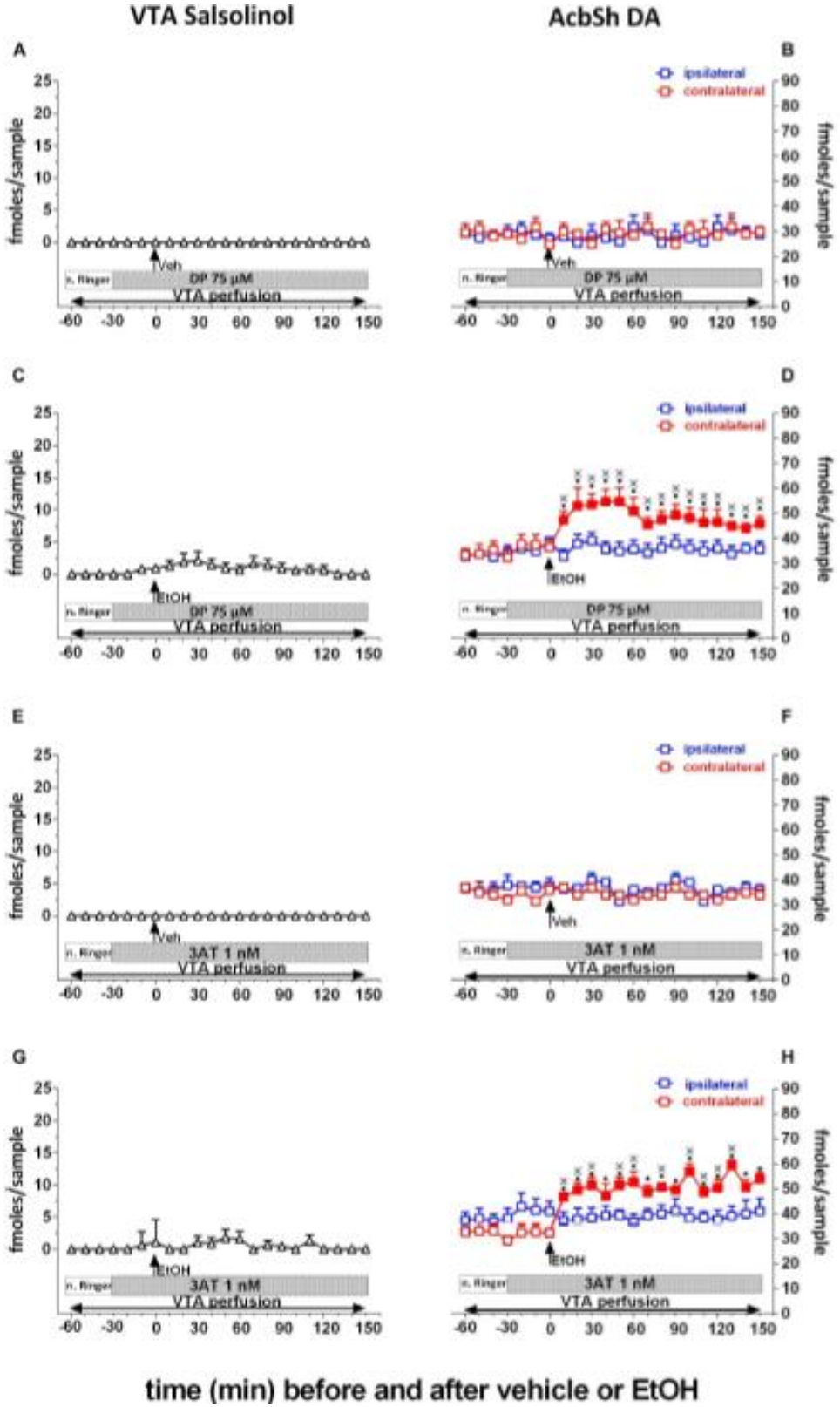
VTA Salsolinol
AcbSh DA
A
B
C
D
E
F
G
H
fmoles/sample
ipsilateral
contralateral
Veh
EtOH
DP 75 µM
3AT 1 nM
VTA perfusion
time (min) before and after vehicle or EtOH

**Figure 27.** Effects of intragastric administration of vehicle (tap water, 10 ml/kg) (A, B, E, F) or ethanol (EtOH) (1 g/kg, 20% v/v) (C, D, G, H) in the presence of reverse dialysis application in the pVTA, beginning 30 min before EtOH administration, of D-penicillamine (DP) (75 mM) (A–D) or 3-amino-1,2,4-triazole (3AT) (1 nM) (E–H) on pVTA salsolinol (A,C,E,G) and on ipsilateral and contralateral AcbSh DA (B,D,F,H) dialysates. Horizontal bars depict the contents of the pVTA perfusion fluid along the experiments. Vertical arrows indicate the last pVTA or AcbSh microdialysis sample before vehicle or EtOH administration. Filled symbols indicate samples representing $p < 0.001$ vs. basal; $p < 0.01$ vs. vehicle administration; x$p < 0.01$ vs. ipsilateral area (Bassareo et al., 2021).

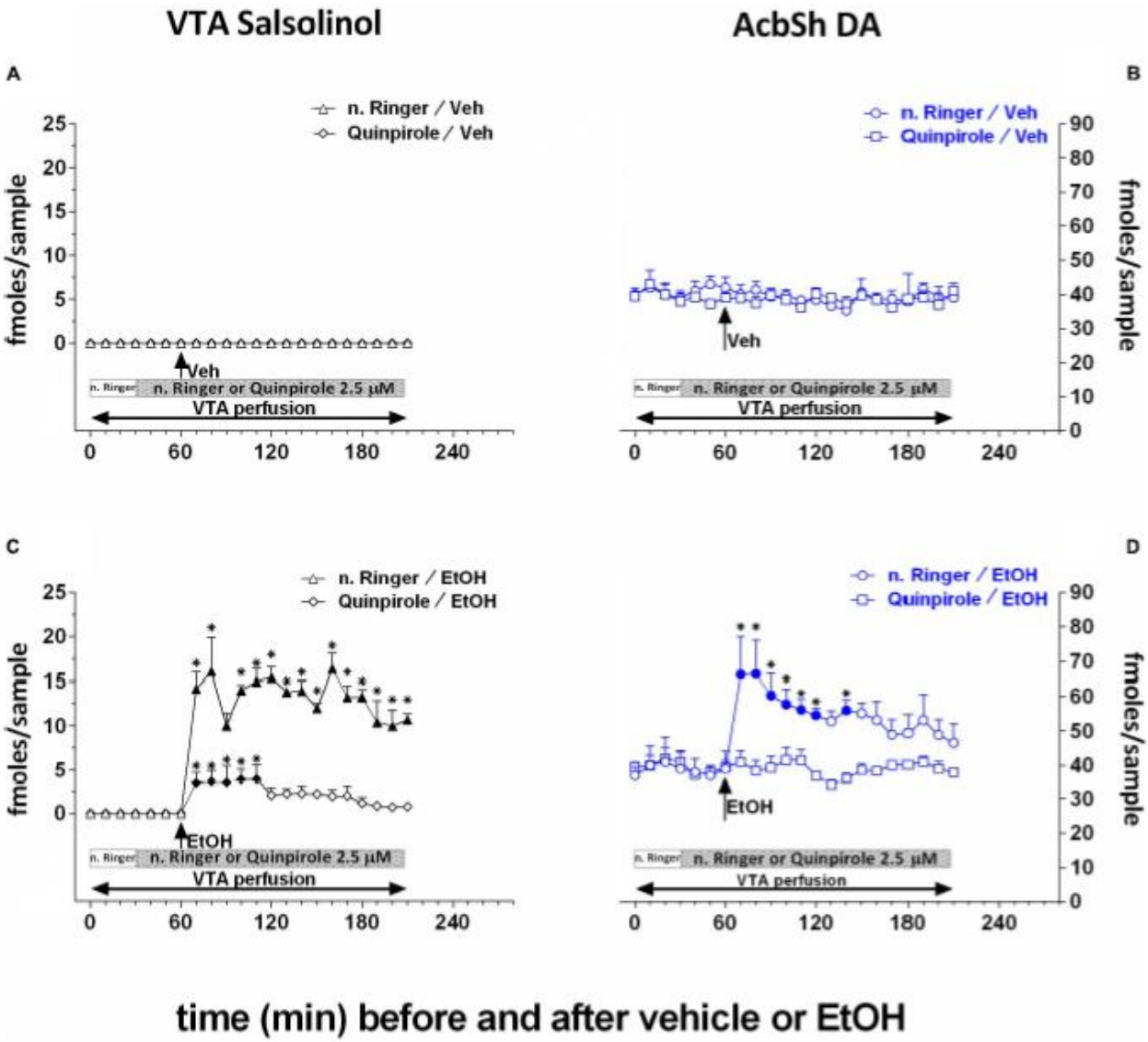
VTA Salsolinol
AcbSh DA
A
B
C
D
n. Ringer / Veh
Quinpirole / Veh
n. Ringer / EtOH
Quinpirole / EtOH
fmoles/sample
Veh
EtOH
n. Ringer
n. Ringer or Quinpirole 2.5 µM
VTA perfusion
time (min) before and after vehicle or EtOH

**Figure 28.** Effects of intragastric administration of vehicle (tap water, 10 ml/kg) (A, B) or ethanol (EtOH) (1 g/kg, 20% v/v) (C, D) in the presence of reverse dialysis application in the pVTA, beginning 30 min before vehicle or EtOH administration, of normal Ringer (n. Ringer) or quinpirole (2.5 mM) on pVTA salsolinol [(A,C); triangles: n. Ringer; lozenges: quinpirole] and on ipsilateral AcbSh DA dialysates [(B,D); circles: n. Ringer; squares: quinpirole]. Horizontal bars depict the contents of the pVTA perfusion fluid along the experiment. Vertical arrows indicate the last pVTA or AcbSh microdialysis sample before vehicle or EtOH administration. Filled symbols indicate samples representing $p < 0.001$ vs. basal; $^{*}p < 0.05$ vs. vehicle (Bassareo et al., 2021).

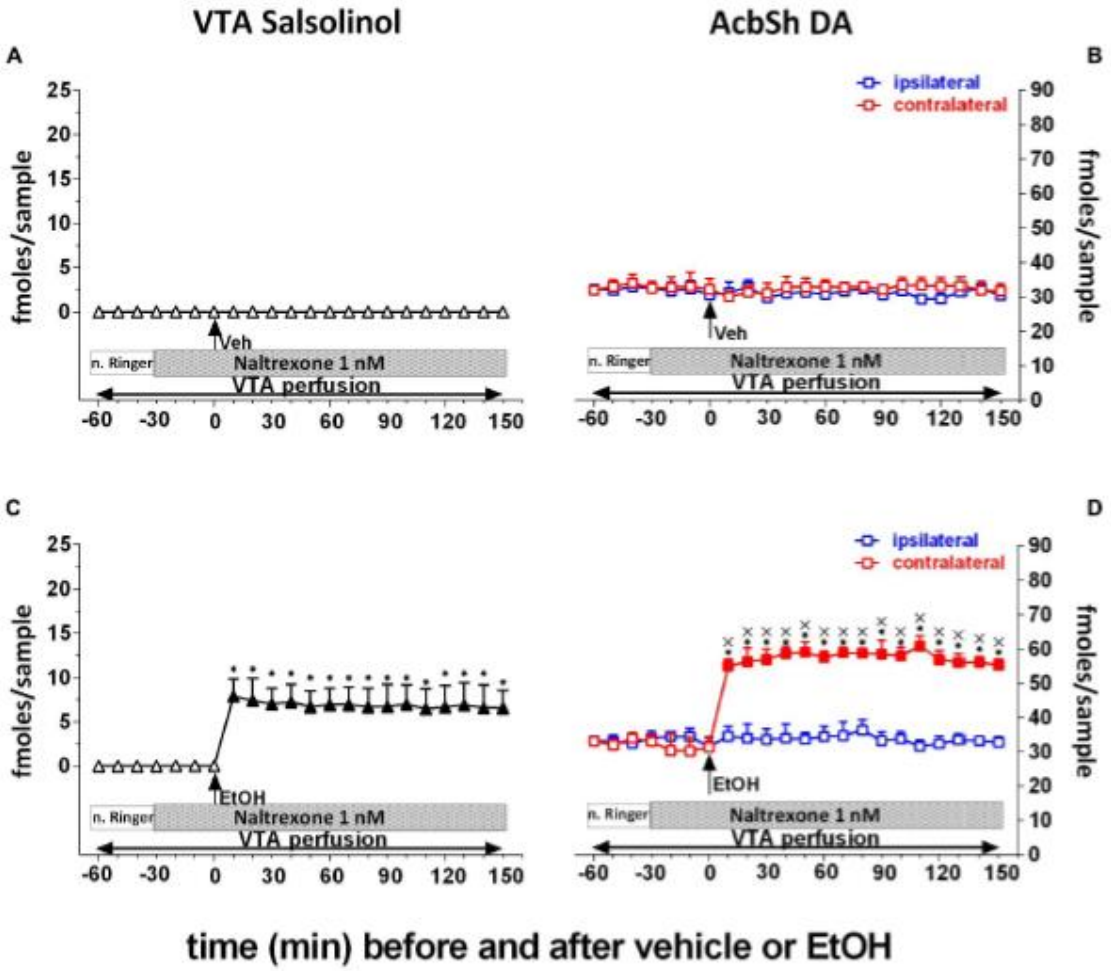


**Figure 29.** Effects of intragastric administration of vehicle (tap water, 10 ml/kg) (A, B) or ethanol (EtOH) (1 g/kg, 20% v/v) (C, D) in the presence of reverse dialysis application in the pVTA, beginning 30 min before EtOH administration, of naltrexone (1 nM) on VTA salsolinol (A,C) and on ipsilateral and contralateral AcbSh DA (B,D) dialysates. Horizontal bars depict the contents of the pVTA perfusion fluid along the experiment. Vertical arrows indicate the last

pVTA or AcbSh microdialysis sample before vehicle or EtOH administration. Filled symbols indicate samples representing $p < 0.001$ vs. basal; *$p < 0.01$ vs. vehicle administration; xp < 0.01 vs. ipsilateral area (Bassareo et al., 2021).

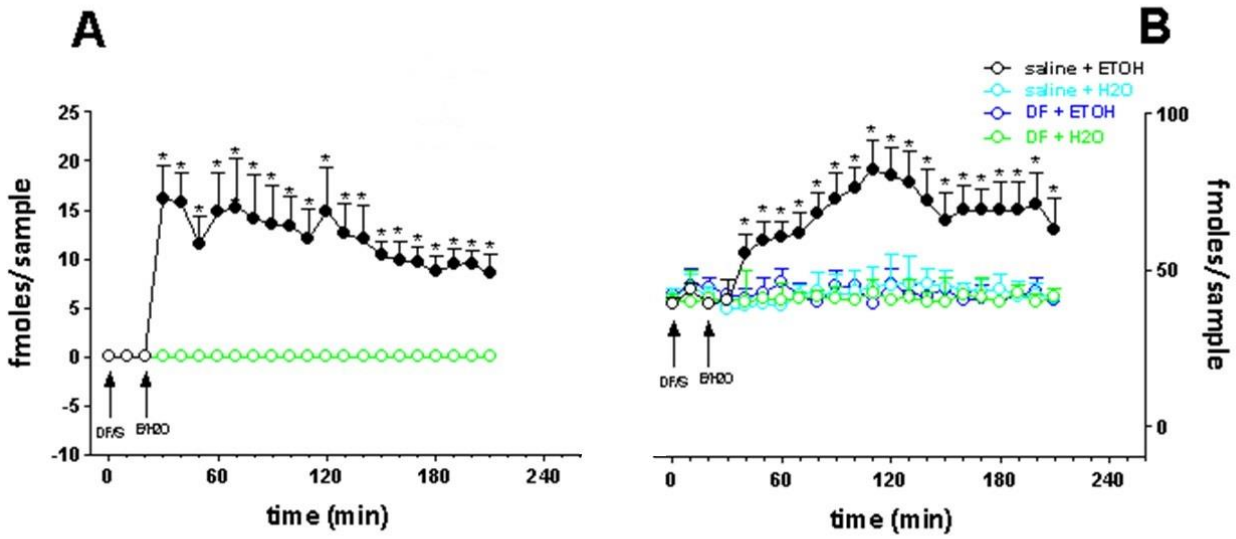


**Figure 30.** Effects of intragastric administration of vehicle (tap water, 10 ml/kg) or ethanol (EtOH) (1 g/kg, 20% v/v) in the presence of reverse dialysis application in the pVTA of normal Ringer (n. Ringer) on VTA salsolinol (A) and on AcbSh DA (B) dialysates. Vertical arrows indicate the last pVTA or AcbSh microdialysis sample before vehicle or EtOH administration and the moment of administration of saline or DF (DF/S), which was exactly 30 minutes before ethanol administration. Filled symbols indicate samples representing $p < 0.001$ vs. basal; *$p < 0.01$ vs. vehicle administration and saline pre-treatment.

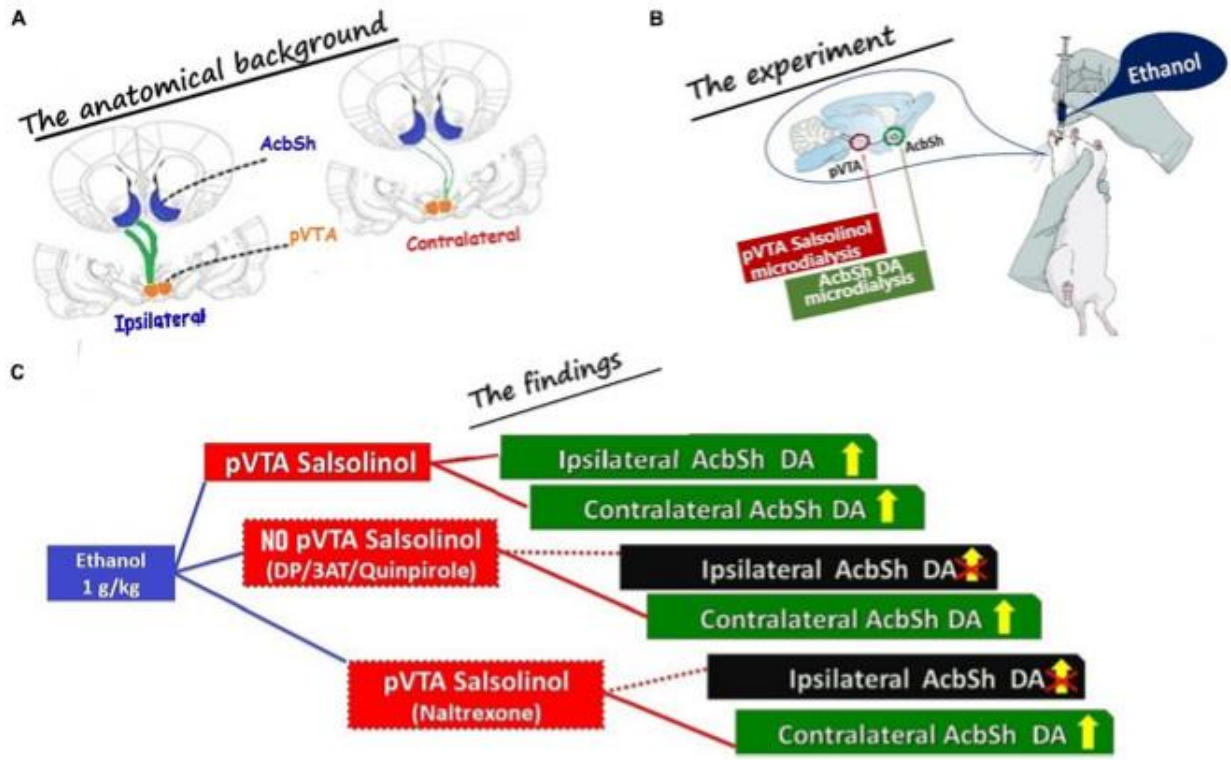


**Figure 31.** Schematic drawing summarizing (A) the anatomical background exploited during the microdialysis experiments (The anatomical background); (B) the main experimental conditions (The experiment): (i) ethanol was administered systemically, (ii) salsolinol was sampled from the posterior ventral tegmental area (pVTA), and (iii) DA was sampled from the shell of the nucleus accumbens (AcbSh) of the same (ipsilateral) or of the opposite (contralateral) side with respect to the pVTA; and (C) the main results (The findings; shown in Figures 3–6). The projections from the pVTA to the AcbSh of the same side (ipsilateral) are shown as thick green lines to indicate that these are prevailing for > 95% (Jaeger et al., 1983; Geisler and Zahm, 2005; Ikemoto, 2007; Breton et al., 2019) on those aiming at the AcbSh of the opposite side (contralateral) shown as green thin lines to indicate that these are only a minority (<5%). Dotted red lines are used in (C) to "connect" the box indicating the pVTA where the formation of salsolinol was prevented (DP or 3AT or quinpirole) and indicating the ipsilateral AcbSh where DA release was not stimulated by systemic ethanol. Similarly, a dotted red line is also used in (C) to "connect" the box indicating the pVTA, where there is formation of salsolinol and m

opioid receptors are blocked by naltrexone, and indicating the ipsilateral AcbSh where DA release was not stimulated by systemic ethanol (Bassareo et al., 2021).