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Faculdade de Farmácia

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Mestrado Integrado em Ciências Farmacêuticas

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Resumo

Atualmente, de entre os problemas de saúde mundial encontra-se o consumo excessivo de álcool, principalmente decorrente de *Binge Drinking*, que é o consumo de grandes quantidades de álcool num curto espaço de tempo. Além dos 4 tratamentos farmacológicos possíveis, muitos estudos têm sido realizados de forma a aumentar as possibilidades terapêuticas, de onde se destaca o fármaco baclofeno, a mais recente adição aos fármacos autorizados na França para este tratamento. Nos últimos anos, vários estudos sobre a eficácia do baclofeno foram realizados, principalmente sobre a forma racémica RS(±)-baclofeno, mas resultados divergentes foram encontrados. Após o isolamento dos 2 enantiómeros deste fármaco, o R(+)-baclofeno e o S(-)-baclofeno, descobriu-se que a eficácia reside no enantiómero ativo R, o que levantou a questão se realmente o melhor tratamento seria com a forma racémica RS. Assim, neste estudo procurou-se caracterizar os efeitos enantiosseletivos do baclofeno num modelo animal de consumo crónico binge-like em ratos Long Evans machos e fêmeas, sendo o foco as diferenças entre os 2 géneros, uma vez que ainda não existem estudos neste sentido. R(+)-baclofeno, S(-)-baclofeno e a forma racémica, que é a única ainda administrada aos pacientes, foram avaliados quanto à sua eficácia na diminuição do consumo de álcool. De seguida, avaliou-se o efeito do RS(±)-baclofeno no sistema dopaminérgico, uma vez que a libertação de dopamina na via mesolímbica sofre alterações com o consumo de álcool e o baclofeno, agonista dos recetores GABA-B, atua nas mesmas vias.

Os resultados obtidos sugerem que a variabilidade da resposta à forma racémica é devido aos diferentes efeitos dos 2 enantiómeros, sendo que uma diminuição no consumo de álcool e uma reposta positiva à terapêutica são devido à sensibilização do enantiómero R, e, pelo contrário, um aumento do consumo pode ser devido ao enantiómero S. Estas diferenças aparentam resultar de uma resposta diferencial do sistema dopaminérgico. Mais interessante ainda, foi demonstrado que a forma racémica RS não tem efeito nas fêmeas e o efeito do enantiómero R é ainda baixo comparado com os machos. Estes resultados sugerem que o tratamento com baclofeno atualmente disponível origina resposta terapêutica variável nas mulheres.

Palavras-chave: Binge drinking, consumo de álcool, sistema dopaminérgico, diferenças entre sexos

Abstract

Currently, among the world's health problems is excessive alcohol consumption, especially Binge Drinking, which is the consumption of large amounts of alcohol in a short time. In addition to the 4 possible pharmacological treatments, many studies have been conducted to increase the therapeutic possibilities, with the highlight in the drug baclofen, the latest addition to the list of drugs authorized in France for this treatment. In recent years, several studies to determine the efficacy of baclofen have been performed, mainly on the racemic form RS (±)-baclofen, but divergent results have been found. After the isolation of the 2 enantiomers of this drug, R (+)-baclofen and S (-)-baclofen, it was found the efficacy of the drug lies in the active enantiomer R. That raised the question if really the best treatment would be with the racemic form RS. Thus, this study sought to characterize the enantioselective effects of baclofen in an animal model of chronic binge-like consumption in male and female Long Evans rats, focusing on the differences between the two genders, since there are no studies in this regard. R (+)-baclofen, S (-)-baclofen and the racemic form, which is the only one still administered to patients, were evaluated for their effectiveness. Then, the relation of the dopaminergic system was also evaluated, since the release of dopamine in the mesolimbic pathway changes with alcohol consumption and baclofen, being a GABA-B receptor agonist, acts in the same pathways.

The results provide important evidence, as they suggest that the variability of patients' response to racemic form is due to the different effects of the 2 enantiomers and a decrease in alcohol consumption with response to therapy are due to sensitization to the R enantiomer and, on the contrary, an increase in consumption may be due to S enantiomer. A differential response of the dopaminergic system seems to be the reason their differences appear. More interestingly, it has been shown that the racemic form RS has no effect on females and the R enantiomer has little effect compared to males. These results suggest the currently available treatment leads to variable therapeutic response in women.

Key-words: Binge drinking, alcohol consumption, dopaminergic system, sex differences;

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Abreviations

AUD- Alcohol Use Disorder

BAC- Blood Alcohol Concentration

BD-Binge Drinking

BEC- Blood Ethanol Concentration

FSH- Follicle stimulating hormone

GABA- γ-aminobutyric acid

GISAH- WHO Global Information System on Alcohol and Health

LH- Luteinizing hormone

NIAAA- National Institute on Alcohol Abuse and Alcoholism

SICAD- Intervention Service on Addictive Behaviors and Addictions

VTA- Ventral Tegmental Area

WHO- World Health Organization

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1. Introduction

1.1 Alcohol Consumption and Abuse

Alcohol (ethanolic alcohol) is an important substance of abuse nowadays. It is an organic compound, toxic and addictive, affecting the central nervous system. Its' harmful use worldwide includes a big range of consequences, mainly long-term damage like addiction, increase of infectious diseases, liver cirrhosis, cardiovascular diseases and cancer. Additionally, people with addiction can not only cause damage to themselves as to others, including violence or traffic accidents, thus being one of the leading risk factors for health worldwide. According to Global status report on alcohol and health in 2018, the harmful use of alcohol resulted in an estimated 3 million deaths (5.3% of all deaths) globally in 2016 (1). Therefore, alcohol use disorder (AUD) is a chronic brain disease, characterized by compulsive alcohol use, loss of control over alcohol consumption and a negative emotional state when not drinking (2). There are several factors who have an impact on alcohol consumption, like the gender (men drink more than women; risk to be dependent higher in men), age and health of the person, as well as the religion and lifestyle options. The country where someone lives in is also taking into account, since the culture may also influence the type and quantity of alcohol consumed. Briefly, AUD is influenced by genetic and environmental factors (3), which happens over time and leads to neuronal transformations (4).

In Portugal, the alcohol consumption in 2016, 12.3 liters per capita, was higher than the average of the European Union, 11.3 liters of pure alcohol; however, it is possible to observe in recent years a continuous decrease. In France, the alcohol consumption in 2016 was also higher, but closer to the average of the European Union. (5). Global Information System on Alcohol and Health (GISAH) projections point to a decline in per capita alcohol consumption by 2025 in Portugal (from 12.3liters to an average of 11 liters in 2025), and stability in the WHO Europe Region (about 9.8 liters in 2025) (6).

1.2 The binge drinking problem

The definition of Binge Drinking (BD) varies according to the different institutions and no global definition has been accepted. The World Health Organization (WHO) defines binge drinking as a heavy episodic drinking, that is, drinking at least 60 grams or more of pure alcohol on at least one occasion in the past 30 days. On the other hand, NIAAA refers binge drinking as a pattern of drinking that leads the blood alcohol concentration (BAC) level of at least to 0.08 g/dL. This level is usually reach after 4 drinks for women and 5 drinks for men, in about 2 hours (2). Clearly, this second definition recognizes different thresholds for men and women, while

specifying a time period in which it occurs. Many researchers follow this definition, although some may determine binge drinking frequency using different time periods (the number of times binge drinking occurred in the past two weeks, or within the past month). For instance, The Substance Abuse and Mental Health Services Administration (SAMHSA), which conducts an annual national survey on drug use and health, defines binge drinking as at least 4 drinks for women and 5 for men, taken on the same occasion and at least on 1 day in the past month (7). Furthermore, the term “heavy episodic drinking” can sometimes be use synonymously to binge drinking in order to describe a pattern of heavy drinking over a defined time period.

Therefore, due to these differences in the definition and considering that the amount of alcohol in a standard drink can be different between different countries, the cut-off points differ as well. Consequently, the standardization in comparative research becomes difficult. Certain researchers proposed that the levels to reach binge drinking should be defined across all countries as about 60-70 grams of ethanol for men and 40-60 grams of ethanol for women per drinking occasion (8).

In conclusion, binge drinking is then characterized by both intensity and frequency of the drinking episode, including as well periods between binges with no consumption (7). This is where binge drinking differs from alcohol dependence (9). Additionally, binge drinkers have different psychological profiles (9,10), depending on gender and personality, which implies different prevention and treatment approaches (11). A large proportion of consumers begin drinking at a young age, which can cause several health problems. Adolescence is a time identified with several changes, including behavioral, hormonal and physical alterations. The brain undergoes ample development during adolescence and the effects of alcohol consumption are different from those seen on adults. Adolescents are for this reason more vulnerable to neurotoxicity induced by ethanol (12,13), with alteration of the neural network leading to diminutions in neuropsychological performance. Also associated with ethanol is the inhibition of neurogenesis in the hippocampus and changes in others brain regions. Alterations in the white and gray matter associated with BD also occurs, important since during adolescence there is an ongoing development of white matter and maturation of fiber tracts (14–16). Therefore the youth is more vulnerable to long-term consequences than an adult, mainly at a cognitive and psychological level, including blackouts, hangover and the need of increasingly drinking more to achieve the same intoxicated level (17). Regarding the blackout episodes, they become more frequent even after a decrease in the binge drinking, possibly related to hippocampal dysfunctions, due to these early exposure (18). Similarly, adolescent and young adult binge drinkers have a bigger probability to develop an AUD (7) and other adverse effects, since young people are more likely to drink at least 5 drinks once a week than older people (19). In general, comparing young adults to previous generations, they have a

higher percentage of frequent binge drinking, with more drinks per binge drinking episode (6-7 drinks) than the binge threshold of 4 (for women) and 5 (for men) drinks (20).

1.2.1 Gender differences

It is important to highlight differences between sex regarding ethanol's pattern. Women normally drink less than men, have higher probability to remain lifetime abstainers and less probability of develop AUD (21). However, in recent generations it is showed the gender gap is becoming reduced. Factors like genetic and social-cultural may explain the variation in alcohol use, specially between countries (1). Women also have aggravated alcohol-related consequences (22), principally liver cirrhosis and hepatitis, when compared to men (23), which makes the increase in female alcohol consumption particularly alarming. Furthermore, women have a higher risk of developing alcohol-related cancer and also cardiovascular diseases (2,24). Regarding binge drinking, there is also a considerable increase among women, with a 14% estimated relative change from a study with meta-analysis of 6 surveys, while men showed no significant difference (25), as it is demonstrated in the figure below.

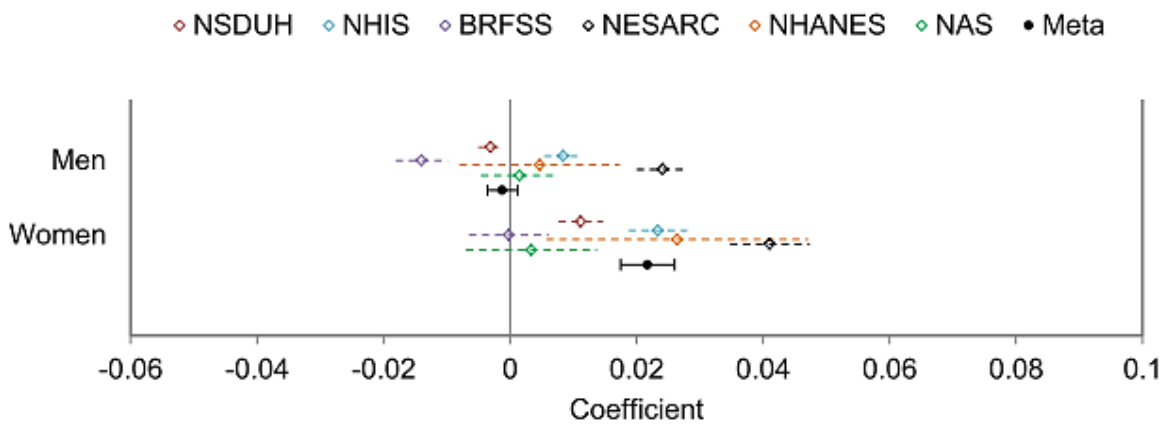

Figure 1- Prevalence of binge drinking from each survey and from meta-analysis of all surveys. Forest plot of regression coefficients; error bars represent 95% confidence intervals. The abbreviations meaning are: NSDUH, National Survey on Drug Use and Health; NHIS, National Health Interview Survey; BRFSS, Behavioral Risk Factor Surveillance System; NESARC, National Epidemiologic Survey on Alcohol and Related Conditions; NHANES; National Health and Nutrition Examination Survey; NAS, National Alcohol Survey; Meta, meta-analysis of all surveys above. Adapted from (25).

At a neuronal level, women can develop more aggravated cognitive deficits as well (26). After binge drinking occurrences, women experience more brain damage; specially in the hippocampus, a region particularly vulnerable to alcohol damage (27). Females, adolescents and adults, with AUD are therefore more vulnerable to the neurotoxic effect of ethanol than men, with several studies showing negative effects (volume loss and demyelination) in the corpus callosum, the largest white matter structure in the cerebrum (28). Studies with diffusion

tensor imaging (method for assessing white matter structure and integrity) using fractional anisotropy to measure the degree of diffusion, showed differences in the white matter integrity in different regions of the brain, associated with gender (29). There are only few animals studies of alcohol use that directly compare males and females, despite evidences of sex differences in ethanol consumption, due to concerns that hormones and the estrous cycle can cause variability and change results (30). However, there's evidence that female rats are not more variable than males both in trait variability and neuroscience research (31). In operant alcohol self-administration, there are mixed studies, either no evidence has been found about differences between males and females, or it was found that females respond and consume more than males (30,32).

Regarding the female rats, there is a possibility that estrous cycle can alter the consumption and pharmacodynamics of ethanol or vice-versa (33). The estrous cycle is regulated by the release of gonadotropins, LH and FSH, from the pituitary gland, and the release of steroids, mainly estrogens, from the ovaries. Furthermore, the estrous cycle can be monitored by the observation of change of cell types in vaginal smears. Studies demonstrated ethanol disrupted the normal estrous cycle (34), while others verified the gender differences in the pharmacokinetics of ethanol, but with estrous cycle just slightly affecting ethanol pharmacokinetics (35).

1.3 Therapeutic Approaches

Goals of the pharmacological treatment are the treatment of withdrawal syndrome, caused by the changes in neurotransmitters that cause the symptoms of hyperexcitability associated with discontinuation of consumption, specifically the treatment of tremors, hyperactivity, nausea, vomiting, psychomotor agitation and seizures, as well as on ingestion control, that is, the maintenance of abstinence, reduction of ingestion and prevention of relapse. Treatment is based on personalized pharmacological therapy, psychotherapy, social intervention and self-help groups.

Currently there are 4 approved medications for alcohol dependence in Portugal: Acamprosate, Dissulfiram, Naltrexone and Nalmefene. Acamprosate and Naltrexone are the first line treatment, while Disulfiram and Nalmefene are used in second line. In France the same medications are used, and since 2018, baclofen was added to this short list of anti-alcohol dependence medications.

Acamprosate is a functional glutamate antagonist and modulates the gabaergic and glutamatergic system. It reduces the consumption of alcohol and is effective in the maintenance of the abstinence, being therefore of first line for maintenance of the abstinence.

Naltrexone is a μ opioid receptor (modulators of the dopaminergic mesolimbic pathway) antagonist, which attenuates the rewarding effect associated with alcohol intake and decreases the craving for intake, significantly reducing the risk of resuming high intakes. Consequently, it is the first line for maintenance of the abstinence or to decrease the intake. Nalmefene is a drug structurally similar to naltrexone; it's a μ, δ opioid receptor antagonist and k receptor partial agonist, which may be relevant in the mediation of the motivational effects of alcohol dependence. It should be taken as soon as the patient anticipates a risk of alcohol consumption; however, there is insufficient evidence to define its role in therapeutics. Disulfiram, unlike the other approved drugs, is an aversive drug, that is, it discourages the intake by indirect effect. It inhibits the enzyme aldehyde dehydrogenase, leading to an unpleasant physiological reaction, such as sweating, headache, dyspnea, hypotension, flushing, palpitations, nausea and vomiting, remaining for 30min to several hours. The intensity of this reaction depends on the amount of alcohol ingested and, in higher alcohol consumption, can even lead to seizures, coma and death. That is why this drug is only given to patients after a careful medical examination and administered only under medical supervision, with the patient fully aware of the implications of alcohol consumption with this drug.

Although all these medications demonstrate evidence of efficacy in some degree, there are problems with their extent. For this reason, research is made to develop new pharmacological treatments and there are other drugs not approved for the treatment of AUD, but that are being used off-label, such us Baclofen, Topiramate, Gabapentin, Varenicline and Ondansetron, all of the above used in Portugal (36). The summary of all therapies can be seen in the table 1.

Baclofen, a GABA-B agonist, is a drug approved by the Food and Drug Administration (FDA) for the treatment of spasticity due to muscle relaxation and consequent pain relief in spinal cord or other neurological lesions (37). The γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain, being present in the central and peripheral nervous system. It controls synaptic transmission and neuronal excitability, regulating behavior and brain reward processes (38). Baclofen, being a GABA-B agonist, activates the GABA-B receptor subtype, a G-protein coupled receptor to potassium and calcium channels, and to adenylyl cyclase (39). This GABA-B receptor plays an important role in several processes, since it regulates systems of neurotransmission (40), as well as transduction pathways, memory and inflammation (41). At a neuronal level, it appears in high density more at the medial habenula, thalamus, cerebellum, cortex and colliculus and in the opposite appears more in low density at the while ventral tegmental area and mesolimbic dopaminergic projections (42). Other studies reach the conclusion that the GABA-B highest densities is in the molecular layer of the cerebellum, the interpeduncular nucleus, frontal cortex, anterior olfactory nucleus and thalamic nuclei (43).

Therefore, the action of baclofen is supposed to be superior in that same brain structures that contain high densities of the GABA-B receptors.

Table 1- Pharmacological treatments for AUD.

Type of prescription	**Active substance INN**	**Pharmacotherapeutic group (Central Nervous System)**	**Therapeutic indications in the Summary of Product Characteristics**
On label	Acamprosate	Drug Addiction Treatment Medicines	Abstinence maintenance therapy in alcohol patients dependents.
	Dissulfiram		Abstinence maintenance therapy in alcohol patients dependents.
	Naltrexone		Abstinence maintenance and decrease the intake.
	Nalmefene		Reduction in alcohol consumption in high risk patients.
On label (France) Off label (Portugal)	**Baclofen**	Muscle relaxants of central action	• Spasticity of skeletal muscles in multiple sclerosis; • Spastic situations arising from medullary, infectious, degenerative, traumatic, neoplastic or unknown pathologies.
Off label	Topiramate	Antiepileptics and anticonvulsants	• Migraine prophylaxis; • Adjuvant therapy for partial seizures with or without secondary generalization or generalized primary tonic clonic seizures; • Treatment of seizures associated with Lennox-Gastault syndrome.
	Gabapentin	Antiepileptics and Anticonvulsants; Analgesics and antipyretics.	• Adjunctive or monotherapy in the treatment of partial epileptic seizures with or without secondary generalization. • Treatment of peripheral neuropathic pain.
	Varenicline	Medicines used for nicotine addiction	• Smoking cessation.
	Ondansetron	Antiemetics and Anti-vertigins	• Control of nausea and vomiting induced by cytotoxic chemotherapy and radiotherapy; • Prevention of postoperative nausea and vomiting.

The mechanism of action for the usual treatment, namely the myorelaxant properties, is to reduce the excitability of spinal reflexes, by decreasing the excitatory transmitter from the presynaptic terminals of afferent fibers (44). Baclofen has two routes of administration, oral or intrathecal. The intrathecal administration is used when the patient is not responding to the oral treatment (45). The oral administration is the most common one, with the recommended daily dose with a range from 15mg to 80mg. Also, it has to be administered three to four times per day, since baclofen has a half-life of only three to four hours. This is probably the reason why the main side-effects dose-related in the baclofen treatments appear, being correlated to the peak of the drug concentration in the blood, at 30 minutes (46). Usually, patient undergoing this treatment with a higher dose of the drug report dizziness, sedation, motor weakness, confusion and fatigue (47). Some of these side effects can be explained by an activation of brain areas containing GABA-B receptors in bigger density; since baclofen activate mostly the receptors on brainstem and hypothalamus, the ones that control basic states of vigilance and so the patient can suffer from fatigue and sedation.

The most recent evidences that baclofen could be used for the treatment of distinct addictive disorders are important to highlight (48). It was found in preclinical studies that Baclofen can indeed inhibit the reinforcing effects of cocaine (49), amphetamine (50), nicotine (51) and alcohol (48,52,53). Few studies reported negative results (54,55). However, the mechanism of action for AUD remains uncertain.

A likely explanation for the differences seen in the baclofen literature is the fact that its effect is due to his racemic composition, being enantiomer dependent (56,57). Thus it is important to consider the enantioselective aspects of drug action, specially between different behavioral assays, when a therapeutic effect is desired. The two enantiomers, R(+), the more active one, and S(-), the less active, show opposite effects in rats. Studies demonstrate R(+)- baclofen suppressed alcohol intake, while S(-)-baclofen stimulated it. Consequently, the pharmacological activity of RS(±)- baclofen is supposed to be based on the R(+)- baclofen (58).

The first time Baclofen's effects were described for the treatment of AUD in humans was in 2005, after one French physician with AUD treated himself with high doses of the drug and described a cure toward his dependence (59). Since then, Baclofen is used off-label in several countries, and widely in France, that has temporary use authorization due to observational data, with some patients showing improvements (60). Several other studies reported the baclofen effectiveness in reducing alcohol intake (61,62).

Regarding preclinical models, it was also demonstrated, in operant self-administration experiments, that Baclofen reduces the motivation to drink alcohol. In these conditions, rats

need to press a lever a given number of times to receive alcohol, and with baclofen the number of lever presses was reduced, decreasing consequently the quantity of ethanol self-administered. This indicates the drug decreases the rats' motivation to drink, which corresponds to human craving for drinking (63). Baclofen was also successfully tested in binge drinking models (45,64–66). Moreover, the two enantiomers were used in a binge-like ethanol model, with results that demonstrated the effects of R(+)- baclofen in decreasing the binge consumption, and the effects of by S(-)-baclofen in increasing the same consumption (57).

1.4. Dopaminergic system and binge drinking Exposure

Nervous system is specialized in the rapid transmission of information, in the form of action potentials (nerve impulses) from cell to cell in active zones of contact between a nerve ending and other neuron, called synapse. The chemical synapses are dominant in the human nervous system, characterized by the presence of a chemical mediator – the neurotransmitter, which ensures the transmission of the nerve impulse from pre-synaptic to post-synaptic cell, since it acts on receptor proteins in the post-synaptic membrane, in order to excite/inhibit/modify the next neuron. In chemical synapse, an impulse propagates through the axon to the pre-synaptic terminal, where it causes the opening of voltage-dependent calcium channels that allow this ion to pass inside. Calcium ions promote vesicle fusion with the pre-synaptic membrane with the aid of SNARE / Synaptotagmine proteins and the release of neurotransmitters, stored in vesicles, to the synaptic cleft. Neurotransmitters diffuse into that cleft and bind to their receptors in the membrane of the post-synaptic cell, increasing permeability of the ion channels present there. Finally, the increased ion permeability originates depolarization (or hyperpolarization) of the post-synaptic membrane cell, resulting in a potential of action post-synaptic if the threshold is exceeded (67,68).

Regarding synaptic transmitters, they could be divided into 2 groups: the small-molecule, rapidly acting transmitters (acute responses of nervous system), as it is showed in table 2, and neuropeptides, slowly acting transmitters.

Dopamine, as a neurotransmitter, has an essential role (69,70). It's involved in several functions through signaling cascades, including cognitive, motor control, motivation and reward. Therefore, an alteration in the dopaminergic system is related to different diseases, like for example Parkinson and Huntington disease, depression, schizophrenia, and behavioral addiction (71). There are 3 major dopaminergic pathways: nigrostriatal (from the substantia nigra, projecting to the dorsal striatum), mesolimbic (transmits from the ventral tegmental area (VTA), a key area of the brain reward system, to the ventral striatum, composed by nucleus accumbens and olfactory tubercle) and mesocortical (transmits dopamine from the VTA to the

prefrontal cortex). Reinforcement and, in more dangerous cases, addictions, happen when there is an increased activity in the projections to nucleus accumbens (72). So, both the mesolimbic and mesocortical initiate in the VTA and, while the mesolimbic progress to the limbic system (via nucleus accumbens), the mesocortical pathway progress to the frontal cortex (73) (figure 2). The mesolimbic has an important role in reward and emotion and the mesocortical is important in several cognitive functions (74).

Table 2- Identification of small-molecule neurotransmitters. Adapted from Textbook of Medical Physiology, 2006.

Classe I	**Acetylcholine**
Classe II- The Amines	Norepinephrine
	Epinephrine
	Dopamine
	Serotonin
	Histamine
Class III: Amino Acids	Gamma-aminobutyric acid (GABA)
	Glycine
	Glutamate
	Aspartate
Classe IV	Nitric oxide (NO)

Dopamine transmission is widely affected by drugs of abuse, such as alcohol. Chronic binge drinking can induce alterations in neural systems controlling motivational processes, which in turn changes neurotransmitter processes in which molecules such as dopamine participate (brain's reward and stress system), creating the reinforcing effects (positive and negative). The positive reinforcing is when there is an increase of the dopamine's release in the brain, associated with an increase in the drinking behavior, producing a feeling of pleasure and alcohol-induced euphoria. The negative reinforcing, in other hand, is when there is a decrease in dopamine's release in the striatum to ease a negative emotional state (41). There are studies and evidences that says the brain of a person with an addiction has a modified mesolimbic dopamine system; a decrease in the natural activity of this system, with a decline of the dopamine's release (70). In that way, therapies which increases the dopamine release and therefore its action could be beneficial to treat the disease and could yield some clinical improvements; however it depresses different portions of the prefrontal lobes and other related areas; so only the normal balance of dopamine, between inhibition and excitation, should be restored. In relation to baclofen, preclinical studies suggest it constrains dopamine transmission (75) and some proposes this effect depend on the dose: small doses increases

dopamine activity whereas high doses inhibits it, both in animals (76) and humans (77). Consequently, baclofen can have anti-reward effects and adjust the alcohol related behavior. As a GABA-B agonist, dose-dependent baclofen activates the receptors located in the ventral tegmental area and prevents the dopamine release in the nucleus accumbens. Microinjection of this drug directly to the nucleus accumbens has shown to decrease binge drinking as well (65,78). There are a few inconsistent results about the effects of baclofen in binge drinking model, but that can be explain by the fact the two enantiomers act in mesolimbic dopamine system in a different way (56).

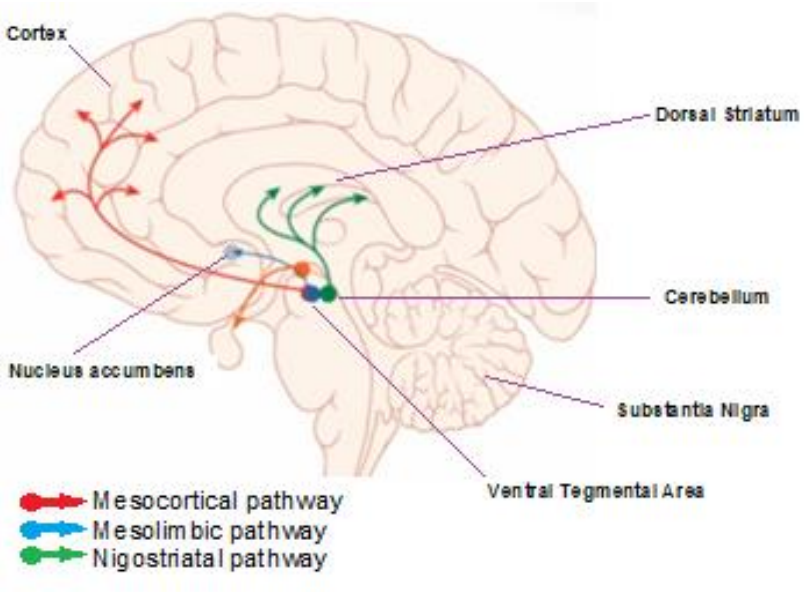

Figure 2- Dopaminergic pathways of the human brain. Adapted from (79).

1.5. Aims of the study

As previously mentioned, binge drinking can lead to several brain modifications, mainly in chronic binge drinkers, with marked differences between males and females. Preclinical evidences indicate Baclofen can decrease ethanol consumption in a variety of behavioral models. However, there have been associated some mixed results due to his racemic composition. Of interest is that it appears to act different if the effects are studied in males or females.

Therefore, in this study the aim is to:

i) Test the hypothesis that baclofen can be use in chronic binge drinkers to decrease the ethanol consumption;

ii) Investigate the efficacy of the different enantiomers of baclofen;

iii) Compare males vs females results regarding the effects on dopaminergic system and hence to the ethanol consumption.

2. Materials and Methods

2.1 Animals

30 Long–Evans rats (Janvier Laboratories, France), 15 males and 15 females, were individually housed under a light-dark cycle of 12 hours, with lights on at 7:00 AM. Feed and water were available *ad libitum*. All animal procedures were in accordance with the European Community Regulations for Animal Use in Research (CEE N° 86/609) and all approved legislation, approved by the local ethics committee (#2145).

2.2 Reagents

Ethanol (96 percent) was purchased from VWR (France). R(+)-baclofen, S(-)-baclofen and RS(±)-baclofen were dissolved in saline (0.9 %) at the doses of 1.5 mg/kg. All previous and sucrose (2 %), dissolved in regular tap water, were purchased from Sigma-Aldrich.

2.3 Voluntary Alcohol Consumption: Self-administration

This procedure was generated with a protocol followed in this laboratory, as described in (80). Briefly, to generate BD behavior, the rats were first submitted to a two-bottle choice protocol for 4 weeks: rats have access to one bottle of tap water and one of alcohol (20 % ethanol solution) every other day (81); i.e., with 24 or 48 hours of ethanol-deprivation periods in between the ethanol-drinking sessions The placement (left or right) of each solution was alternated between each session to control for side preference (80). After these 4 weeks, rats were placed in Skinner box, with chambers containing two levers: an active lever, for which each press (fixed ratio of 1) leads to the delivery of 0.1 ml of the 20% ethanol solution, and an inactive lever, that has every response counted but no programmed events occur. The sessions were 1-hour daily, 5 days per week. Then, rats were trained to self-administer the same amount of ethanol under a fixed ratio of 3 (3 lever presses to get 0.1ml of 20% ethanol) for 1 hour, and after reduced in daily 30-minute sessions. After the rats reached a stable baseline of ethanol self-administration (approximately 3 weeks), the session time was reduced to 15 minutes, because very short sessions of operant self-administration increases the consumption of alcohol and the speed of drinking, therefore can facilitate the acquisition of BD behavior in rats (80). Rats continued to self-administer the 20 percent ethanol solution daily (0.1 ml per delivery under a fixed ratio of 3).

Alcohol intake is expressed as grams of pure ethanol consumed per kg body weight per session.

2.4 Sucrose Self-administration

To evaluate the integrity of the dopaminergic system and if there is a sensitization of the reward pathway, it was assessed the behavior towards other reward substance not addictive, in this case, sucrose. Sucrose and sweet tastes activate the reward system and increase dopamine levels in nucleus accumbens, having the same reward circuit as alcohol so intermittent exposure to sucrose can produce effects similar to those of drugs of abuse, such as modifications in brain reward circuits and addiction-like behavior.

A solution of sucrose 2% was delivered in the self-administration session, instead of the usual ethanol 20%, with a fixed ratio of 3 as well for 15 minutes. The day after, in substitution of sucrose it was delivered a solution of sucrose 2% plus ethanol 20%. The active lever presses and rewards were recorded.

2.5 Estrous cycle phase determination and comparison with ethanol consumption

Vaginal smears were collected to determine whether hormonal fluctuations impacted ethanol consumption. This procedure to evaluate the stage of the estrous cycle for each rat was made once a day, between 9:00am and 10.00am (2hours after the lights on), for 6 consecutive days, since the majority of the techniques examine vaginal smears at 24h intervals. The ethanol self-administration began only in the afternoon, few hours after this procedure, to avoid interference with behavior. Vaginal smears were obtained with a pipette, filled with a small amount of saline solution (0.9%). Then the tip was gently inserted into the vagina of the rat and slowly rotated clockwise, carefully for not inserting it too deep or with aggressiveness, because inadvertent pressure into the cervix can origin pseudopregnancy (82). Next, the saline solution was quickly released and then instantly drawn back and the vagina was flushed like this three times before putting the sample into an Eppendorf tube. The tubes were coded by a different person from the one who did the sample collection to avoid bias, making this a double-blind experiment. Immediately after this procedure, the samples were transferred onto a microscope slide and viewed under a light microscope at x100. The final step was to do the cells counting, with three different types of cells: leukocytes (small speckling), nucleated epithelial cells (small and round, with a nucleous), and cornified epithelial cells (irregular shape, anucleated), and then proceed to evaluate the phase of the estrous cycle the females were in. Figure 3 shows both

the type of cells present in all phases and typical examples of the 4 phases of the estrous cycle (83).

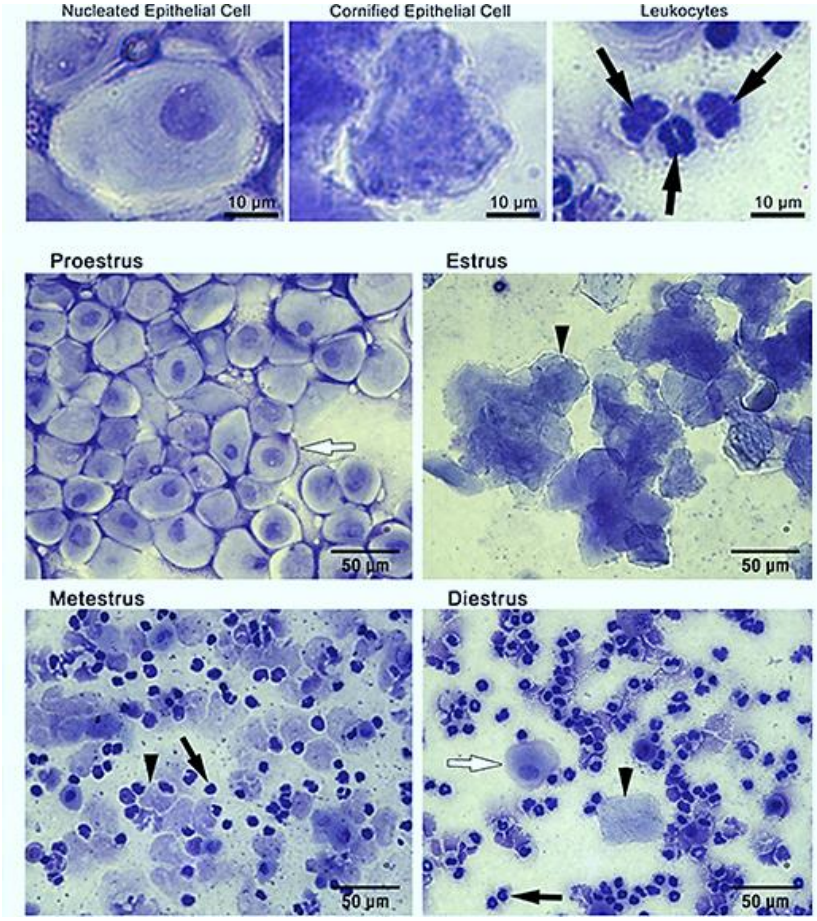

Figure 3 - Estrous stage identification by cytological assessment. Adapted from (83).

2.6 Pharmacological Treatment: Baclofen Injections

The administration of saline (0.9%), RS(±)-baclofen, R(+)- baclofen and S(-)-baclofen were made by intraperitoneal injections.

Animals were divided in two experimental groups, one receiving the treatment and the other as a control group, receiving only saline solution, in a conterbalanced manner. This experiment lasted for two consecutive weeks. In the first week, the control group (15 rats) received the saline solution and the treatment group (15 rats) received the racemic baclofen. In the second week, the control group from before became the treatment group (same rats) and the enantiomers were applied, instead of the racemic. The treatment group from the session before became the control group, only receiving the saline. 30 minutes after the injections the rats proceed to binge drinking, doing the self-administration of ethanol like usually. The rats received the following treatment schemes:

Table 3 - Experimental procedure schematics.

	Week 1		Week 2	
Rats	**Solution day 1**	**Solution 48h later**	**Solution day 1**	**Solution 48h later**
G1 (9 males and 6 females	RS-Baclofen	Saline	R-Baclofen	S-Baclofen
G2 (6 males and 9 females	Saline	RS-Baclofen	S-Baclofen	R-Baclofen

2.7 Blood collection

Blood collection was done 30 and 90 minutes after the self-administration session for all 30 rats, subsequently to the baclofen Injections. They were placed in a different division, within their home cages and one by one they were sedated with 5 percent isoflurane for 2 minutes. At that moment, blood was collected from the lingual vein in heparinized tubes (+- 200 ‑ µl).

Samples were centrifuged and stored on ice. Blood levels of baclofen (ng/mL) were determined by HPLC (High Performance Liquid Chromatography) by Pr Labat Laboratory (Unité Fonctionnelle Toxicologie Biologique - Hôpital Lariboisière (AP-HP) – Paris, France).

2.8 Ex vivo fast-scan cyclic voltammetry

After established the BD behavior and perform all the experiments previously described, rats were euthanized and brains were collected to perform the fast-scan cyclic voltammetry.

This technique can provide real-time measurements of changes in the monoamine neurotransmitters in the rat brain, more specifically dopamine, serotonin and norepinephrine. In this case, it was used to monitor the release of dopamine ex vivo. It is a technique based on voltage-dependent oxidation and reduction reactions (84,85).

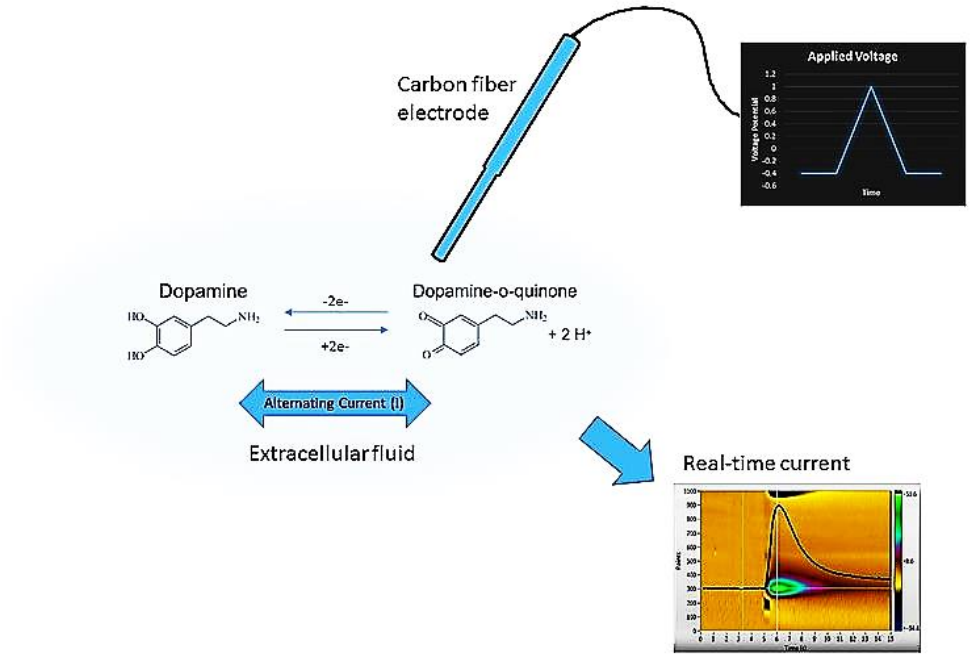

Figure 4- Monitorization of dopamine release by oxidation and reduction processes in the fast-scan cyclic voltammetry. Adapted from GRAP Lab.

Voltametry assays were performed following previously described procedure (80). Briefly, brain slices were cut and immerse in a buffer solution of artificial cerebrospinal fluid, with supply of oxygen and maintenance of osmolarity. While these slices were continuously in perfusion with the solution, fast scan cyclic voltammetry was used to monitor the extracellular dopamine released, by a stimulation of 200 µA for 0.5 ms every 3 min. The scan rate was 400 V/s with a sampling interval of 100 ms and the scan range was from −0.4 to +1.3 V (vs Ag/AgCl). After, the monitorization of the dopamine concentration was made with a standard curve, for each microelectrode.

3. Results

3.1 Baclofen treatment

3.1.1 Baclofen Injections

To gain further insight into the effects of the different baclofen enantiomers regarding ethanol consumption, injections in the 30 rats were done with RS(±)-baclofen and the 2 enantiomers, R(+)-baclofen and S(-)-baclofen and the BD pattern was evaluated.

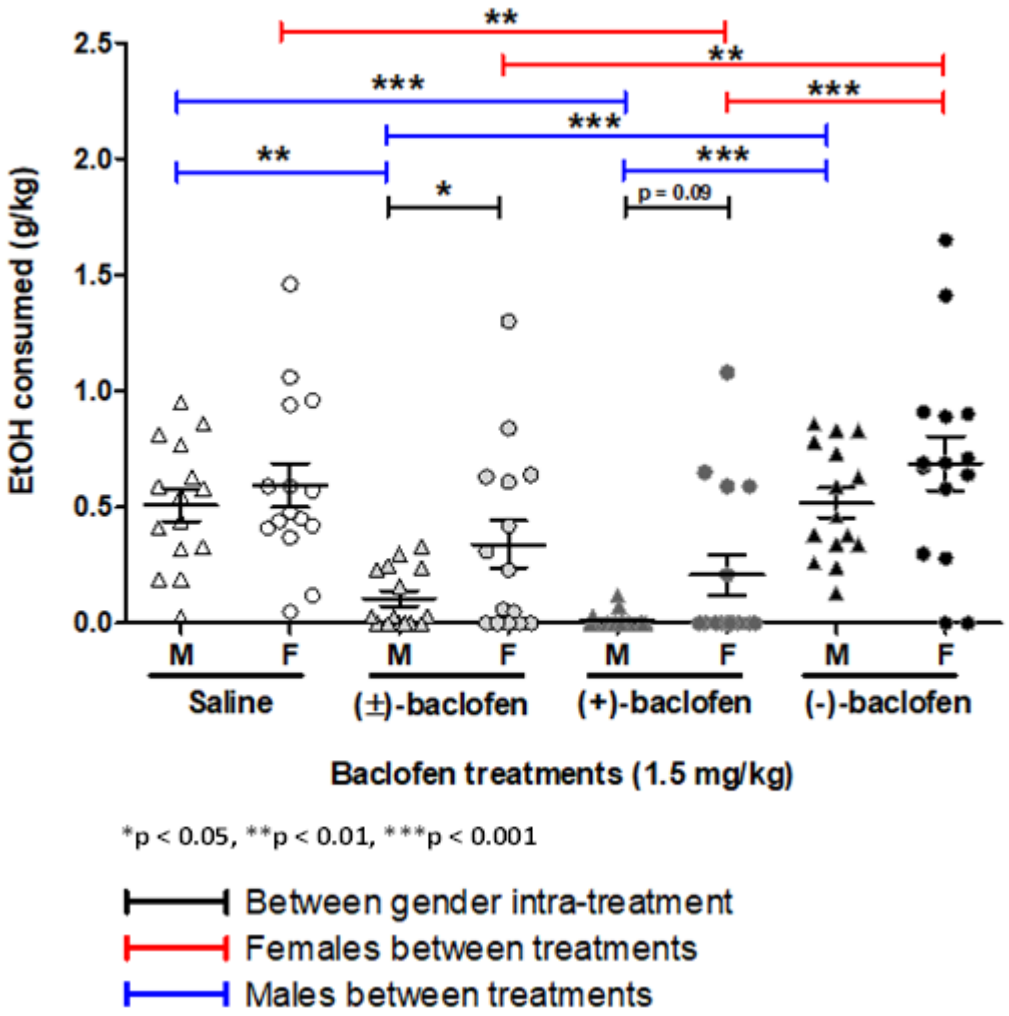

Figure 5- Baclofen treatments and efficacy on the reduction of ethanol consumption, depending on gender. Analyze with two-way ANOVA indicated a main effect of the factor gender ($F_{(1,84)}$=7.035, $p<0.05$) and of the baclofen treatment ($F_{(3,84)}$=20.27, $p<0.001$), and no interaction between both factors ($F_{(3,84)}$=0.34, $p>0.05$).

As can be observed in Figure 5, the RS(±)-baclofen and the R(+)-baclofen promoted a decreased in ethanol consumption in the male rats, while the S(-)-baclofen did not have any effect. As for the female rats, the RS(±)-baclofen had no effect on ethanol consumption and the R(+)- baclofen was the only form with a reduce in ethanol consumption, despite showing less efficacy than in males. Besides, it appears to be a large variability within the 2 groups regarding the ethanol consumed so an analysis was performed to see if the tendency observed on the average is similar for all the rats (Fig.6).

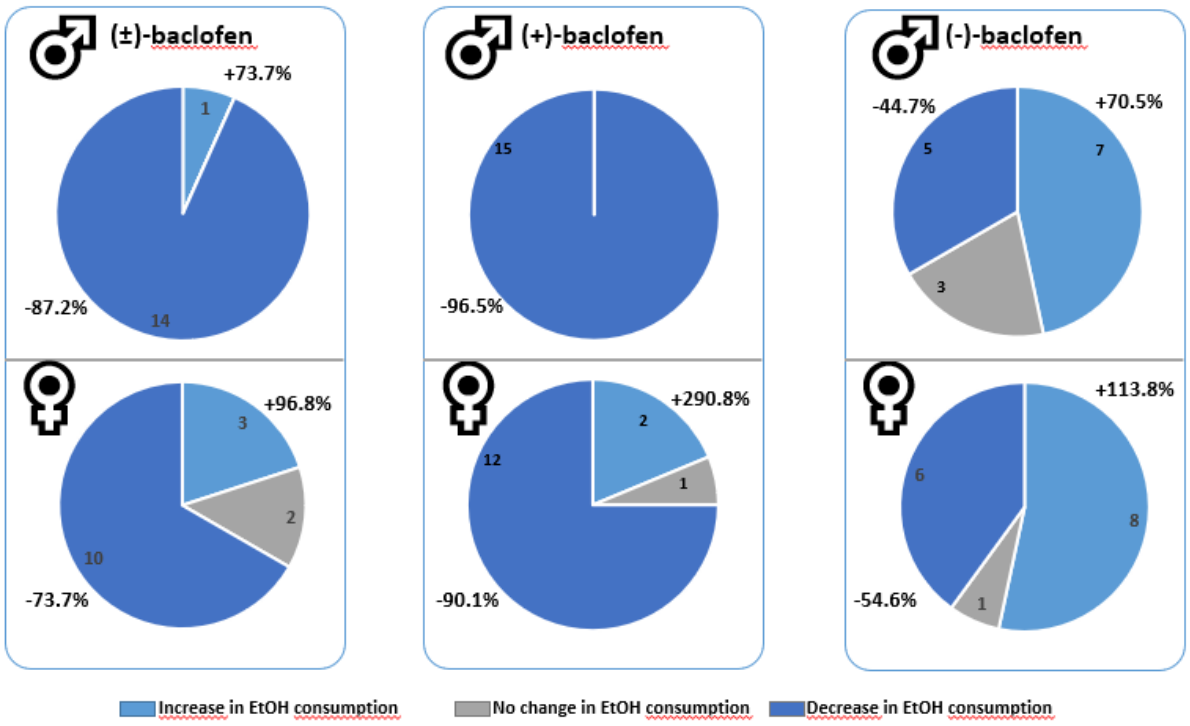

Figure 6- Variation of ethanol consumption due to response to the different baclofen enantiomers depending on gender. Baclofen was injected 30 minutes prior to the test (1.5 mg/kg i.p., 1 mL/kg).

After RS(±)-baclofen injections, within the males almost all 15 rats decrease their consumption, with the same response happening for the R(+)-baclofen. Considering now the females, 2 had no change in their BD consumption pattern and 3 of them even increased the ethanol consumption after the injection of RS(±)-baclofen.

When we compare the BD after the S(-)-baclofen injections, we can see almost no difference, as half of the animals increased their consumption and half decreased.

3.1.2 Blood Baclofen Concentration

In order to verify the efficacy of baclofen (comparison between consumption of the 20% ethanol solution after injections and the baseline), blood collection was carried out.

As figure 7 shows, there appears to be no difference between genders in relation of baclofen's metabolization, since the concentration 30 minutes after is almost the same. This suggests that the difference in the efficacy of baclofen in males and females is not due to the metabolization of this drug in the organism.

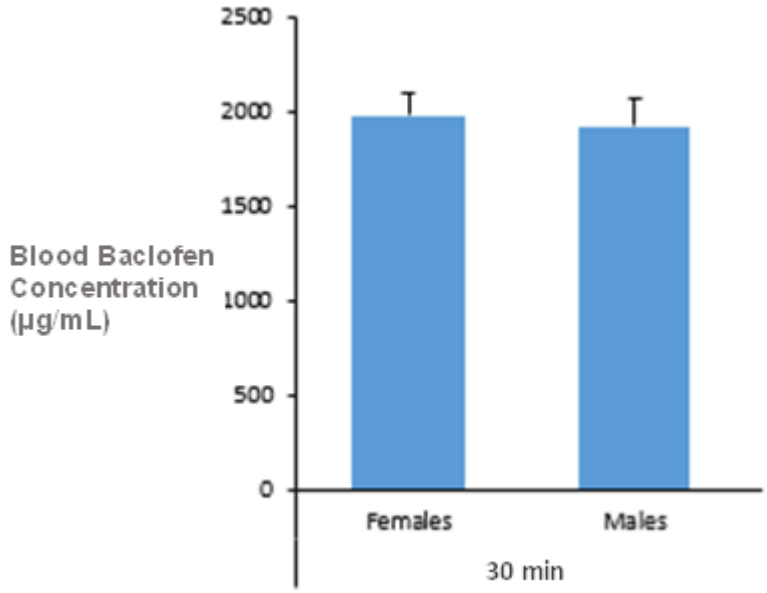

Figure 7- Injection of the racemic RS(±)-baclofen; 1.5 mg/kg i.p and blood sampling 30 minutes (peak of baclofen in the blood) after these injection. Values are expressed as median ± S.E.M.

The next step was a correlation analysis. As contemplated at the graphic above (Fig.8A), in the male group (n=14) there is no correlation between the level of baclofen in the blood and the efficacy in the reduction of ethanol consumption, sustaining that the efficacy is optimal for any blood concentration of baclofen. However, within the female group (n=13) interesting results were seen: the higher the concentration of racemic baclofen in the blood, the lower its effectiveness, that is, the greater is the ethanol consumption (Fig.8B). The efficacy of baclofen was evaluated comparing the results of the self-administration 30min after the injections with the baseline of ethanol consumption.

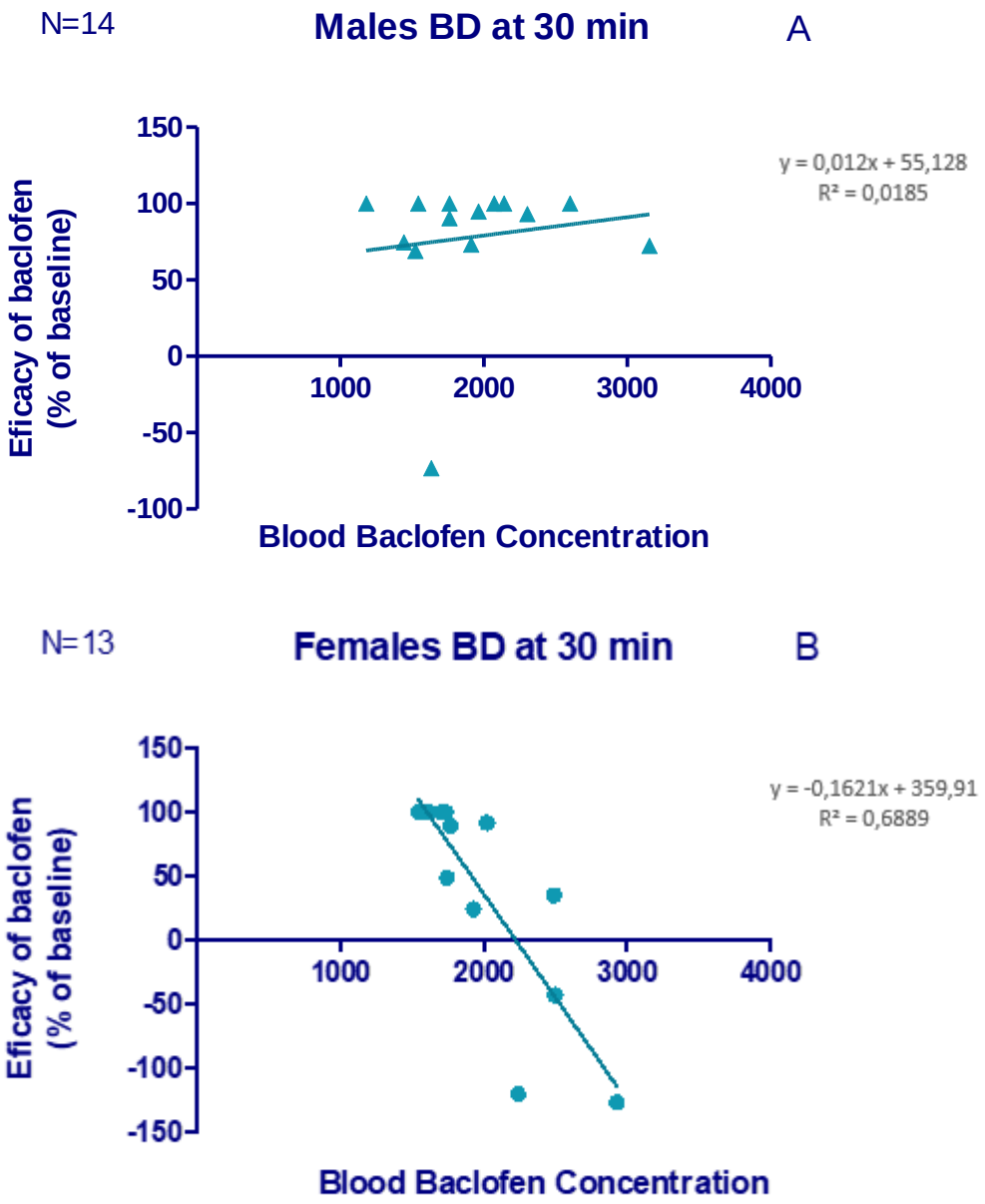

Figure 8- Evaluation of the BD pattern 30 minutes after the injections in males (A) and females (B).

3.1.3- Voltammetry: Dopaminergic release in brain slices

To evaluate how baclofen modulates the concentration of dopamine release from terminals in the nucleus accumbens core, fast-scan voltammetry was carried out from our adult chronic binge drinkers' rats. Differences between genders were the main focus, since at a behavior level there are some alterations.

Interestingly, results show a significant increase in the levels of dopamine release in rats that were treated with S(-)-baclofen, whereas the racemic baclofen and the R(+)-baclofen did not affect the levels of this release (Fig.9). The Kruskal-Wallis test conducted on these data

revealed an effect of the treatment and the Dunn's comparison indicated a significant difference between the S(-)-baclofen and the RS(±)-baclofen in both genders. In addition, only in females, the release in dopamine under S(-)-baclofen is also significantly different from the one observed with the R(+)-baclofen treatment ($p < 0.05$).

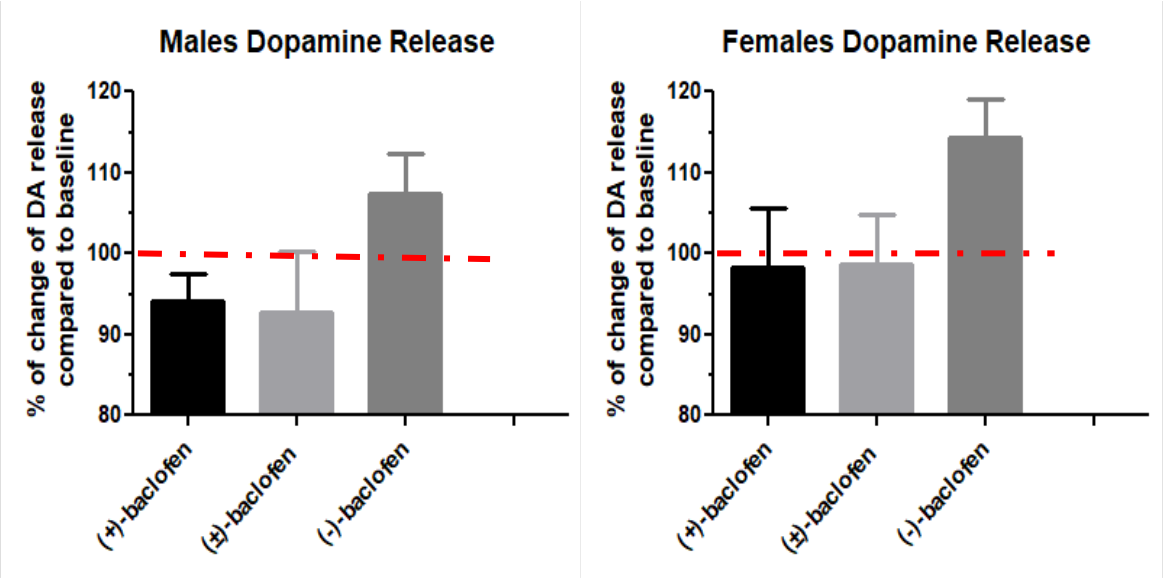

Figure 9- Measurement of the amplitude of the peak of electrically stimulate dopamine release after different baclofen's treatment in males and females.

3.2 Sucrose Self-administration

Sucrose Self-administration was evaluated after the rats had developed chronic binge, months later of initiating the ethanol self-administration and before the baclofen experiment. First, it was recorded the number of active lever presses in all rats for the 2 days. For the day with only sucrose 2% consumption, it was recorded the active lever presses and compare with a day with only ethanol 20%, for both males and females, as seen in figure 10.

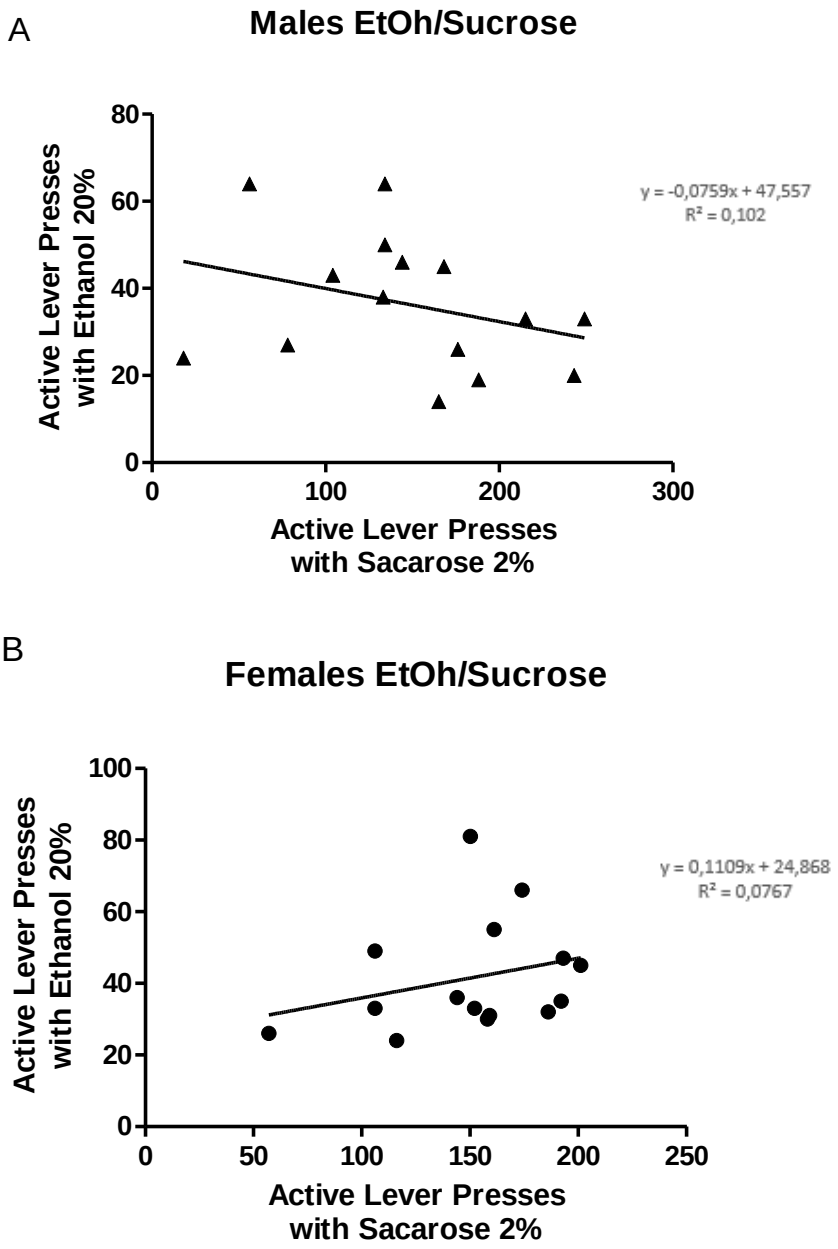

Figure 10- Comparison between gender (A and B), at a day when all rats received only sucrose 2% vs a day when they received the normal 20% ethanol solution (both days under normal conditions; SA procedure with fixed ratio of 3, for 15minutes). Both graphics show no significant correlation between ethanol and sucrose.

Sucrose was introduced as a control, to assess the behavior towards other reward substance not addictive, since it can produce similar effects on the reward system, activating common pathways. As described here, the consumption of sucrose does not correlate with the consumption of ethanol, showing there is not a sensibilization of the reward pathway; meaning there is no difference in the ability of the rats to perceive the rewarding properties.

3.3 Estrous cycle determination and comparison with ethanol intake

According to cell types observed in vaginal smears and their proportion, there are four stages that can be observed in a cycle, called proestrus, estrus, metestrus and diestrus (86) lasting 4 to 5 days. One whole estrous cycle is the time from one estrus phase to another estrus. Some articles refer to Diestrus as first Diestrus and Metestrus as last Diestrus (87), as others classify the estrous cycle with one more phase, as proestrus, estrus, metestrus I, metestrus II and diestrus (88). Some authors also classify the estrous cycle in one more phase, anestrus, characterized with ovarian inactivity (89).

The determination of the phase was evaluated by light microscopy and according cells density criteria, as mentioned before, and assuming 4 phases (proestrus, estrus, diestrus and metestrus). Proestrus is characterized by the prevalence of round nucleated epithelial cells with uniform size; Estrus, where ovulation occurs, with irregularly shaped, un-nucleated cornified cells; Diestrus by a predominance of leukocytes and finally Metestrus, by leucokytes and round nucleated epithelial cells.

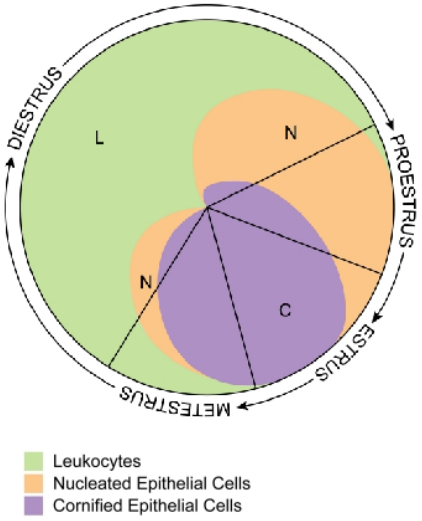

Figure 11- Visual representation; shows the proportion of each cell type present at the 4 stages of the estrous cycle of rats. Adapted from (90).

For each female rat, every cell type was counted, and the phase determined. Most of the vaginal smears displayed typical cells known to be present at a specific phase so it could easily be identified. However, there were a few without a 100% accuracy in phase determination and some irregular estrous cycle were detected. Subsequently, association between the phase of the estrous cycle for each female and the ethanol consumption for the respective day was studied. The results show total daily ethanol intakes (grams per kilogram of body weight) were not different across all estrus cycle phases, remaining almost unchanged, as it is observed in figure 12. Therefore, these results show there is no correlation between the estrous cycle and

the voluntary alcohol consumption, meaning the ovarian hormone fluctuations do not affect ethanol consumption patterns.

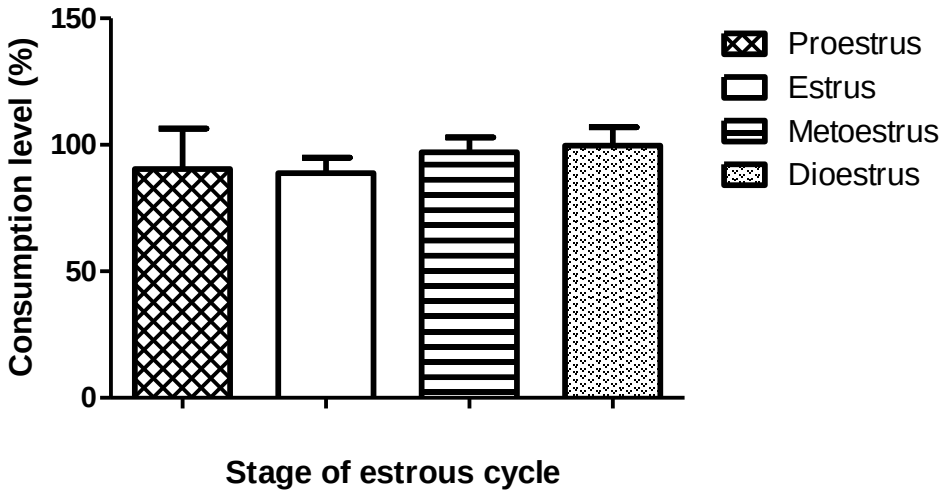

Figure 12- Association between EtOH consumption and estrous cycle. For each day, vaginal smears were collected and ethanol consumption, after the normal self-administration, was evaluated and compared with the corresponding phase of the cycle. Consumption level in percentage was calculated with the average of consumption, including these 6 consecutive experiment days.

4. Discussion

Recently, Baclofen has been the subject of several studies regarding AUD. Even in France, it has been approved as a treatment of AUD in some specific cases (47). However, there is not definitive results about his efficacy in all the different human studies performed (55,61). The current study searched to characterize the enantioselective effects of baclofen in a binge-like chronic model of ethanol drinking in Long Evans rats, especially in a gender approach.

The clinically used racemic compound can be separated into separate enantiomers, who have produced different profiles in behavioral assays, with an efficacy thought to reside with the active R(+)-baclofen enantiomer (58). The main experiment with the baclofen treatment shows it alters ethanol self-administration depending of the enantiomers. Overall, these data show that injections with the R(+)-baclofen lead to a decreased in ethanol consumption for both genders, even though is more efficient in males. As for the RS(±)-baclofen, it displayed a significant decrease only within the males, while in the females has essentially no effect. One conclusion that can be drawn from this observation is that R(+)-baclofen is more efficient than RS(±)-baclofen, which was expected considering other studies (57,58).

As mentioned before, gender is a factor that plays an important role in ethanol consumption (23,91,92). So, an identification of subpopulations was made and, as expected, there is some important variability at an individual level. Examining the RS (±)-baclofen and the R(+)-baclofen, almost all the treated male rats decreased their ethanol consumption, with the S(-)-baclofen being the one bringing more variability, with only 5 rats decreasing their consumption after this treatment. With the females the results are different, since in all 3 forms of baclofen there is more variability.

To analyze these data and see what can explain such differences between these 2 groups, blood baclofen concentration was investigated, but results showed no difference between gender, so the different efficacy cannot be explained by this. Besides, along the correlation analysis, it is possible to conclude that the blood baclofen concentration does not affect the efficacy in the males and in the females is inversely proportional. These results support the idea there is a strong difference between males and females regarding the response to the baclofen's treatment

To further investigate these genders differences estrous cycle had to be evaluated as well. It was monitored in this study and there is a prediction that it did not affected ethanol consumption and so explanations of the results due to the cycle can be excluded.

Besides all that, it was important to evaluate the integrity of the reward pathway , since some alterations due to the chronic BD could alter and explain the variability, but no difference appeared, with results proving that there was no sensitization of this pathway and so this is not a factor that influence extracellular dopamine concentration.

Dopamine systems is known to an important part in the effects of drugs of abuse. A part of the neural pathway mediating motivated behaviors comprises dopamine neurons and their projections from the VTA to the nucleus accumbens. Therefore, factors that influence an increase in extracellular dopamine concentration at the nucleus accumbens play an important role in treatments (75). Regarding the voltammetry, it was possible to assess different effects on the dopaminergic release, depending on gender as well. All 3 forms of Baclofen were compared to the baseline, i.e, 100%. R(+)-baclofen and RS(±)-baclofen induce a decrease in dopamine release in males, but not in females, while the S(-)-baclofen promoted an increased in the dopamine release in both groups, even though the increase was greater in female rats, which implies there can be a distinctive response from the dopaminergic system, with baclofen balancing the reward network (41).

5. Conclusion and Future Perspectives

The purpose of the current study was to determine the efficacy of the different baclofen enantiomers on the reduction of chronic BD of adult rats and evaluate the results among males and females. In order to investigate if the dopaminergic pathway is influence differently by the enantiomers and what differences can appear between gender, two main experiments were performed.

The baclofen treatments have shown there are significant differences between the 3 forms of this drug, with the R(+)-baclofen being the most effective one in decreasing the ethanol consumption in almost the entire population of rats whereas the S(-)-baclofen has opposite effects within the same population. Indeed in some animals it will decrease ethanol consumption but on the other part of the animals it will induce an increase in ethanol consumption (sometimes more than 2 times the baseline levels of consumption). Moreover, the effects are divergent in the genders, with the RS(±)-baclofen only promoting an effect in males and R(+)-baclofen being more effective in them.

The results of voltammetry demonstrated that there is a significant increase in dopamine release regarding rats treated with S(-)-baclofen and gender differences as well, for females RS(±)-baclofen and R(+)-baclofen shown no effect, and for males induced a small decrease. All these differences in sensitivity support the role of the dopamine response in ethanol consumption, which suggests it can be due to differential response of the dopaminergic system.

Consequently, this study has a relevant clinical implication, since the racemic form RS(±)-baclofen is the one currently administered to patients and these results demonstrate the reason why the therapeutic response is not the same in all of them; proposing the variability may be due to the sensitivity of the 2 enantiomers, mainly the S(-)-baclofen that can promote an increase in both ethanol intake and dopamine release in both genders. . And maybe most importantly, these data demonstrated that the racemic form is not efficient in female's rats, but it is still prescribed to women in need of treatment to cure their alcohol dependence with probably no efficacy neither as suggested by a study presented recently during the RSA meeting in Minneapolis. Additionally, the most efficient form, the R(+)-baclofen, is demonstrated to still be less efficient in females and, alongside the RS(±)-baclofen having no effect in them, leads the question if the current treatment is relevant for women.

Further gender studies involving larger number of patients are needed to better understand the effects different baclofen enantiomers can have in the dopaminergic system and the factors that can lead to alteration in ethanol intake, principally in BD behavior. In any case, there is

currently a great investment and work in this area, but there is more to discover about BD and GABA-B receptors, to see if they continue to be an interesting target for female treatments.

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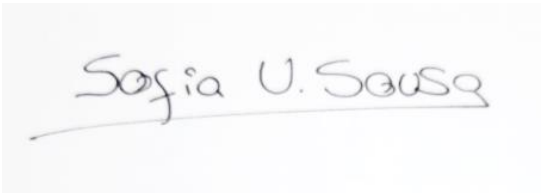
Annexes

Declaração de Compromisso de Anti-Plágio

Eu, Sofia Vilelas de Sousa, estudante n.º 10287 declaro por minha honra que o trabalho da minha autoria, intitulado Efficacy of the different baclofen enantiomers on the reduction of binge drinking in a rodent model: a gender study é original e que todas as minhas citações estão corretamente identificadas; no caso de ter utilizado frases de trabalhos de outros autores, ou, se as redigi com palavras diferentes, as fontes destas foram referenciadas devidamente. Tenho consciência de que a utilização de elementos alheios não identificados constitui uma grave falta ética e disciplinar.



Lisboa 11 de Dezembro de 2019