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IN VIVO CHARACTERIZATION OF THE ANTI-ADDICTIVE PROPERTIES OF COR659 IN RODENTS

Scientific Disciplinary Sector(s)

BIO/14 - Pharmacology

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1. Introduction

1.1. Alcohol Use Disorder

According to the Diagnostic and Statistical Manual of Mental Disorders – DSM (5th edition, 2013), alcohol use disorder (AUD) is a chronic brain disease characterized by compulsion in seeking and drinking alcohol, loss of control over its intake, and relapse after long periods of abstinence. It has been estimated that in 2018, 14.4 million people were affected by AUD in USA, 9.2 million men and 5.3 million women, aged 18 and older (SAMHSA, 2018). Comparable numbers have been collected in recent surveys aimed at assessing AUD prevalence among the European population (EISAH, 2019). These alarming data become more concerning in light of the approximately 401.000 adolescents aged between 12-17 years with a diagnosis of AUD (SAMHSA, 2018). Moreover, a drinking pattern called binge drinking - that consists in an excessive amount of alcohol consumed in a short period of time - has been pointed out as a risk factor for developing AUD (Miller et al., 2007; Addolorato et al., 2018, Jacob et al., 2020). In spite of a well-documented correlation between AUD and health, social, and family issues, alcohol is still one of the substances of abuse most used all over the world. Its behavioral effects typically depend on the dose ingested: at low doses people experience increased sociability (alcohol is often considered a social icebreaker or lubricant), disinhibition, and relief from anxiety, while progressively higher doses lead to judge and motor impairment, sedation, coma, and even death (Jacob et al., 2020; LaHood, 2020). Strategies to treat AUD include psychotherapy and pharmacological support. Currently, four drugs have been approved for treating AUD: disulfiram, acamprosate, naltrexone, and nalmefene (the latest available only in Europe). Disulfiram inhibits aldehyde dehydrogenase enzyme promoting the accumulation of acetaldehyde, the primary metabolite of alcohol. High

concentrations of acetaldehyde are responsible for several biological responses such as nausea, vomiting, headache, tachycardia, and sweating that contribute to limiting alcohol drinking. Acamprosate mechanism of action is not entirely understood but it appears to act as an antagonist of the glutamatergic N-Methyl-D-aspartate (NMDA) receptors and agonist of the ionotropic gamma-(γ)-aminobutyric acid receptors A ($GABA_A$), restabilizing a balance between the excitatory and inhibitory neuronal systems. Lastly, naltrexone and nalmefene are an antagonist and an antagonist/partial agonist of opioid receptors, respectively, that likely act attenuating the rewarding properties of alcohol (Witkiewitz et al., 2019). Still, relatively little is known about alcohol mechanism of action and molecular targets. One of the first theories, the *"lipid theory"*, postulated at the beginning of the 20th century, suggested that because of the small size and amphiphilic properties, the alcohol sedative/hypnotic effects could be due to its ability to interfere with the lipid membranes of the central nervous systems (CNS) (Meyer, 1899; Overton, 1901). Later, it was demonstrated that only at very high concentration of alcohol equal to 500 mg% (approximately 108.5 mM) could disrupt the lipid bilayer; alcohol effects like anxiolysis, disinhibition, motor incoordination, and sedation, typically induced by doses of alcohol <500 mg%, could not be explained by the lipid theory (Peoples et al., 1996). Nowadays, neurotransmitters, receptors, enzymes, and other molecules have been proposed as molecular targets by which alcohol affects the CNS (Matošić et al., 2016; Abrahao et al., 2017). Importantly, the rewarding effects of alcohol are mediated by several neurotransmitter systems. For instance, neurotransmitters like dopamine and endogenous opioids contribute to mediating the reinforcing properties of alcohol in the CNS, exerting critical roles especially during the acute reinforcing effects of alcohol drinking. In addition, recent studies demonstrated that the reinforcing properties of alcohol are also mediated by ghrelin, or "hunger hormone", that is produced by the stomach,

intestine and pancreas, and in small amounts in the brain. Ghrelin receptors have been found in different areas of the brain reward circuitry, and preclinical and clinical studies demonstrated that administration of ghrelin antagonist markedly reduced alcohol consumption in both laboratory animals and humans (Jerlhag, 2019; Farokhnia et al., 2019).

Chronic use of alcohol can lead to alcoholism, also referred to as alcohol addiction or AUD. Addiction can be described as constituted by three main stages: (i) binge/intoxication - the stage at which alcohol or another drug of abuse is consumed occasionally up to intoxicating amounts, and induces the perception of its rewarding (positive) effects, (ii) withdrawal/negative affect - the stage at which negative emotional states are perceived when access to alcohol or the drug of abuse is prevented, and (iii) preoccupation/anticipation - when alcohol or the drug of abuse is sought again after periods of withdrawal (Koob & Volkow, 2010, 2016). Interestingly, each stage of this "addiction cycle" involves different brain regions and also different neurotransmitters. Specifically, basal ganglia are involved during the binge/intoxication stage, and the reinforcing properties of alcohol are mostly mediated by dopamine and opioid systems. These same systems appear to be dysregulated during abstinence from chronic alcohol use (reduced activity). At this stage of withdrawal/negative affect, activation of stress neurotransmitters such as corticotropin-releasing factor (CRF), norepinephrine, and dynorphin, occurs in the extended amygdala. As a result, the contribution of both processes characterizes the negative emotional states that drive compulsive drug-taking (negative reinforcement). Lastly, the third stage of preoccupation/anticipation is characterized by altered executive function of the prefrontal cortex (PFC) and increased glutamatergic neurotransmission that promotes the perpetuation of this vicious cycle.

1.2. Mesocorticolimbic system

The mesocorticolimbic system is constituted by dopaminergic neurons located in the ventral tegmental area (VTA) that project their axons to specific brain regions: nucleus accumbens (NAc), amygdala, and PFC. The VTA is a heterogenous brain region composed by dopaminergic (60-65%), GABAergic (30-35%), and a smaller proportion of glutamatergic neurons (Swanson, 1982; Nair-Roberts et al., 2008).

Several studies demonstrated that natural rewards (i.e., food, water, sex) and substances of abuse, including alcohol, can stimulate VTA dopaminergic neurons, increasing dopamine release in the NAc and the dorsal caudate nuclei (Di Chiara & Imperato, 1988; Di Chiara 2002; Klawonn & Malenka, 2018). This increased concentration of dopamine, especially in the NAc shell, is responsible for positive feelings, euphoria, and reinforcement, and contributes to the development of alcohol and drug abuse. Activity of the dopaminergic neurons in the VTA is modulated both by GABAergic and glutamatergic inputs (Fig. 1) and takes part in the reward processing. For example, optogenetic studies demonstrated that activation of VTA GABA neurons produces aversion while their inhibition produces preference (van Zessen et al., 2012; Tan et al., 2012). GABAergic neurotransmission can, by an indirect mechanism, interfere with the release of dopamine induced by alcohol or drugs of abuse in the NAc, thus preventing their reinforcing properties. The modulation of the GABAergic neurotransmission therefore represents a potential pharmacological target.

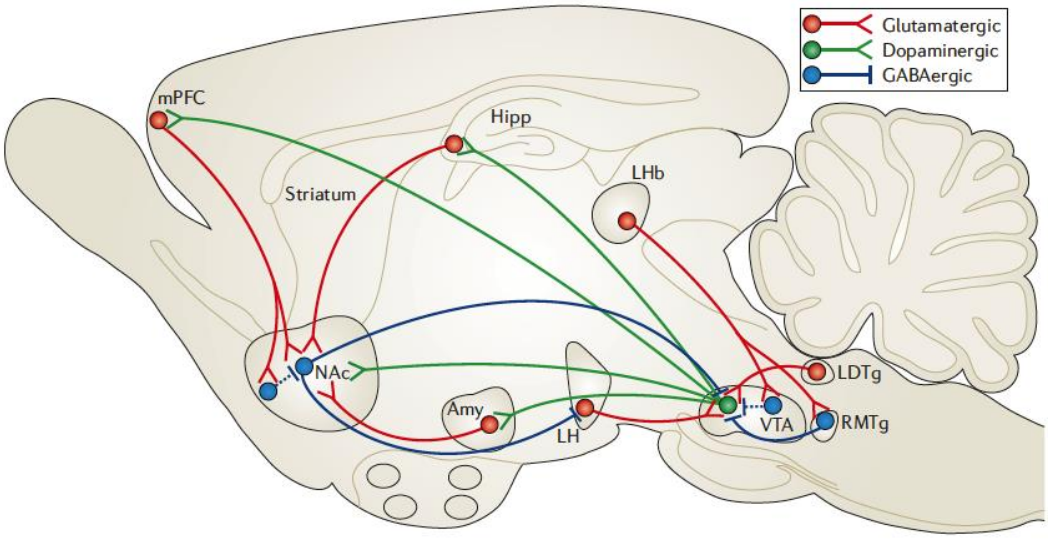

Fig. 1. Schematic representation of the brain reward circuitry in the rodent brain.
RMTg: rostromedial tegmentum; **VTA**: ventral tegmental area; **LDTg**: lateral dorsal tegmentum; **LHb**: lateral habenula; **LH**: lateral hypothalamus; **Amy**: amygdala: **Hipp**: hippocampus; **NAc**: nucleus accumbens; **mPFC**: medial prefrontal cortex. Figure from Russo & Nestler, 2013.

1.3. GABAergic receptors

GABA represents the main inhibitory neurotransmitter in the CNS and exerts its effects by interacting with three different receptor types called $GABA_A$, $GABA_B$, and $GABA_C$. The main difference between these three receptor types lies in their distinctive structure. Specifically, $GABA_A$ and $GABA_C$ are ionotropic receptors and their activation by the endogenous/exogenous agonist results in a change of chlorine concentrations inside and outside cells; because of this mode of activation, $GABA_A$ and $GABA_C$ receptors are involved in fast synaptic inhibition (Sieghart 2006; Kumar et al., 2009). Conversely, $GABA_B$ is a G-protein-coupled receptor capable of modulating calcium and potassium channels involved in slow inhibition, either presynaptic or postsynaptic (Frangaj et al., 2018; Park et al., 2020). The $GABA_B$ receptor is a heterodimer constituted by the association of two subunits, called $GABA_{B1}$ and $GABA_{B2}$, each of which is

composed by an N-terminal extracellular domain, a 7-helix transmembrane, and a cytoplasmic tail. Moreover, while $GABA_{B1}$ binds orthosteric ligands, $GABA_{B2}$ couples with G proteins (Fig. 2) (Frangaj et al., 2018; Park et al., 2020). $GABA_B$ receptors are widely distributed in the CNS. Particularly, high density of $GABA_B$ receptors has been found in thalamic nuclei, cerebellum, amygdala and cortex, and also in hippocampus, habenula, substantia nigra, VTA, NAc, globus pallidus, and hypothalamus (Castelli & Gessa 2016).

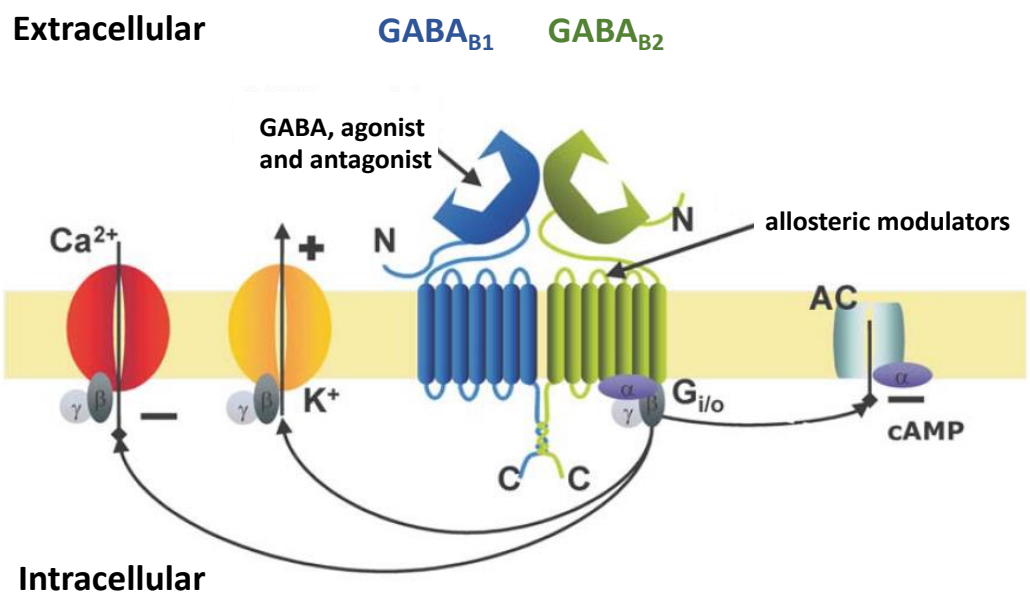

Fig. 2. Schematic representation of the $GABA_B$ receptor. Each $GABA_B$ receptor is a heterodimer constituted by the association of two subunits, $GABA_{B1}$ and $GABA_{B2}$. $GABA_{B1}$ presents the orthosteric binding site for exogenous/endogenous agonist and for exogenous antagonist (i.e., phaclofen, saclofen and 2-hydroxy saclofen) in the extracellular surface; $GABA_{B2}$ contains the site for allosteric modulation and is coupled with Gi/0 protein by which it modulates the activity of specific effectors: (i) adenylate cyclase (AC), (ii) potassium, and (iii) calcium channels. Figure adapted from Filip & Frankowska, 2008.

1.4. Positive allosteric modulators of the GABA$_B$ receptor

Allosteric modulators bind to a region of a receptor (the allosteric or allotopic site) that is topographically distinct from the binding site of the endogenous agonist (the primary or orthosteric site on the receptor). As a result of the interaction of an allosteric modulator to a receptor, the conformation of the receptor changes and, consequently, its affinity for the endogenous or exogenous agonist increases (positive allosteric modulation, PAM) or decreases (negative allosteric modulation, NAM) based on the molecule properties (Schwartz & Holst, 2007). Lack of intrinsic activity, inability to induce receptor downregulation, and high selectivity for specific receptor subtypes are the main useful characteristics that contributed to the development and characterization of allosteric modulators as potential therapeutic agents. The hypothesis, based on which GABAergic neurotransmission, mainly by its GABA$_B$ receptor, is involved in a wide range of diseases, including AUD and other substance use disorders (SUD), has been supported by multiple preclinical studies that showed how administration of baclofen, an orthosteric agonist of the GABA$_B$ receptor, effectively suppressed several alcohol- and drugs of abuse-related behaviors in rodents (Colombo & Gessa, 2018).

In 1976, the very first study that demonstrated baclofen ability to abolish alcohol-induced locomotor hyperactivity in mice was published (Cott et al., 1976), using a validated animal model of alcohol- and drug-induced euphorigenic effects in humans (Wise & Bozarth, 1987; Phillips & Shen, 1996). Since then, several other studies extended what observed by Cott and colleagues, confirming the involvement of the GABA$_B$ receptor in the stimulating effects induced by alcohol (Humeniuk et al., 1993; Shen et al., 1998; Chester at al., 1999; Broadbent et al., 1999; Boehm et al., 2002; Quintanilla et al., 2008; Holstein et al., 2009). The pharmacological profile of baclofen was then further investigated in different alcohol-related behaviors employing animal models that

well represented different aspects of AUD in humans. For instance, it has been demonstrated that baclofen was able to suppress (i) alcohol drinking under the 2-bottle "alcohol versus water" choice regimen (Daoust et al., 1987; Colombo et al., 2000; Stromber et al., 2004; Quintanilla et al., 2008; Villas et al., 2012; Peters et al., 2013); (ii) binge-like drinking (Kasten et al., 2015, 2016; Crabbe et al., 2017), (iii) Alcohol Deprivation Effect (ADE) (Colombo et al., 2003; 2006a; Vengeliene et al., 2018), (iv) operant oral alcohol-self administration, either under fixed ratio (FR) (Lorrai et al., 2016; Maccioni et al., 2005, 2012; Liang et al., 2006; Besheer et al., 2004; Walker et al., 2007) or progressive ratio (PR) schedules of reinforcement (Besheer et al., 2004; Walker et al., 2007; Maccioni et al., 2012), and (v) reinstatement of alcohol-seeking behavior (Maccioni et al., 2008a; Vengeliene et al., 2018) in rodents. Preclinical data collected to date on the anti-alcohol properties of baclofen possess remarkable translational value, since most of the effects observed in animals have subsequently been reproduced in AUD patients. Specifically, several clinical surveys reported substantial reductions in alcohol drinking and craving for alcohol after treatment with baclofen (Agabio et al., 2018; de Beaurepaire et al., 2019), making baclofen a promising pharmacotherapy for AUD. In this regard, recent studies by Weerts and colleagues demonstrated that baclofen efficiently reduced lever responding, amount of self-administered alcohol, and number of alcohol drinks in non-human primates (Duke et al., 2014). Authors showed also that when baclofen was administered during alcohol abstinence, it was not able to reduce alcohol drinking; conversely, when baclofen was administered during ongoing alcohol access it significantly reduced alcohol intake in baboons (Holtyn et al., 2017). These results collected in non-human primates were in close agreement with those found in a human study (Addolorato et al., 2007; Garbutt et al., 2010). However, narrow therapeutic index, scarce ability to pass the blood-

brain barrier (BBB), short length of action and rapid induction of tolerance, beyond several side effects (i.e., sedation, loss of muscle tone), strongly limit baclofen clinical use.

In light of these considerations, researchers moved their attention to identifying and developing allosteric modulators of the $GABA_B$ receptor, particularly PAMs. It has been indeed demonstrated that $GABA_B$ PAMs possess several advantages over the orthosteric agonist baclofen, for example (i) lack of intrinsic activity, so they enhance receptor activity only when and where needed physiologically, (ii) presence of a wide therapeutic window in which the drug can be safely used, and (iii) lack of tolerance development, that allow repeated administrations without need to increase dosage. Several studies indicated that $GABA_B$ PAMs possess disparate pharmacological effects *in vivo* that include anxiolytic, antidepressant, and antipsychotic properties, as demonstrated for CGP7930, GS39783, rac-BHFF, and ADX71441 (Frankowska et al., 2007; Cryan et al., 2004; Mombereau et al., 2004; Kalinichev 2017). In addition, $GABA_B$ PAMs display a promising use in treating drug addiction. In this respect, drugs like CGP7930 and GS39783 have been showed to prevent alcohol- (Kruse et al., 2012), amphetamine- (Wierońska et al., 2011), cocaine- (Lhuillier et al., 2007), and nicotine- (Lobina et al., 2011) induced locomotor hyperactivity in mice.

Most of the $GABA_B$ PAMs, i.e., CGP7930, GS39783, BHF-177, rac-BHFF, CMPPE, and ADX71441, have also been studied for their ability to reduce, and, in some cases, suppress, (i) alcohol self-administration (Maccioni et al. 2007, 2008b, 2009, 2010, 2012, 2015, 2018, 2019; Liang at el., 2006; Augier et a., 2017), (ii) cocaine (Smith et al., 2004; Filip et al., 2007b), and (iii) nicotine (Paterson et al., 2008; Vlachou et al., 2011) intravenous self-administration in rats. In addition, GS39783 and ADX71441 have been reported to reduce alcohol drinking in mice (Linsenbardt & Boehm, 2014; Hwa et al., 2014). Also, CGP7930, GS39783, BHF177, CMPPE

and ADX71441 have been shown to prevent reinstatement of cocaine- (Filip & Frankowska 2007a) and alcohol- (Leite-Morris et al., 2009; Augier et al., 2017; Maccioni et al.; 2019) seeking behavior in rats. Notably, in 2018, ASP8062, a $GABA_B$ PAM, entered a Phase 1 clinical trial, supporting the translational potential of this class of drug as for the pharmacotherapy for AUD and SUD (ClinicalTrials.gov ID: NCT04003402).

1.5. COR659

In this context, our laboratory started a collaboration with the Department of Biotechnology, Chemistry, and Pharmacy of the University of Siena (Italy), aimed at synthetizing, screening, and characterizing, both *in vitro* and *in vivo*, new chemical entities with potential PAM activity at the $GABA_B$ receptor. Evaluating the structural differences of GS39783, CGP7930, BHF177, and rac-BHFF, four well-characterized $GABA_B$ PAMs, the essential features that the novel molecule should possess to exert the desired pharmacological activity were identified. Subsequently, through virtual screening in a database, one chemical entity was found to satisfy all the required steric and electronic characteristics previously identified. This molecule was a 2-(acylamino) thiophene, renamed COR627 (compound #5571990 from the ChemBridge EXPRESS-Pick Database); COR627 and its simplified analog, COR628, represented the prototypes from where we started in search of novel $GABA_B$ PAMs (Castelli et al., 2012). A first result of our collaboration was the finding that changing the structure of COR627 by modifying the substituents connected to the thiophene ring led to obtaining a series of molecules with $GABA_B$ PAM activity (Castelli et al., 2012; Mugnaini et al., 2013). Specifically, these molecules were found to be able to potentiate GABA and baclofen-stimulated [35S] guanosine 5′-O-(3-thio) triphosphate([35S]GTPγS) binding to native $GABA_B$ receptors; importantly, both compounds

were devoid of any effect when given alone. Moreover, *in vivo* studies demonstrated that administration of *per se* ineffective doses of these compounds effectively potentiated the sedative/hypnotic effects of baclofen in DBA mice (Castelli et al., 2012; Mugnaini et al., 2013). In the wake of these initial results, several additional compounds were identified and synthesized. This new series included methyl 2-(4-chlorophenylcarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate, called COR659, which was selected as the most interesting and promising compound for the following reasons: i) it was one of the most potent compounds in potentiating the $GABA_B$ receptor activity, ii) it was not characterized by any intrinsic activity, iii) when tested *in vivo*, at *per se* ineffective doses, it effectively potentiated the sedative/hypnotic effect of baclofen in DBA mice, and iv) it possessed effectiveness even after oral administration (Castelli et al., 2012; Mugnaini et al., 2013). Additionally, calculation of COR659 drug-like properties using Cheminformatics software (http://www.molinspiration.com/) strongly supported its selection as a lead compound. In this analysis, the following physico-chemical parameters were evaluated: (i) logarithm of the octanol-water partition coefficient (ClogP); (ii) molecular weight (MW); (iii) number of hydrogen bond acceptors (HBA); (iv) number of hydrogen bond donors (HBD); (v) topological surface area (tPSA measured in Å2); and (vi) number of violations of Lipinski's rule of five (Lipinski et al., 2001). Based on ClogP, MW, and tPSA values of COR659, its elevated oral bioavailability and blood-brain barrier permeability were confirmed (Castelli et al., 2012; Mugnaini et al., 2013).

Initial *in vivo* studies demonstrated that acute administration of COR659 effectively reduced (i) alcohol and sucrose self-administration in Sardinian alcohol-preferring (sP) rats (Maccioni et al., 2017), and (ii) self-administration of a chocolate flavored beverage in Wistar rats (Maccioni et al., 2017). Conversely, acute treatment of COR659 did not affect regular food pellets self-

administration in Wistar rats (Maccioni et al., 2017). Subsequent studies demonstrated that pretreatment with SCH50911, an antagonist of the $GABA_B$ receptor, partially prevented the reducing effects of COR659 (Maccioni et al., 2017). Conversely, we found that pretreatment with AM4113, a neutral antagonist of the cannabinoid CB_1 receptor, blocked the reducing effect of COR659 on chocolate self-administration but not on alcohol self-administration in rats, suggesting that COR659 would have been able to act on a further receptor system (Maccioni et al., 2017). This hypothesis was later confirmed by *in vitro* studies that demonstrated that micromolar concentrations of COR659 (comparable with the doses tested in all *in vivo* studies conducted to date) displaced [3H]CP55940 from the cannabinoid CB_1 receptor binding site and inhibited WIN 55,212-2-induced stimulation of [35S]GTPγS binding via cannabinoid CB_1 receptors (Ferlenghi et al., 2020). Thus, COR659 is a PAM of the $GABA_B$ receptors with a composite mechanism that includes an antagonist/inverse agonist pharmacological profile at the cannabinoid CB_1 receptors.

1.6. Animal models of AUD: Sardinian alcohol-preferring (sP) rats.

sP rats represent one of the few rat lines of selectively bred for their high alcohol preference and consumption. The development of this line started in 1981 at the University of Cagliari, by Professors Fabio Fadda and Gian Luigi Gessa, from a heterogenous stock of outbred male and female Wistar rats. At the age of 75 days, rats were singly housed and exposed for 14 consecutive days to the standard 2-bottle "alcohol [10% (v/v)] versus water" choice regimen with unlimited access for 24 hours a day. Alcohol and water intake were recorded every day. Rats that displayed the highest daily alcohol intake were mated to start the sP line; on the contrary, rats that displayed the lowest alcohol drinking were mated to start the opposite line of non-preferring alcohol rats [Sardinian alcohol-non preferring (sNP)]. In an attempt to obtain the best bidirectional breeding,

two main criteria needed to be satisfied by rats that would be mated: (1) sP rats should have consumed daily an amount of alcohol equal to or higher than 4 g/kg and displayed a preference for alcohol equal to or greater than 2:1, while (2) sNP rats should have drunk daily an amount of alcohol equal to or lower than 1 g/kg and their preference for alcohol should have been equal to or lower than 0.2:1. After 40 generations, all sP and sNP rats satisfied the above mentioned criteria, and the selective breeding program was considered completed. In addition, at the end of the breeding program, sP rats displayed a consolidated drinking behavior of 6-7 g/kg, while their counterpart sNP rats consumed less than 0.5 g/kg under the standard 2-bottle "alcohol [10% (v/v)] versus water" choice regimen with unlimited access for 24 hours a day (Colombo et al., 2006b). As a point of interest, sP rats well satisfy the criteria proposed to define an adequate animal model of alcoholism (Cicero, 1980). In fact, sP rats voluntarily consume great amounts of alcohol that result in psychopharmacological blood alcohol levels (BALs) (Agabio et al., 1996) and central effects, including anxiolysis (Colombo et al., 1995) and stimulation of locomotor activity (Colombo et al., 1998). Moreover, sP rats are willing to "work" to obtain alcohol, and this behavior is pharmacologically manipulable (Lorrai et al., 2016). Also, sP rats displayed relapse-like drinking behavior after prolonged alcohol deprivation (ADE procedure) (Agabio et al., 2000; Serra et al., 2003), and reinstatement of alcohol-seeking behavior subsequent to extinction of lever-responding for alcohol (Maccioni et al., 2008, 2019). Lastly, sP rats exposed to a novel protocol of alcohol drinking under the 4-bottle "alcohol [10%, 20%, 30% (v/v)] versus water" choice regimen, with limited (1 h) and unpredictable daily access, consumed intoxicating amounts of alcohol during the last hours of the dark phase of the light/dark cycle. These alcohol intakes resulted in (i) pharmacologically relevant BALs (>100 mg%) and (ii) severe signs of intoxication (motor incoordination), satisfying the criteria for binge drinking in humans (Colombo et al., 2014;

2015). Since their phenotypic selection started in 1981, sP rats have been extensively characterized and, to date, they represent one of the most useful, reliable, and validated animal models to study several aspects of human alcohol drinking.

2. Aim of the Thesis

Based on this background, the aim of the present thesis is to provide a further characterization of the anti-addictive properties of COR659, a novel PAM of the $GABA_B$ receptor, in animal models of AUD and SUD. To accomplish this aim, the following research questions were addressed:

a) Does repeated treatment with COR659 induce the development of tolerance to the reducing effect of COR659 on alcohol self-administration?

b) Is treatment with COR659 able to suppress alcohol drinking in an animal model that mimics human alcohol craving and relapse?

c) Can COR659 interfere with binge-like drinking?

d) Does treatment with COR659 prevent locomotor stimulation induced by drugs of abuse?

3. Materials and Methods

The experimental procedures employed in the present study fully complied with European Directive no. 2010/63/EU and subsequent Italian Legislative Decree no. 26, March 4, 2014, on the "Protection of animals used for scientific purposes" and have been approved by the Italian Ministry of Health [authorization no. 342/2016-PR (including Supplementation of Feb. 22, 2018) and 338/2019-PR].

3.1. Animals

In Experiments 1, 2, and 3, independent groups of male sP rats bred in house (Neuroscience Institute, National Research Council of Italy, Section of Cagliari, Cagliari, Italy) from the 85th-90th generation were employed; while in Experiments 4 and 5, male C57BL/6J and CD1 (Charles River, Calco, Italy) mice were used, respectively. Rats and mice were located in two different colony rooms, both under an inverted 12:12 h light/dark cycle, at a constant temperature of 22 ± 2 °C and relative humidity of 60%. Water and food were always available except when otherwise reported.

3.2. Drugs

Alcohol 95% was obtained from Silvio Carta s.r.l., Oristano, Italy, and alcohol solutions [10%, 15%, 20%, and 30% (v/v)] were made using tap water. COR659 (synthesized at the Department of Biotechnology, Chemistry, and Pharmacy, University of Siena, Siena, Italy), was suspended in saline with 1% (w/v) Tween 80 and administered intraperitoneally 30 min before the start of the alcohol drinking session (Test Day of each experiment) or locomotor activity experiment. Administration volume of COR659 was 2 ml/kg in rats and 12.5 ml/kg in mice. All COR659 doses used in this study were previously demonstrated to be unable to alter spontaneous locomotor activity in either sP rats (Maccioni et al., 2017) or mice (this laboratory, unpublished results).

3.3. Apparatus

3.3.1. Operant chambers for alcohol self-administration and reinstatement of alcohol seeking behavior

Modular chambers (Med Associates, St. Albans, VT, USA), located in ventilated and sound-attenuated cubicles, were used. The front panel of each operant chamber was fitted with two retractable levers, one dual-cup liquid receptacle, two stimulus lights (one green and one white) mounted above each lever, and one tone generator (65 dB). Fluid delivery into the receptacle occurred via two 60-ml syringes connected by polyethylene tubes to two different pumps located outside each chamber. One of the syringes contained an alcoholic solution [10% (v/v) during the acquisition phase and 15% (v/v) during the maintenance and testing phases] and the other one contained water. Every time the rat pressed one of the levers and achieved the number of responses required (RR) to obtain a reward, the following events occurred concurrently: 1) one of the pumps, depending on which lever was pressed, was activated determining the delivery of 0.1 ml of fluid into the receptacle; 2) the light above the lever was switched on for the time period of fluid delivery; 3) the tone generator was activated. Right and left levers were respectively associated with alcohol and water for half of the animals, while for the other half the opposite condition was applied in a counterbalanced way, to avoid position preference. The rear panel of each operant chamber was equipped with a white light located close to the ceiling, that turned on or off indicating the start or the end of each alcohol self-administration session.

3.3.2. Cages for locomotor activity assessment

Mouse spontaneous locomotor activity was assessed in squared open field arenas [480 X 480 X 400 (h) mm]. Each motility cage was made of plexiglas, equipped with photocells (Motil, TSE, Bad Homburg, Germany), and connected to a computer. Photocells were spaced every 40-mm and their interruptions, due to mouse movements, were recorded as "motility counts". Motility cages were located in a sound-proof room close to the mouse colony room.

3.4. Experimental procedures

3.4.1. <u>Experiment 1</u> – Effect of repeated treatment with COR659 on alcohol self-administration in male sP rats

At the age of 45-days, sP rats were exposed to the two-bottle "alcohol [10% (v/v)] versus water" choice regimen with unlimited access for 24 h/day over 10 consecutive days in their home-cage. Under this regimen, sP rats consume alcohol in a stable manner, mostly during the dark phase of the light/dark cycle, averaging approximately 6-7 g/kg/day (Colombo et al., 2006). The main purpose of this alcohol pre-exposure phase was to acclimate rats to the taste and pharmacological effects of alcohol, resulting in a considerable reduction of the subsequent auto-shaping phase once they were introduced to the operant chambers (Lorrai et al., 2016). Subsequently, rats were trained to lever-respond for alcohol in the operant chambers during the first 4 to 6 h of the dark phase of the light/dark cycle, from Monday to Friday. At the beginning of the operant training, rats were water-deprived in their home-cage for 12 h before the start of each self-administration session in order to facilitate the acquisition of an operant responding behavior. During the first 4 daily sessions, rats were exposed to a fixed ratio (FR) 1 (FR1) schedule of reinforcement for 10% alcohol

(v/v), while, over the subsequent 4 sessions, FR was progressively increased to FR2 and FR4. At this point, alcohol concentration was switched from 10% (v/v) to 15% (v/v; final concentration). Rats were then allowed to lever-respond for water (FR1) or alcohol (FR4) every other day during the 4 subsequent self-administration sessions; from then onwards, animals that well discriminated between the water and alcohol levers were moved to the maintenance phase during which both levers were concomitantly available and session length was set at 30 min. After 20 sessions of alcohol self-administration of the maintenance phase, rats that displayed the most stable and consistent lever-responding behavior were promoted to the testing phase. Specifically, 4 independent groups of rats (n=10 each) matched for the number of lever-responses on the alcohol lever and the amount of self-administered alcohol over the last 3 self-administration sessions of the maintenance phase, were injected intraperitoneally (i.p.) with COR659 at the doses of 0, 2.5, 5 and 10 mg/kg, once a day for 10 consecutive days, 30 min before the start of each daily alcohol self-administration session (Treatment phase). The dose range of COR659 was chosen based on previous experiments that demonstrated its ability to reduce alcohol self-administration in sP rats when administered acutely (Maccioni et al., 2017). At the end of the 10-day treatment, rats were allowed to self-administer alcohol for 5 additional consecutive sessions, providing a way to evaluate their recovery from COR659 anti-alcohol effects (Post-treatment phase). The number of lever-responses for alcohol and the amount of alcohol (g/kg) self-administered by rats during each phase (Pre-treatment, Treatment, and Post-treatment Phase) were statistically analyzed by separate 2-way (treatment, time) ANOVAs with repeated measures on factor “time”, followed by Tukey post hoc test for multiple comparisons.

3.4.2. ***Experiment 2*** *– Effect of acute treatment with COR659 on reinstatement of alcohol seeking in male sP rats*

At the end of the maintenance phase, an independent set of 44 sP rats were exposed to consecutive daily sessions (60-min long) of extinction responding during which lever-responding was unreinforced. Also, the syringe pumps, stimulus lights, and tone generator were off. An extinction criterion of 10 or fewer responses on the alcohol lever per session for 2 consecutive sessions was established. Once the extinction criterion was reached, rats were divided into 4 groups of n=11 each, matched for the number of responses on the alcohol lever over the first 3 sessions of the extinction responding phase. The day after, rats were treated with COR659 (0, 2.5, 5 and 10 mg/kg) 30 min before the start of the reinstatement session. This session was characterized by exposing rats to a stimulus complex (previously associated with alcohol availability) 10 times within 20 seconds. Specifically, the following events occurred: activation of a tone, turning on of the stimulus lights, and availability every other time of 0.1 ml alcohol (15%, v/v) in the receptacle for a total number of 5 presentations. The log-rank (Mantel Cox) test and 1-way ANOVA were run to evaluate the number of extinction responding sessions needed to reach the extinction criterion; in addition, a 2-way [phase (extinction/reinstatement); treatment] ANOVA with repeated measures on the factor "phase" followed by Tukey's post hoc test for multiple comparisons was performed.

*3.4.3. **Experiment 3** – Effect of acute treatment with COR659 on binge-like drinking in male sP rats*

Two independent sets (n=64 each) of 60-day old sP rats were employed to investigate the alcohol reducing effects of 2 different dose ranges of COR659, low (0, 2.5, 5, and 10 mg/kg) and high (0, 10, 20, and 40 mg/kg), on a recently developed model of binge-like drinking in sP rats, resulting

from their exposition to the 4-bottle "alcohol [10%, 20%, and 30% (v/v)] versus water" choice regimen protocol. Previous studies demonstrated that sP rats, when exposed to limited 1-h daily drinking sessions, occurring during the dark phase of the light/dark cycle, with multiple alcohol concentrations and unpredictability of time of access to alcohol, consume excessive amounts of alcohol (>2 g/kg), especially when the session occurs over the last hours of the dark phase of the light/dark cycle (Colombo et al., 2014, 2015).

Briefly, this procedure is constituted by two different subsequent stages, called Phase 1 and Phase 2. During Phase 1, rats had unlimited access (24 h/day) to 4 bottles in their home-cage: one containing water and the other ones containing 3 different alcohol solutions [10%, 20%, and 30% (v/v)] over a period of 12 consecutive days. At the end of Phase 1, the rats were exposed to the above mentioned "4-bottle choice" regimen again, but with limited access of 1 h per day during the dark phase of the light/dark cycle, for 12 consecutive days (Phase 2). Over the 12 days of Phase 2, time of alcohol drinking session was determined in a semi-random way so that the rats experienced alcohol drinking during all 12 hours of the dark phase of the light/dark cycle. For one set of rats the time sequence used was: 3rd, 8th, 11th, 2nd, 9th, 6th, 10th, 4th, 1st, 12th, 5th, and 7th hour of the dark phase of the light/dark cycle; while the time sequence for the second set of rats was 6th, 3rd, 8th, 1st, 5th, 11th, 4th, 12th, 10th, 2nd, 7th and 9th hour of the dark phase of the light/dark cycle. Once Phases 1 and 2 were completed, rats from each set were matched for their body weight, alcohol intake, and time sensitivity over Phase 2 and allocated into four groups of n=16 rats. The day after, called "Test Day", animals from the first set were injected i.p. with COR659 at the dose of 0, 2.5, 5, and 10 mg/kg; while animals from the second set were injected i.p. with COR659 at the dose of 0, 10, 20, and 40 mg/kg. Time of drug delivery was 30 min before the start of the alcohol drinking session at the 12th-h of the dark phase of the light/dark cycle. Alcohol (g/kg) and

water (ml/kg) intake was measured by weighing the bottles immediately before and after each drinking session (0.01-g accuracy) and analyzed by separate 1-way ANOVAs with repeated measures for factor "time" during Phase 2. In addition, regression analysis of the mean of alcohol intake versus time of the drinking session over Phase 2 was performed and the correlation coefficient was calculated. On the Test Day, data on alcohol and water intake was analyzed by separate 1-way ANOVAs followed by Tukey's post hoc test for multiple comparisons, when statistical significance was reached.

3.4.4. Experiment 4 – Effect of acute treatment with COR659 on binge-like drinking in male C57BL/6J mice

Forty-eight C57BL/6J mice, 6-weeks old, were singly housed and, after 1-week acclimation, exposed to the "Drinking in the Dark" (DID) procedure. Typically, C57BL/6J mice under this procedure consume intoxicating amounts of alcohol and achieve high BALs (≥80 mg%), providing a useful animal model of excessive alcohol drinking in a limited time period, also known as binge-like drinking behavior. In the present study, according to the protocol set by Rhodes (Rhodes et al., 2005), starting 3 hours into the dark phase of the light/dark cycle, mice were allowed to access to a bottle containing 20% (v/v) alcohol instead of water for a limited time of 2-h over three consecutive days (Days 1-3, acquisition phase). On the fourth day (Day 4), mice previously matched for their body weight and alcohol intakes over the acquisition phase were allocated into 4 groups (n=12 each) and injected i.p. with COR659 at the doses of 0, 10, 20, and 40 mg/kg, 30 min prior the start of the alcohol drinking session. Length of the alcohol drinking session during Day 4 was extended from a 2- to 4-h period. Moreover, immediately at the end of the 4-h drinking session, 50 microliters of blood were taken from the tip of the tail of each mouse. Samples were

centrifuged, and plasma analyzed to determine BALs using an Analox Alcohol Analyzer (GLM5 series, Analox Instruments, Lunenburg, MA). Alcohol intake (g/kg) over the 3-day acquisition phase was measured weighing the 20% (v/v) alcohol bottle immediately before and after the 2-h drinking session. Data were analyzed by a 2-way (group, days) ANOVA with repeated measures for factor "day". On Day 4, alcohol intake was measured at 2- and 4-h time intervals of the drinking session. Alcohol intake and BALs data were analyzed by separate 1-way ANOVAs followed by Tukey's post hoc test for multiple comparisons.

3.4.5. Experiment 5 – Effect of acute treatment with COR659 on locomotor hyperactivity induced by cocaine, amphetamine, nicotine, and morphine

Fifty-two male CD1 mice were divided into 6 independent groups for each of the following experiments: cocaine-, amphetamine-, nicotine-, and morphine-induced locomotor hyperactivity. Assessment of locomotor activity took place in motility cages and the motility session had a duration of 2 h. Briefly, each mouse was placed into a motility cage and allowed to freely move for 30 min. Mice were removed from the motility cages and treated with COR659 at the doses of 0, 10 and 20 mg/kg, and then re-exposed to the motility cages for an additional 30 min. The number of motility counts over the total 60-min period provided the baseline of spontaneous locomotor activity. Mice were removed from the motility cages again and now treated with one of the drugs of abuse (cocaine, amphetamine, nicotine, or morphine), and re-exposed to the motility cages for a final 60-min period. Locomotor activity tests were conducted during the first 6 hours of the light phase of the light/dark cycle. At the end of each session, the motility cages were cleaned with a 70% (v/v) alcoholic solution. For each experiment, the measured variable was the number of motility counts recorded automatically by the apparatus. Moreover, data on motility counts over

the 60-min period following administration of cocaine, amphetamine, nicotine, or morphine were divided into six 10-min intervals and statistically analyzed by a 2-way (treatment, time) ANOVA for repeated measures on the factor "time", followed by Tukey post hoc test for multiple comparisons.

4. Results

4.1 Experiment 1 – Effect of repeated treatment with COR659 on alcohol self-administration in male sP rats

After 20 sessions of alcohol self-administration (maintenance phase), sP rats that showed the most stable and consistent lever-responding behavior were selected and tested under 10-days repeated treatment with COR659 at the doses of 0, 2.5, 5, and 10 mg/kg. Experimental groups were made evaluating the number of lever-responses for alcohol and the amount of self-administered alcohol over the 3-days prior to the start of the pharmacological treatment (Pre-treatment phase). The number of lever-responses for alcohol over the 10-days treatment period was analyzed by 2-way (treatment, time) ANOVA with repeated measures for both factors revealing a significant effect of treatment [$F(3,36)=12.79$; $p<0.0001$] and time [$F(9,324)=3.28$; $p<0.001$] but no significant interaction between the factors [$F(27,324)=1.35$; $p>0.05$] (Fig. 3, panel A). Conversely, 2-way ANOVA of the amount of self-administered alcohol indicated a significant effect of treatment [$F(3,36)=12.54$; $p<0.0001$] and time [$F(9,324)=3.27$; $p<0.001$], and a significant interaction between the two factors [$F(27,324)=1.55$; $p<0.05$] (Fig. 3, panel B). Moreover, Tukey's post hoc test revealed that reductions in the number of lever-responses for alcohol and the amount of self-administered alcohol were statistically significant on Days 1-3 in the rat group treated with 5 mg/kg

COR659 and on Days 1-9 in the rat group treated with 10 mg/kg OR659, in comparison to the vehicle-treated rat group (Fig. 3). At the end of the 10-day treatment, 5 days elapsed before lever-responding for alcohol and the amount of self-administered alcohol in the rat groups treated with 5 and 10 mg/kg COR659 returned to control values (Fig. 3). Number of lever-responses for water during treatment and post-treatment phases was negligible (less or equal to 5) and not affected by treatment with any dose of COR659 (data not shown).

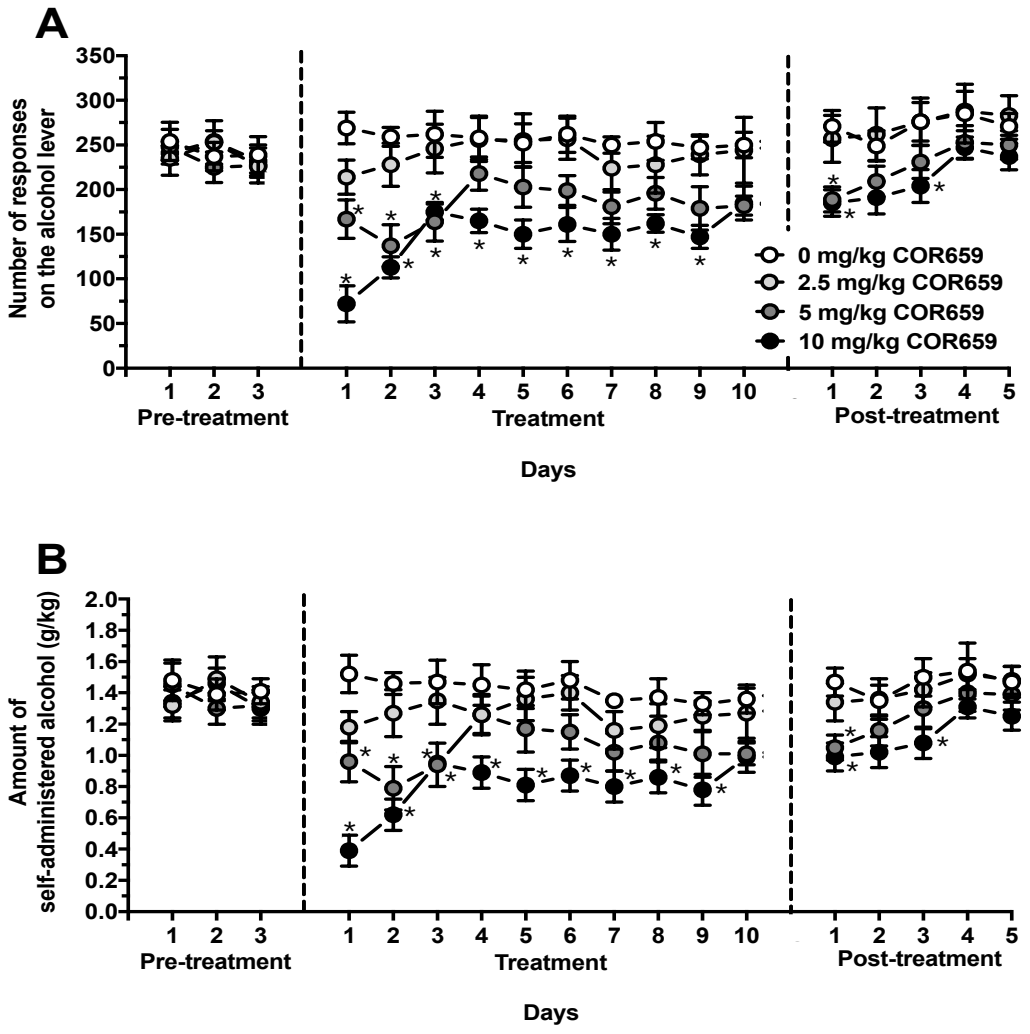

Fig. 3. Effect of repeated 10-day treatment with COR659 on **A)** number of lever-responses for alcohol **B)** and amount of self-administered alcohol in sP rats. COR659 was intraperitoneally injected 30 min before

the start of each daily 30-min alcohol self-administration session. Each point represents the mean ± SEM of n=10 rats. *p<0.05 in comparison to the vehicle-treated rat group (Tukey's post hoc test).

4.2 <u>Experiment 2</u> – Effect of acute treatment with COR659 on reinstatement of alcohol seeking in male sP rats

Before being tested on the reinstatement seeking behavior paradigm, sP rats – matched for the number of lever-responses for alcohol and the amount of self-administered alcohol over the last 3 days of the maintenance phase – underwent to an extinction responding phase characterized by the unavailability of alcohol and water in the operant chamber. Statistical analysis demonstrated that the 4 rat groups subsequently treated with 0, 2.5, 5, and 10 mg/kg COR659, extinguished their lever responding behavior in a similar way [log-rank (Mantel Cox) test, $\chi 2= 1.14$; $p>0.05$] (Fig. 4, panel A). Moreover, the number of extinction responding sessions required to achieve the established extinction criterion (≤10 responses on the alcohol lever per session for 2 consecutive sessions) was virtually identical and equal to 6.6 ± 0.8, 8.5 ± 0.7, 8.4 ± 0.9, and 8.9 ± 0.9 (mean ± SEM), in the 4 rat groups subsequently treated with 0, 2.5, 5, and 10 mg/kg COR659, as confirmed by 1-way ANOVA analysis [$F(3,40)=1.66$; $p>0.05$]. In the subsequent reinstatement session, presentation of the alcohol-associated stimulus complex effectively triggered lever-responding for alcohol in vehicle-treated rats ($p<0.0005$, Tukey's post hoc test) (Fig. 4, panel B). Statistical analysis of the number of lever-responses by 2-way [phase (extinction/reinstatement); treatment] ANOVA with repeated measures on the factor "phase" showed a significant effect of both "presentation of the alcohol-associated stimulus complex" [$F(1,40)=5.60$; $p<0.05$] and "treatment" [$F(3,40)=79.30$; $p<0.0001$] factors, and a significant interaction [$F(3,40)=41.55$; $p<0.0001$].

Moreover, Tukey's post hoc test indicated that treatment with any dose of COR659 reached statistical significance ($p<0.0005$) (Fig. 4, panel B).

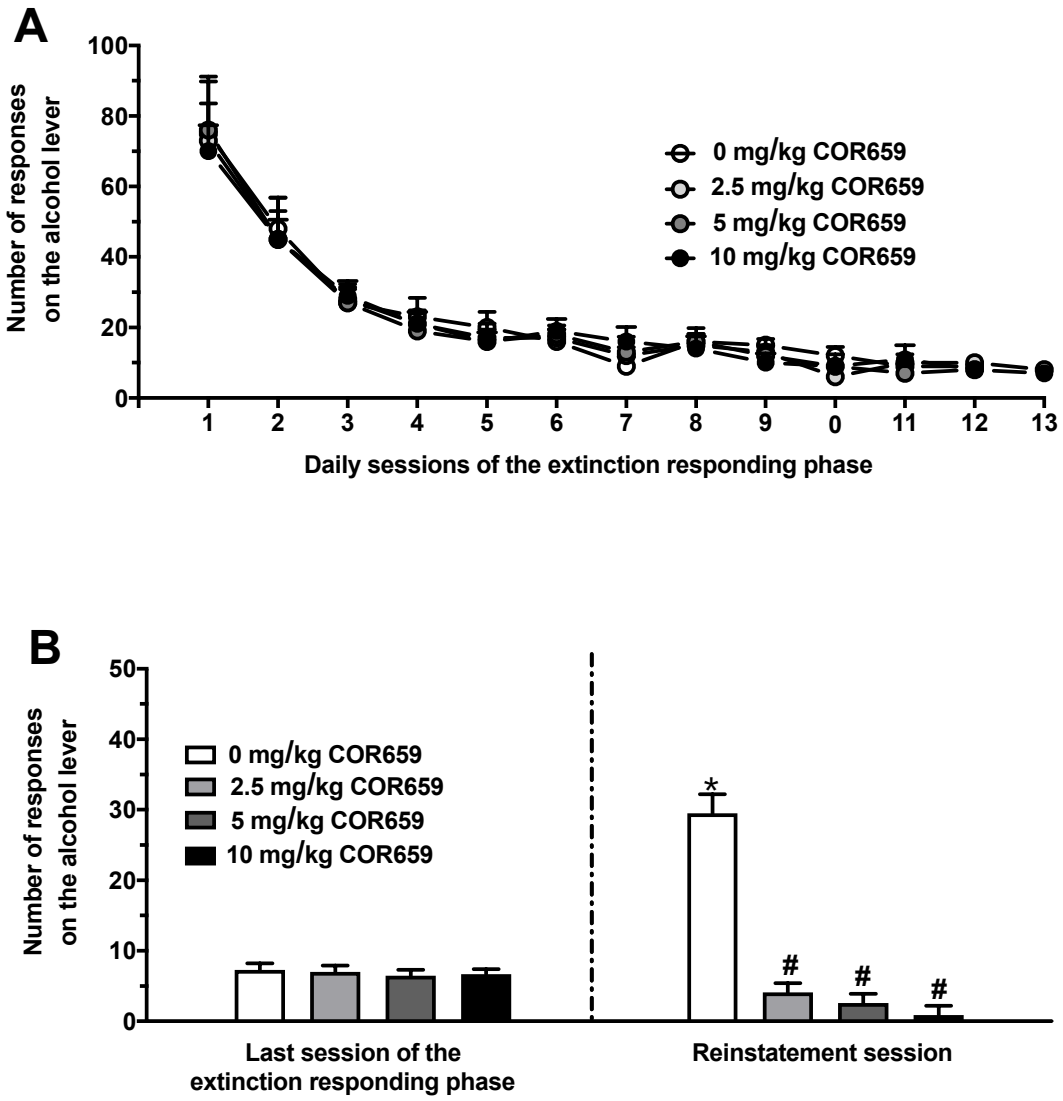

Fig. 4. Effect of acute treatment with COR659 on cue-induced reinstatement of alcohol-seeking behavior in sP rats. **A)** Profile of lever-responding over the extinction responding phase of the 4 rat groups subsequently treated with 0, 2.5, 5, and 10 mg/kg COR659. Since each rat achieved the extinction criterion in different days, each point represents the mean ± SEM of n= 2-11 rats. **B) Left panel.** Each bar represents the mean ± SEM of the number of responses on the alcohol lever over the last session of the extinction responding phase of the 4 rat groups subsequently treated with 0, 2.5, 5, and 10 mg/kg COR659. **Right panel.** The day immediately after each rat achieved the extinction criterion, they were exposed to a

reinstatement session. The presentation of a complex of stimuli previously associated with alcohol availability reinstated the lever-press behavior for alcohol of the rat vehicle group (*p<0.0005). Treatment with COR659, 30 min before the start of the session, suppressed the reinstatement of alcohol seeking behavior at any dose (#p<0.0005 in comparison to vehicle-treated rat group in the reinstatement session). Each bar represents the mean±SEM of n=11 rats.

4.3 Experiment 3 – Effect of acute treatment with COR659 on binge-like drinking in male sP rats

4.3.1 Low doses of COR659

Phase 1. All rats (n=64) promptly acquired alcohol drinking behavior over the 12 consecutive drinking sessions of unlimited (24-h) access. Specifically, the amount of alcohol consumed daily by the rats increased progressively, reaching stable values of approximately 6 g/kg over the last 3 days; conversely, daily water intake gradually decreased (data not shown).

Phase 2. When switched to daily limited 1-h drinking sessions, with unpredictable time schedule, sP rats displayed a time-sensitive alcohol drinking characterized by low alcohol consumption over the first hours of the dark phase of the light/dark cycle, and high alcohol consumption over the last hours of the dark phase of the light/dark cycle. Specifically, when the drinking session occurred at the 1st-h of the dark phase, alcohol intake averaged 1.17 ± 0.07 g/kg; conversely when the drinking session occurred at the 12th-h of the dark phase, alcohol intake average 2.67 ± 0.15 (Fig. 5, left panel). In addition, evidence that the sP rats consumed alcohol in a time-sensitive manner was confirmed by the high positive correlation coefficient calculated between mean alcohol intake over the 12 consecutive drinking sessions of limited 1-h access and time of access to alcohol (r^2= 0.93; slope= 0.1371; intercept= 1.051; p< 0.0001, Fig. 5, left panel).

Test Day. On Test Day, rats previously matched for their body weight, alcohol intake and time sensitivity over Phase 2 were allocated into 4 groups (n=16), and injected i.p. with COR659 at the doses of 0, 2.5, 5, and 10 mg/kg, 30 min before the start of the drinking session at the 12th-h of the dark phase of the light/dark cycle. Notably, vehicle-treated rats consumed intoxicating amounts of alcohol (averaging 2.45 ± 0.12 g/kg) and statistical analysis revealed a weak significance of the treatment factor [$F(3, 60)=3.838$; $p<0.05$]; in fact, only the dose of 10 mg/kg COR659 significantly reduced alcohol intake ($p<0.05$, Tukey's post hoc test) (Fig. 5, right panel). Water intake was not affected by any dose of COR659 [$F(3,60)=2.215$; $p>0.05$].

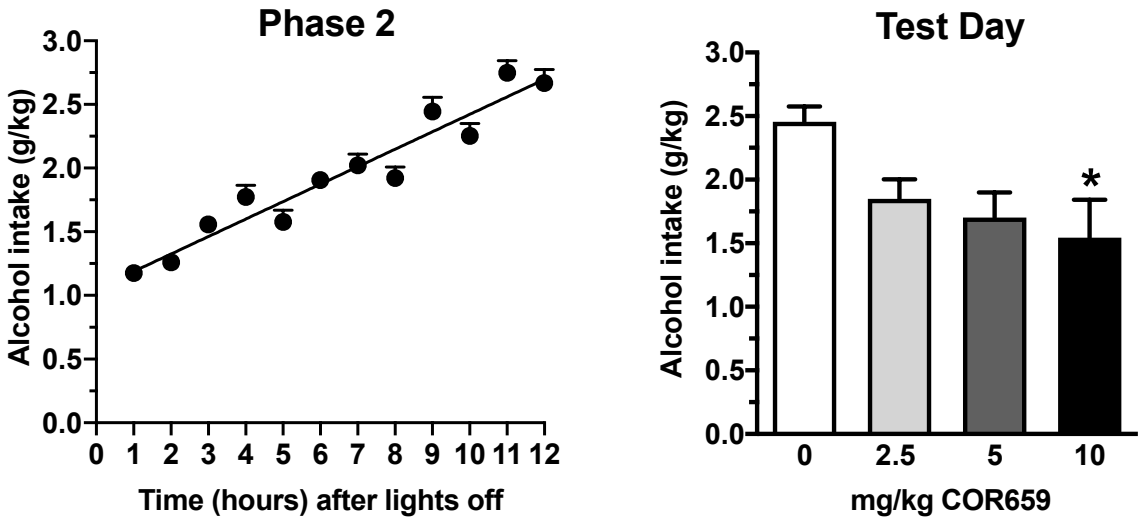

Fig. 5 – Left panel. Alcohol intake of sP rats over 12 consecutive days of Phase 2. Rats displayed high sensitivity to time schedule of alcohol drinking when exposed to limited 1-h sessions with concurrent availability of multiple alcohol concentrations and unpredictable access to alcohol during the dark phase of the light/dark cycle. Each point represents the mean ± SEM of n = 64 rats. **Right panel.** On Test Day, rats were treated with COR659 at the doses of 0, 2.5, 5, and 10 mg/kg, 30 min before the start of the drinking session at the 12th-h of the dark phase of the light/dark cycle. Each bar represents the mean ± SEM of n = 16 rats (*$p<0.05$).

4.3.2 High doses of COR659

Phase 1 and 2. Data from Phase 1 and 2 were virtually identical to those collected in the previous experiment. Rats (n=64) acquired alcohol drinking behavior, they increased and then stabilized the amount of alcohol consumed from an average of 4.5 g/kg to 6.4 g/kg, respectively, and they gradually decreased water intake over the 12 consecutive sessions of Phase 1 (data not shown). During Phase 2, alcohol intake of the rats was sensitive to time schedule, so that when the drinking session occurred at the 1st-h of the dark phase rats consumed an average of 0.86 ± 0.04 g/kg alcohol, conversely when the drinking session occurred at the 12th-h of the dark phase rats consumed an average of 2.24± 0.12 g/kg (Fig. 6, left panel). Moreover, linear regression of the mean of alcohol intake over the 12 consecutive drinking sessions of limited 1-h access and time of access to alcohol were highly positively correlated (r^2= 0.89; slope= 0.12; intercept= 1.05; p< 0.0001) (Fig. 6, left panel).

Test Day. On Test Day, rats previously matched for their body weight, alcohol intake, and time sensitivity over Phase 2 were allocated into 4 groups (n=16) and injected i.p. with COR659 at the doses of 0, 10, 20, and 40 mg/kg, 30 min before the start of the drinking session at the 12th-h of the dark phase of the light/dark cycle. Vehicle-treated rats consumed intoxicating amounts of alcohol (averaging 2.65 ± 0.22 g/kg) and statistical analysis revealed a high significance of the factor "treatment" [F(3, 60)=10.49; p<0.0001]. Specifically, all 3 doses of COR659 effectively reduced alcohol intake in the rat groups treated with 10, 20, and 40 mg/kg, respectively (**p<0.005, ***p<0.0005, and ****p<0.0001, Tukey's post hoc test) (Fig. 6, right panel). Water intake was not affected by any dose of COR659 [F(3,60)=0.7083; p>0.05].

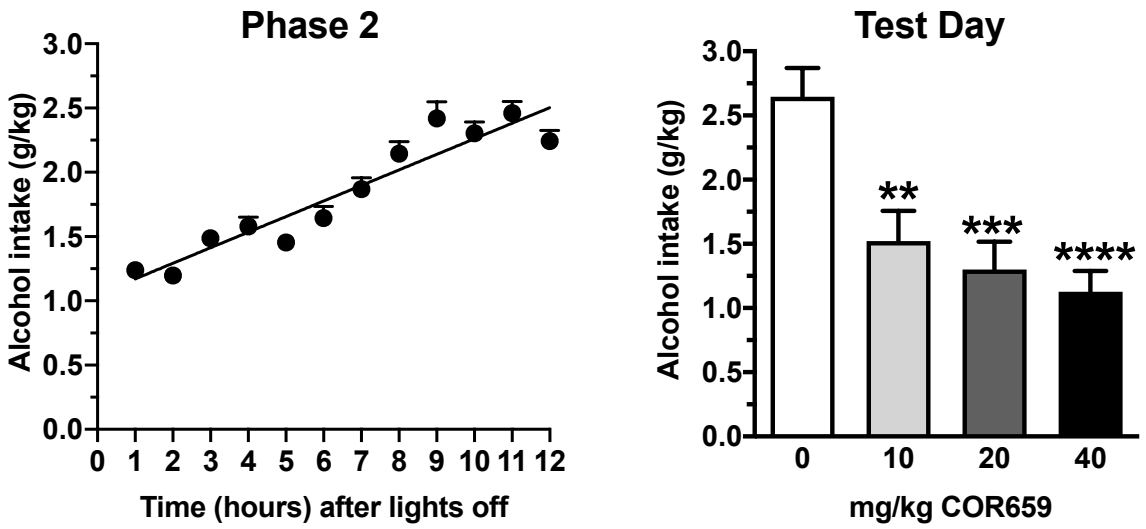

Fig. 6 – Left panel. Alcohol intake of sP rats over 12 consecutive days of Phase 2. Rats displayed high sensitivity to time schedule of alcohol drinking when exposed to limited 1-h sessions with concurrent availability of multiple alcohol concentrations and unpredictable access to alcohol during the dark phase of the dark light cycle. Each point represents the mean ± SEM of n = 64 rats. **Right panel.** On Test Day, rats were treated with COR659 at the doses of 0, 10, 20, and 40 mg/kg, 30 min before the start of the drinking session at the 12th-h of the dark phase of the dark light cycle. Each bar represents the mean ± SEM of n = 16 rats (**p<0.005, ***p<0.0005, ****p<0.0001, Tukey's post hoc test).

4.4 <u>Experiment 4</u> – Effect of acute treatment with COR659 on binge-like drinking in male C57BL/6J mice

Alcohol intake among the 4-experimental C57BL/6J mouse groups was stable and virtually identical over the 3-day acquisition phase (Fig. 7, panel A). On Day 4, 30 min before the start of the drinking session, mice were injected i.p. with COR659 at the doses of 0, 10, 20 and 40 mg/kg. Thirty minutes later, mice were allowed to drink from a bottle of 20% (v/v) alcohol. Statistical analysis indicated that after a 2-h drinking session COR659 potently reduced alcohol intake with a magnitude of 47%, 70%, and 85% in the mouse groups treated with 10, 20, and 40 mg/kg, respectively [$F(3,44)=13.32$; $p<0.0001$] (Fig. 7, panel B). Reducing effects of COR659 on alcohol

intake persisted even at the end of the 4-h drinking session, as indicated by 1-way ANOVA [F(3,44)=23.48; p<0.0001] (Fig. 7, panel C). Immediately at the end of the 4-h drinking session, BALs were measured. Notably, the vehicle-treated mouse group reached BALs of approximately 75 mg% (Fig. 7, panel D), close to the criteria posed for binge drinking in humans (BALs ≥80 mg%) (NIAAA, 2004). Conversely, BALs of the mouse groups treated with the 3 doses of COR659 were extremely low (<10 mg%) and highly significantly different from those recorded in the vehicle-treated mouse group [F(3,44)=21.03; p<0.0001] (Fig. 7, panel D).

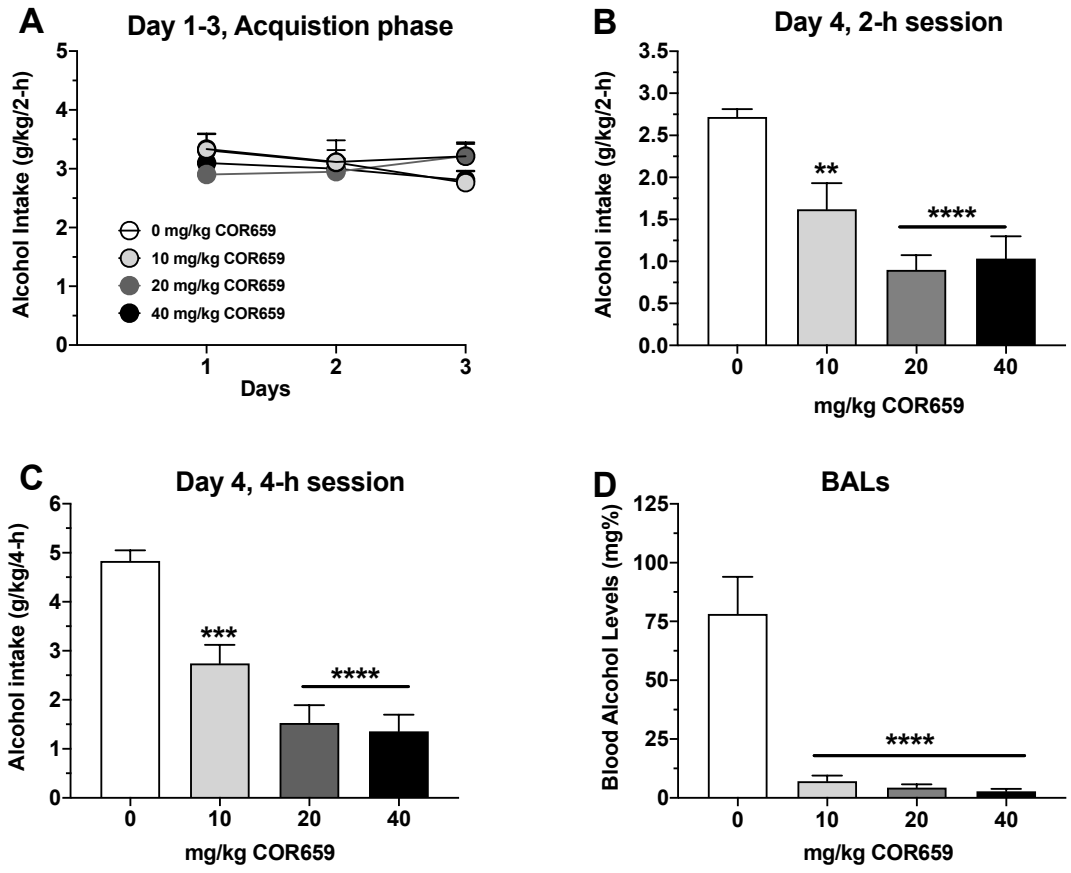

Fig. 7 - Effect of acute treatment with COR659 on drinking in the dark in C57BL/6J mice. **A)** 3 hours after lights off, mice were allowed to drink from a bottle containing 20% (v/v) alcohol for 2-h over 3 consecutive days (acquisition phase). On Day 4, alcohol consumption was measured **(B)** at the end of a 2-h drinking session and **(C)** at the end of a 4-h drinking session. **D)** Blood alcohol levels (BALs) were evaluated

immediately at the end of the 4-h drinking session. Each bar represents the mean±SEM of n=12 mice **p<0.01; ***p<0.0005; ****p<0.0001 (Tukey's post hoc test)].

4.5 Experiment 5 – Effect of acute treatment with COR659 on locomotor hyperactivity induced by different drugs of abuse

4.5.1 Cocaine-induced locomotor hyperactivity

Administration of COR659 plus cocaine vehicle did not alter CD1 mouse spontaneous locomotor activity at any dose, as shown in Fig. 8. Conversely, administration of cocaine plus COR659 vehicle markedly increased mice locomotor activity persisting over the entire 60-min session (Fig. 8). Pretreatment of mice with COR659 at the doses of 10 and 20 mg/kg significantly prevented locomotor hyperactivity induced by acute administration of 10 mg/kg cocaine. Specifically, 2-way (treatment, time) ANOVA for repeated measures on the factor "time" showed a highly significant effect of both factors, treatment [$F(5,54)=13.19$, $p<0.0001$] and time [$F(5,270)=9.24$, $p<0.0001$], and their interaction [$F(25,270)=1.86$, $p<0.01$], on motility counts. Notably, pretreatment with COR659 produced a reduction of cocaine-induced locomotor hyperactivity, in a dose-related manner that persisted across the first 40 min of the session (Fig. 8).

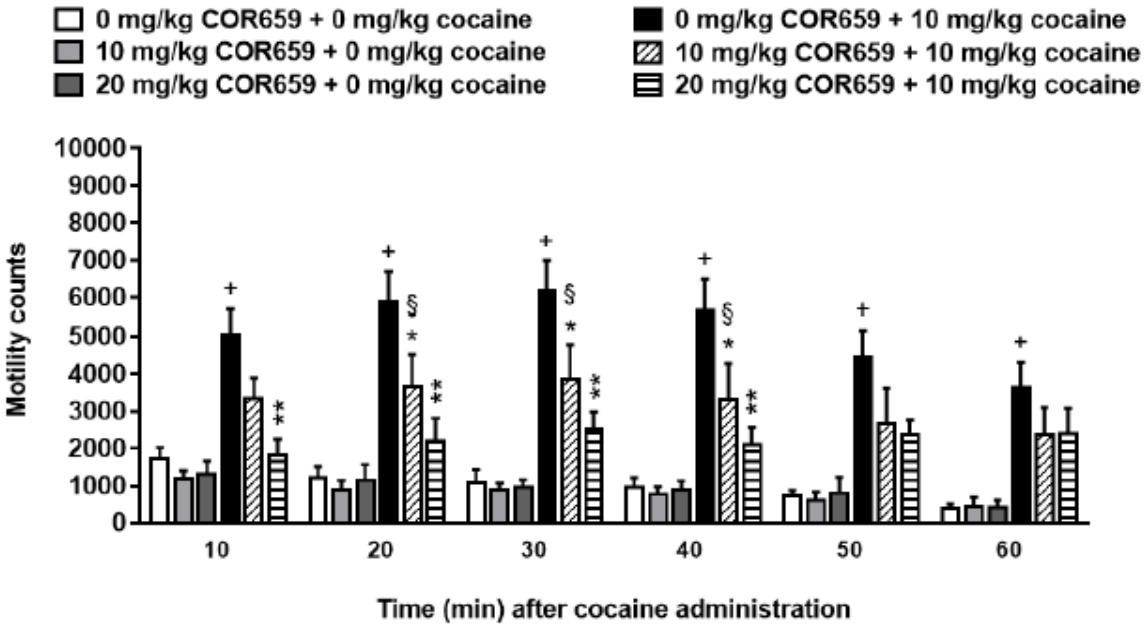

Fig. 8 - Effect of pretreatment with COR659 on cocaine-induced locomotor hyperactivity in male CD1 mice. Data refer to the final 60-min period of the locomotor activity test and are presented as mean ± SEM of the number of motility counts in six 10-min intervals (n=10 mice). +: $p<0.0005$ in comparison to the control mouse group (0 mg/kg COR659 + 0 mg/kg cocaine) at the same time interval (Tukey's post hoc test). ✶: $p<0.05$ and ✶✶: $p<0.0005$ in comparison to the mouse group treated with cocaine alone (0 mg/kg COR659 + 10 mg/kg cocaine) at the same time interval (Tukey's post hoc test). §: $p<0.05$ in comparison to the mouse group treated with the same dose of COR659 (10 or 20 mg/kg COR659 + 0 mg/kg cocaine) at the same time interval (Tukey's post hoc test).

4.5.2 Amphetamine-induced locomotor hyperactivity

Administration of COR659 plus amphetamine vehicle did not alter CD1 mouse spontaneous locomotor activity at any dose, as shown in Fig. 9. Conversely, administration of amphetamine plus COR659 vehicle markedly increased mice locomotor activity persisting over the entire 60-min session (Fig. 9). Pretreatment with COR659 at the doses of 10 and 20 mg/kg significantly prevented locomotor hyperactivity induced by acute administration of 5 mg/kg amphetamine.

Specifically, 2-way (treatment, time) ANOVA for repeated measures on the factor "time" showed a highly significant effect of both 2 factors, treatment [$F(5,50)=20.23$, $p<0.0001$] and time [$F(5,250)=9.53$, $p<0.0001$], and their interaction [$F(25,250=4.45$, $p<0.0001$], on motility counts. Notably, pretreatment with COR659 produced a reduction of amphetamine-induced locomotor hyperactivity that persisted across the first 60 min of the session (Fig. 9).

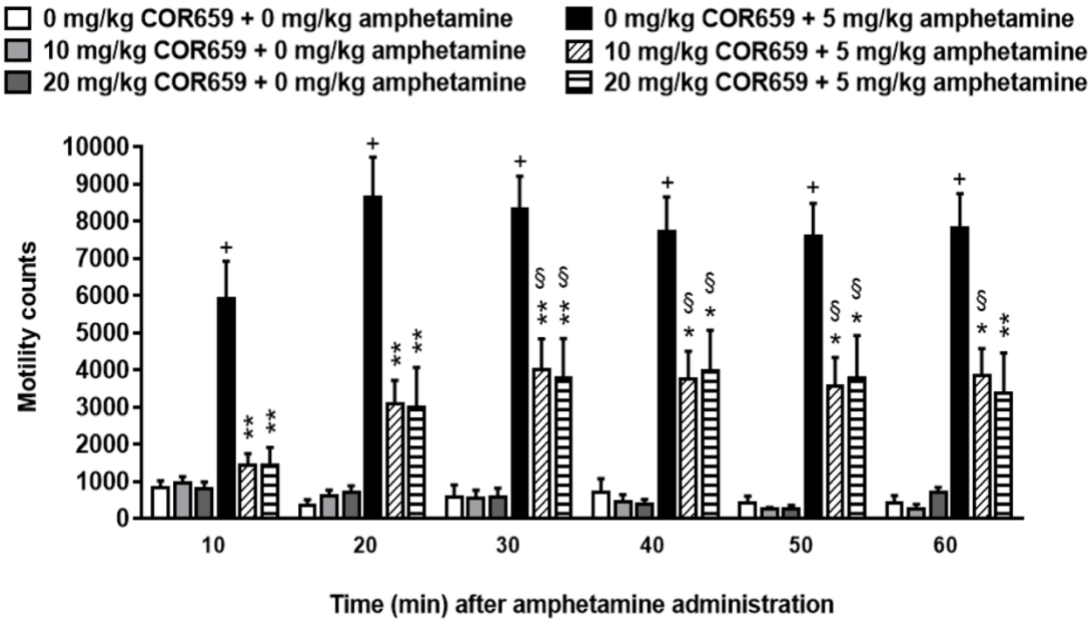

Fig. 9 – Effect of pretreatment with COR659 on amphetamine-induced locomotor hyperactivity in male CD1 mice. Reported data refer to the final 60-min. period of the locomotor test and are presented as mean ± SEM of the number of motility counts in six 10-min intervals (n=9-10 mice). +: $p<0.0001$ in comparison to the control mouse group (0 mg/kg COR659 + 0 mg/kg amphetamine) at the same time interval (Tukey's post hoc test). ✶: $p<0.001$ and ✶✶: $p<0.0001$ in comparison to the mouse group treated with amphetamine alone (0 mg/kg COR659 + 5 mg/kg amphetamine) at the same time interval (Tukey's post hoc test). §: $p<0.01$ in comparison to the mouse group treated with the same dose of COR659 (10 or 20 mg/kg COR659 + 0 mg/kg amphetamine) at the same time interval (Tukey's post hoc test).

4.5.3 Nicotine-induced locomotor hyperactivity

Administration of COR659 *plus* nicotine vehicle did not alter male CD1 mouse spontaneous locomotor activity at any dose, as shown in Fig. 10. Conversely, administration of nicotine plus COR659 vehicle markedly increased mice locomotor activity persisting over the entire 50-min session (Fig. 10). Pretreatment with COR659 at the doses of 10 and 20 mg/kg significantly prevented locomotor hyperactivity induced by acute administration of 0.05 mg/kg nicotine. Specifically, 2-way (treatment, time) ANOVA for repeated measures on the factor "time" showed a highly significant effect of both factors, treatment [$F(5,90)=21.20$, $p<0.0001$] and time [$F(5,450)=9.44$, $p<0.0001$], and their interaction [$F(25,450=1.55$, $p<0.05$], on motility counts. Notably, pretreatment with COR659 produced a reduction of nicotine-induced locomotor hyperactivity that persisted across the first 50 min of the session (Fig. 10).

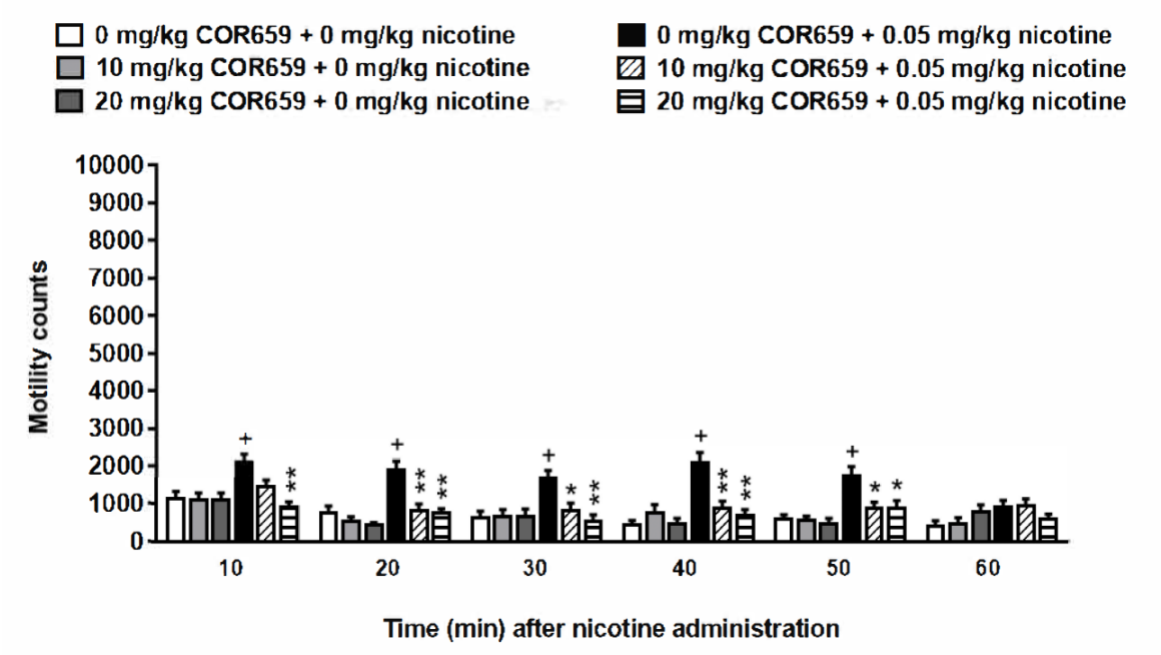

Fig. 10 – Effect of pretreatment with COR659 on nicotine-induced locomotor hyperactivity in male CD1 mice. Data refer to the final 60-min. period of the locomotor test and are presented as mean ± SEM of the number of motility counts in six 10-min intervals (n=16 mice). +: $p<0.005$ in comparison to the

control mouse group (0 mg/kg COR659 + 0 mg/kg nicotine) at the same time interval (Tukey's post hoc test). ∗: P<0.01 and ∗∗: P<0.0001 in comparison to the mouse group treated with nicotine alone (0 mg/kg COR659 + 0.05 mg/kg nicotine) at the same time interval (Tukey's post hoc test).

4.5.4 Morphine-induced locomotor hyperactivity

Administration of COR659 *plus* morphine vehicle did not alter male CD1 mouse spontaneous locomotor activity at any dose, as shown in Fig. 11. Conversely, administration of morphine plus COR659 vehicle markedly increased mice locomotor activity persisting over the entire 60-min session (Fig. 11). Pretreatment with COR659 at the doses of 10 and 20 mg/kg significantly prevented locomotor hyperactivity induced by acute administration of 20 mg/kg morphine. Specifically, 2-way (treatment, time) ANOVA for repeated measures on the factor "time" showed a highly significant effect of both factors, treatment [$F(5,90)=83.11$, $p<0.0001$] and time [$F(5,450)=52.60$, $p<0.0001$], and their interaction [$F(25,450=21.70$, $p<0.0001$], on motility counts. Notably, pretreatment with COR659 produced a reduction of morphine-induced locomotor hyperactivity that persisted over the 60-min session (Fig. 11).

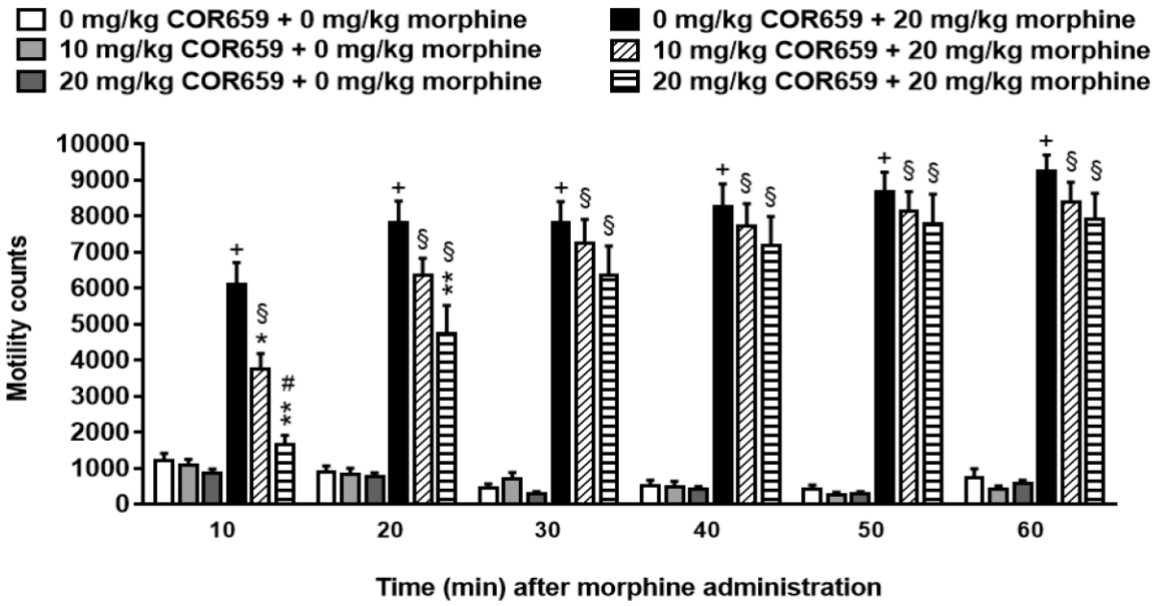

Fig. 11 – Effect of pretreatment with COR659 on morphine-induced locomotor hyperactivity in male CD1 mice. Data refer to the final 60-min. period of the locomotor test and are presented as mean ± SEM of the number of motility counts in six 10-min intervals (n=16 mice). +: P<0.0001 in comparison to the control mouse group (0 mg/kg COR659 + 0 mg/kg morphine) at the same time interval (Tukey's post hoc test). ∗: P<0.005 and ∗∗: P<0.0001 in comparison to the mouse group treated with morphine alone (0 mg/kg COR659 + 20 mg/kg morphine) at the same time interval (Tukey's post hoc test). §: P<0.000 in comparison to the mouse group treated with the same dose of COR659 (10 or 20 mg/kg COR659 + 0 mg/kg morphine) at the same time interval (Tukey's post hoc test). #: P<0.05 in comparison to the mouse group treated with the lower dose of COR659 (10 mg/kg COR659 + 20 mg/kg morphine) at the same time interval (Tukey's post hoc test).

5. Discussion

The results reported in this thesis provide further characterization of the *in vivo* anti-addictive properties of COR659, a novel $GABA_B$ PAM with promising potential as a therapeutic for treating AUD. Our data possess relevance since the $GABA_B$ PAM, ASP8062, has recently entered Phase 1 of clinical studies, with the intent of evaluating its safety and efficacy in treating SUD and its possible interaction with alcohol in healthy subjects.

The first research question of the present study was to assess COR659 efficacy after repeated administration, in an attempt to verify a possible decrease of its anti-alcohol properties when administered chronically. To this end, a set of sP rats was initially trained to lever-respond for alcohol and then treated repeatedly (once a day for 10 consecutive days) with COR659 at the doses of 0, 2.5, 5 and 10 mg/kg. Data from Day 1 showed that treatment with COR659 (corresponding to an acute injection) suppressed, in a dose-related manner, the number of lever-responses for alcohol and the amount of self-administered alcohol, replicating what was observed in a previous experiment (Maccioni et al., 2017). On continuing treatment, administration of COR659,

particularly at the highest dose (10 mg/kg), on Days 2 and 3, was characterized by a slight decrease of its potency in suppressing the number of lever-responses for alcohol and the amount of self-administered alcohol. Nevertheless, the magnitude of the suppressing effects of all 3 doses of COR659 remained relatively stable over the following 7-8 days of treatment, suggesting the development of very limited tolerance to the reducing effect of COR659 on alcohol self-administration. These results are important as they demonstrate COR659's ability to retain its anti-alcohol properties even after repeated treatment. Moreover, these data are in line with those from a previous study demonstrating that repeated treatment with GS39783, once a day for 10 consecutive days, selectively suppressed alcohol self-administration in sP rats, with no development of tolerance (Maccioni et al., 2015). Similar data were also obtained after repeated (once a day for 5 consecutive days) treatment with rac-BHFF (Maccioni et al., 2015). All together, these results indicate that agents belonging to the $GABA_B$ PAM class retain their efficacy in reducing alcohol self-administration even after prolonged treatment, a fundamental condition for a pharmacotherapy for AUD. Moreover, from a translational point of view, lack of tolerance would not require increasing the drug dosage and could contribute to greater compliance by patients.

The second research question was stimulated by recent studies that reported $GABA_B$ PAM ability to reduce reinstatement of alcohol-seeking behavior, a validated animal model that mimics relapse episodes and loss of control over alcohol in AUD patients (Martin-Fardon & Weiss, 2013). It is well-known that several factors can contribute to induce reinstatement of alcohol-seeking behavior in laboratory rodents. These factors include environmental stimuli previously associated with availability of alcohol, alcohol itself, acute administration of given drugs (e.g., nicotine), and exposure to stressful events. For this study, a set of sP rats was initially trained to lever-respond for alcohol under the standard FR4 schedule of reinforcement, where each drop of alcohol earned

was paired with a light above the alcohol lever and a tone; both cues turned on once the rat achieved the correct number of responses required; in this way, the rat learned that availability of alcohol was associated with the occurrence of these signals. Once alcohol self-administration had stabilized, lever-responding was initially extinguished, as both levers were unreinforced, and finally resumed, or reinstated, by the non-contingent presentation of a complex of stimuli (the above-mentioned auditory and visual cues plus a few drops of alcohol solution in the receptacle) previously associated with alcohol availability. Our results from Experiment 2 indicated that presentation of the alcohol-associated stimulus complex effectively reinstated lever-responding for alcohol in vehicle-treated rats, suggestive of a robust relapse-like behavior in control sP rats. Conversely, acute treatment with COR659 prevented sP rats from restoring their lever-responding for alcohol, resulting in a virtually complete suppression of reinstatement of alcohol-seeking behavior at any administered dose. These findings are in line with the results of prior studies demonstrating that (i) acute administration of ADX71441 suppressed reinstatement of alcohol-seeking behavior induced by either presentation of environmental cues or exposure to intermittent footshock (stress-induced reinstatement) in Wistar rats (Augier et al., 2017) and (ii) acute administration of CMPPE suppressed reinstatement of alcohol-seeking behavior in male Wistar (Vengeliene et al., 2018) and female sP (Maccioni et al., 2019) rats. Moreover, CMPPE was also reported to suppress relapse-like drinking behavior in Wistar rats exposed to the alcohol deprivation effect (ADE) procedure (Vengeliene et al., 2018). Together, these results suggest that the anti-relapse properties are an attribute common to several compounds of the $GABA_B$ PAM class.

Results from the present study (Experiments 3 and 4) also demonstrated that COR659 effectively suppressed alcohol intake in sP rats and C57BL/6J mice exposed to two different protocols of

binge-like drinking. Specifically, acute administration of COR659 at the dose range previously found to suppress the reinforcing and motivational properties of alcohol in sP rats (Maccioni et al., 2017) weakly reduced alcohol drinking of sP rats under the 4-bottle "alcohol [10%, 20%, and 30% (v/v)] versus water" choice regimen, as shown in Fig. 5. A possible explanation of this decreased potency of COR659 could reside in the strength of the drive underlying excessive alcohol intake in sP rats when exposed to this specific experimental procedure of binge-like drinking. Indeed, it has been demonstrated that concurrent availability of multiple alcohol concentrations, temporarily limited access to alcohol, and unpredictability of time of access to alcohol induced dramatic escalations in alcohol drinking in sP rats, up to intakes of clearly intoxicating (motor-impairing) amounts of alcohol (Colombo et al., 2014, 2015). Accordingly, higher doses of COR659 would be required to affect such a strongly driven alcohol drinking behavior. The experiment testing the higher dose range of COR659 was designed to address this additional research question. As predicted, administration of doses of COR659 equal to or higher than 10 mg/kg effectively suppressed the intoxicating amounts of alcohol consumed by sP rats in their binge-like mode (Fig.6).

In an attempt to verify potential interspecies drug-sensitivity differences, the suppressing properties of COR659 on binge-like drinking were also evaluated using the DID protocol in C57BL/6J mice (Rhodes et al., 2005). Acute administration of COR659 in the dose-range between 10 and 40 mg/kg (i.e., the same doses tested in sP rats under the 4-bottle "alcohol versus water" choice regimen), effectively suppressed alcohol intake at the first 2-h time interval of the drinking session as well as at the end of the 4-h drinking session, revealing a long-lasting effect of the anti-alcohol properties of COR659 (Fig. 7, panels B and C). BALs measured immediately after the end of the 4-h drinking session were markedly lower in all mouse groups treated with COR659,

reflecting the suppression of alcohol drinking (Fig. 7, panel D). These data confirm and extend to mice and to a widely used procedure of binge-like drinking the results of the previous study with sP rats. The observation of an anti-binge activity of COR659 is in line with several recent studies reporting that acute administration of $GABA_B$ PAMs such as GS39783 (Linsenbardt et al., 2014), ADX71441 (Hwa et al., 2014), rac-BHFF, and ORM-27669 (de Miguel et al., 2018) effectively suppressed binge-like drinking in rats and mice.

Notably, all the above-mentioned effects of COR659 occurred at doses lower than those found to produce sedation and hypolocomotion in sP rats (Maccioni et al, 2017), providing high specificity to the suppressing effects of COR659 on alcohol drinking and self-administration. Therefore, it could be hypothesized that the suppressing effects of COR659 on alcohol drinking and self-administration were not influenced by sedative and motor-incoordination effects. In this regard, a recent study found that the therapeutic index (TI) of COR659 was higher than 16 in sP rats (Maccioni et al, 2017); TI was calculated as the ratio between the ED50 of hypolocomotion and the ED50 of reduction of lever-responding for alcohol (Maccioni et al, 2017). If theoretically translated to humans, this TI is suggestive of a large separation between the expected, or "desired", pharmacological effects and the adverse, or toxic, effects. Nevertheless," drug-drug interaction" studies are now needed to verify whether co-administration of COR659 and alcohol can result in the potentiation of the sedative effects of alcohol (an event that cannot be ruled out *a priori* considering the inhibitory function of the $GABA_B$ neurotransmission).

Lastly, we investigated COR659 anti-addictive properties in a validated animal model that mimics the euphorigenic effects of several drugs of abuse (Wise & Bozart, 1987; Phillips & Shen, 1996). The results obtained suggest that COR659 shares with other agents belonging to the $GABA_B$ PAM class (Lhuiller et al., 2007; Kruse et al., 2012; Lobina et al., 2011; Wierońska et al., 2011) and

with the orthosteric $GABA_B$ receptor agonist, baclofen (Kalivas et al., 1990; Woo et al., 2001; Leite-Morris et al., 2002; Lhuiller et al., 2007; Lobina et al., 2011; Jacobson et al., 2016), the ability to prevent hyperlocomotion induced by psychostimulants like cocaine, amphetamine, and nicotine; additionally, these results demonstrate, for the first time, the ability of a $GABA_B$ PAM to prevent the stimulant effects of an opioid compound in rodents. Acute administration of COR659 effectively blocked locomotor hyperactivity induced by all four drugs of abuse tested in this study, with significant differences in terms of effect duration and magnitude. In fact, COR659 was observed to be (i) more effective in preventing nicotine-induced locomotor hyperactivity and (ii) shorter-acting on morphine-induced locomotor hyperactivity. This is likely due to the relatively limited dose-range of COR659 tested in the present study; it is indeed expected that testing higher doses of COR659 would likely result in an increased duration and magnitude of its preventing effect on locomotor hyperactivity induced by all four of the drugs of abuse studied. Additionally, possible drug-drug interactions between COR659 and some of the stimulating substances may underlie the observed differences in magnitude and duration of COR659 effect; pharmacokinetic studies are needed to address this issue. Yet, when administered in association with the vehicle of each drug of abuse, COR659 did not alter, even minimally, mouse spontaneous locomotor activity, confirming that prevention of drug-induced locomotor hyperactivity was not affected or confounded by any sedative or motor-impairing effect of COR659.

Comparison of preclinical data collected so far on several animal models of AUD revealed that COR659 possesses a better pharmacological profile than baclofen. In fact, although both drugs have been found to efficiently reduce the reinforcing and motivational properties of alcohol in rodents (Maccioni et al., 2017; 2019; Colombo & Gessa, 2018), full activation of the $GABA_B$ receptor by the orthosteric agonist was frequently associated to significant side effects such as

hypothermia, sedation, and myorelaxation (Cryan et al., 2004). Also, it has been reported that repeated treatment with baclofen often induced the development of tolerance to its "desired effects" limiting its use (Beveridge et al., 2013; Chartier et al., 2018). Conversely, here we demonstrate that either acute or chronic administration of COR659 were able to reproduce the anti-alcohol effects of baclofen with no hypolocomotor or sedative effects, as the likely consequence of a more "physiological" activation of the inhibitory $GABA_B$ neurotransmission.

COR659 ability to reduce, and occasionally suppress, several behaviors related to alcohol, drugs of abuse, and positive reinforcers apparently depends on its composite mechanism of action. COR659 in fact is a unique molecule synthetized as $GABA_B$ PAM but exerting a further activity as an antagonist/inverse agonist at the cannabinoid CB_1 receptors. It is reasonable to hypothesize that COR659 anti-alcohol properties might be due to its modulation of the $GABA_B$ receptors located in the brain reward circuitry, and more specifically in the VTA. $GABA_B$ PAM receptors have been identified both presynaptically on GABA and glutamate afferent neurons, and postsynaptically on dopamine efferent neurons (Castelli & Gessa, 2016). By modulation of these receptors, COR659 would potentiate GABAergic neurotransmission and, in turn, reduce the release of dopamine from neural terminals located in the NAc, thereby decreasing the reinforcing properties of alcohol and other abused drugs. In support of this "dopamine" hypothesis on the anti-alcohol properties of COR659, and in general of the $GABA_B$ PAMs, it has been demonstrated that acute intra-VTA microinjections of CGP7930 resulted in a reduction of lever-responding for alcohol and the amount of self-administered alcohol in sP rats. This reduction was site-specific, as demonstrated by a lack of the reducing effect on lever-responding for alcohol and amount of self-administered alcohol after CGP7930 microinjections in the deep mesencephalic nucleus in sP rats and no effect on motor performance (Maccioni et al., 2018). In addition, Leite-Morris and

colleagues demonstrated that (i) intra-VTA microinjections of GS39783 and BHF177 decreased alcohol seeking in Long Evans rats; more importantly, these authors demonstrated that the release of dopamine induced by presentation of cues previously associated with alcohol availability was markedly reduced in the core of the NAc (Leite-Morris et al., 2009, 2013).

It is noteworthy that the data so far collected suggest that the antagonist/inverse agonist action of COR659 at the cannabinoid CB_1 receptor apparently did not contribute to its suppressing effect on alcohol self-administration. Indeed, AM4113 was totally unable to prevent COR659-induced reduction of alcohol self-administration in sP rats (Maccioni et al., 2017). However, several studies demonstrated a role for the endocannabinoid receptor system in mediating the reinforcing, motivational, and rewarding properties of alcohol (Kleckowska et al., 2016; Basavarajappa et al., 2019; Kunos, 2020). Endocannabinoids can be released from dopaminergic neurons located in the VTA, where they inhibit the release of neurotransmitters such as GABA or glutamate by acting as retrograde messengers on presynaptic CB_1 receptors (Melis et al., 2004; Riegel & Lupica, 2004). This retrograde inhibition, occurring at both excitatory and inhibitory synapses, provide a mechanism through which endocannabinoids modulate dopamine neurotransmission and, in turn, regulate dopamine-mediate behavior (Lupica & Riegel, 2005; Maldonado et al., 2006). In this regard, it has been observed that CB_1 knockout CD1 mice showed a reduction in alcohol-induced conditioned place preference (Naassila et al., 2004; Houchi et al., 2005; Thanos et al., 2005) and decreased alcohol drinking when exposed to the 2-bottle "alcohol versus water" choice regimen. The same effect was observed in C57BL/6J CB_1 knockout mice (Poncelet et al., 2003; Wang et al., 2003; Lallemand & de Witte, 2005; Vinod et al., 2008). Moreover, it has been demonstrated that administration of rimonabant, a cannabinoid CB_1 receptor antagonist/inverse agonist, suppressed alcohol self-administration and other alcohol-related behaviors in several rodent

models of AUD (Natividad et al., 2015; Henderson-Redmond et al., 2016), including sP rats (Colombo et al., 2005; Maccioni et al., 2008c). However, the lack of effect of pretreatment with AM4113 on COR659-induced suppression of alcohol self-administration suggested that COR659 action on cannabinoid CB_1 receptor was likely too weak to contribute to its reducing effect on alcohol-related behaviors, and that the involvement of an additional receptor system may contribute to this COR659 effect (Maccioni et al., 2017).

Finally, it is possible that both receptor systems – $GABA_B$ and cannabinoid CB_1 – are likely involved in mediating the effect of COR659 preventing locomotor hyperactivity induced by psychostimulants and morphine. Several studies indeed have demonstrated that rimonabant is able to (i) prevent locomotor hyperactivity induced by cocaine, amphetamine, nicotine, and morphine in rodents (Poncelet et al., 1999; Singh et al., 2004; Kelsey & Calabro, 2008; Polissidis et al., 2014; Gobira et al., 2019) and (ii) block dopamine release induced by alcohol, amphetamine, nicotine and palatable food in rats (Cohen et al., 2002; Melis et al., 2007; Covey et al., 2016).

6. Conclusions

Several studies demonstrated that $GABA_B$ signaling has a key role in mediating the reinforcing properties of alcohol and drugs of abuse at the brain reward circuitry level (Filip et al., 2015; de Beaurepaire, 2019; Maccioni & Colombo 2019; Holtyn & Weerts, 2020). Data from these studies suggest that activation of the GABAergic inhibitory system by an orthosteric agonist or by PAM of the $GABA_B$ receptor, could interfere with several alcohol- and drugs of abuse-related behaviors, suggesting that the $GABA_B$ receptor plays a crucial role in the neurobiological and pharmacological effects of alcohol and drugs of abuse. Based on these lines of experimental evidence, pharmacological activation of the $GABA_B$ receptor may represent a novel and effective

pharmacotherapy for AUD and SUD. To date, clinical data collected with baclofen apparently confirm and support this hypothesis, and hopefully, they will be generalized to $GABA_B$ PAMs, once available for clinical testing.

The results reported in this thesis are in keeping with this hypothesis as they depict the anti-addictive properties of the novel $GABA_B$ receptor PAM, COR659, using several validated animal models of AUD and a model of SUD in rodents. Collectively these results possess translational value because of the predictive validity of the animal models used. With the final objective of assessing whether COR659 may be developed as a possible candidate for treating AUD, several studies are now required to complete the preclinical characterization of COR659. For instance, it will be necessary to evaluate whether COR659: (i) lacks abuse potential, (ii) retains its anti-alcohol properties when administered per os, allowing its possible administration by more common dosage forms (e.g., pills, tablets), (iii) potentiates, when combined with alcohol, its intoxicating effects, (iv) lacks toxicity, allowing safe administration, (v) interferes with alcohol metabolism and elimination, and (vi) alters the taste of alcohol. Lastly, it would be interesting to verify whether COR659 properties extend to animal models representative of the negative emotional states that characterize alcohol withdrawal syndrome, such as anxiety and depression, further supporting the potential of COR659 in AUD therapy.

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