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Resumo

O álcool é uma das bebidas mais consumidas no mundo, sendo que a Europa é a região cujo consumo é mais elevado, de acordo com o "Global Status Report on Alcohol and Health" da WHO (2018). Este consumo não só resulta em custos para a sociedade, como também é um grave problema de saúde dado que as poucas opções terapêuticas que existem no mercado para tratar a dependência do álcool não apresentam elevada eficácia terapêutica. Por estes motivos, é importante investigar o desenvolvimento da dependência do álcool.

Neste projeto foi avaliada a influência da exposição prolongada ao álcool nos níveis de recetores de dopamina e glutamato (recetores D1, D2 e NMDA respetivamente) em três regiões do cérebro de um modelo animal (ratos Wistar) sujeito a exposição prolongada intermitente. Os recetores NMDA foram analisados no *hippocampus*, enquanto que os recetores D1 e D2 foram analisados no corpo estriado dorsal e no "córtex", uma região inicialmente utilizada como controlo. A técnica utilizada para efetuar estas análises designa-se ensaio de radioligandos, baseia-se na ligação de um radioligando ao recetor de interesse e consequente determinação da radioatividade na amostra como medida dos níveis do recetor de interesse.

Os resultados obtidos mostram que, apesar de os ratos demonstrarem sintomas de abstinência relacionados com um estado hiperglutamatérgico após exposição prolongada ao álcool, os níveis do recetor NMDA não se alteraram significativamente em relação ao grupo de controlo, o que significa que pode existir outro mecanismo que conduz ao estado hiperglutamatérgico. No corpo estriado dorsal, os níveis dos recetores D1 e D2 não sofreram alterações estatisticamente significativas, contudo, os níveis do recetor D2 mostraram uma clara tendência de diminuição (quase estatisticamente significativa), o que pode traduzir um incremento tempo-dependente da influência do corpo estriado dorsal no processo de dependência do álcool. Por fim, os resultados na região do "córtex" foram surpreendentes, dado que como se tratava de uma região de controlo, não eram esperadas alterações. No entanto, os níveis do recetor D2 diminuíram após exposição ao álcool, o que sugere que esta região pode ter mais importância no desenvolvimento da dependência do que o esperado.

Palavras-chave: Álcool; Adição; Dopamina; Glutamato; Cérebro.

Abstract

Alcohol is one of the most consumed beverages in the world, namely in Europe, which is the region of the world that has the highest consumption, according to the "Global Status Report on Alcohol and Health" from WHO (2018). This consumption not only results in high costs to the society, but it is also a serious health problem, because the few therapeutic options that exist to treat Alcohol Use Disorder have very limited effectiveness. For these reasons, it is important to study and to better understand the alcohol addiction process.

In our project, we analysed the influence of prolonged ethanol exposure (through prolonged intermittent exposure) on the levels of dopamine and glutamate receptors (D1, D2 and NMDA receptors respectively) in three brain regions of an animal model (Wistar rats). The NMDA receptor was analysed in the *hippocampus* and the D1R and D2R were analysed in the *dorsal striatum* and in the "cortex", a region initially used as a control. To accomplish this, we used a technique designated radioligand binding assay, which is based on the connection between a radioactive ligand and the receptor of interest and the consequent determination of the radioactivity in the sample to determine the receptor levels.

Our results show that upon prolonged alcohol exposure, although the rats still displayed hyperglutamatergic-related withdrawal symptoms, NMDAR levels remained unaltered in the *hippocampus*, which suggest that there might be other mechanisms that lead to enhanced glutamatergic activity. In the *dorsal striatum*, D1R and D2R levels were not different from control, however D2R levels were almost statistically decreased, which might reflect an increased influence of this region in the addiction process as the consumption is prolonged over time. Finally, in the "cortex", D2R levels decreased with ethanol exposure. This result was surprising since we expected that both D1R and D2R levels remained unchanged. These findings suggest that regions thought not to play a role in the addiction process might actually be important.

Keywords: Alcohol; Addiction; Dopamine; Glutamate; Brain.

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This work was performed in the French laboratory GRAP INSERM U 1247 in Amiens, whose director is the Pr. Mickael Naassila. The GRAP (Groupe de Recherche sur l'Alcool et les Pharmacodépendances) exists since 2003 and its work is focused on alcohol addiction.

This work is part of a bigger project aimed at describing the neuro-adaptations that come along alcohol addiction, more precisely to monitor the expression of glutamatergic and dopaminergic receptor in the brain of rats previously subjected to prolonged ethanol exposure.

After 5 years of a Master's degree in Pharmaceutical Sciences, I am lucky enough to say that my favourite project of the degree was my final thesis, and I could not have done it without the help of my friends, family and the opportunity that was given to me in Amiens. I would like to thank my family, namely my parents, who always gave me the best conditions to study and supported me every time; to Mariana Monteiro, who was always there for me even in France and to Hilal Foudi, Quentin and Virginia Desideri and the rest of my friends, who reminded me that life is not always work.

Finally I would like to thank my tutors Catherine Vilpoux, who welcomed me very well in Amiens and Vasco Branco for their guidance, sympathy and for their valuable inputs in this work.

Abbreviations

AF	After Treatment
AM	Amygdala
AUD	Alcohol Use Disorder
BAL	Blood Alcohol Levels
CB	*Cerebellum*
CC	Central Cortex
CrC	Cerebral Cortex
CREMEAP	Comité Régional d'Ethique en Matière d'Expérimentation Animale de Picardie
CP	*Caudate Putamen*
CPM	Counts per Minute
DLS	*Dorsolateral Striatum*
DMS	*Dorsomedial Striatum*
DRN	*Dorsal Raphe Nucleus*
DSM	Diagnostic and Statistical Manual
D1R	Dopamine Receptor 1
D2R	Dopamine Receptor 2
FC	Frontal Cortex
GP	*Globus Palidus*
HIP	*Hippocampus*
HYP	*Hypothalamus*
NAC	*Nucleus Accumbens*
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptors
PFC	Pre-Frontal Cortex
PIE	Prolonged Intermittent Exposure
SNc	*Substancia Nigra pars compacta*
SNr	*Substancia Nigra pars reticulata*
SPT	*Septum*

STN	Subthalamic Nucleus
US	United States
VTA	Ventral Tegmental Area
WHO	World Health Organization
WD	Withdrawal

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1.Introduction

1.1. Alcohol use disorder

Alcohol is the most famous drug in the world, with more than 55% of people aged 25 and over consuming it at least once a month (1). It is known for its stimulating and mind altering effects, thus turning it into one of the most preferred products used by people when socializing (2). Alcohol consumption is responsible for 3 million deaths per year (3), turning it into the fifth risk factor for premature death and disability in the U.S (4). Also a weighted analysis of the harmful effects of several drugs concluded that alcohol is the most harmful drug (5). Alcohol consumption is defined as the consumption of any beverage containing the chemical ethanol (CH_3CH_2OH). Ethanol exerts its effects in a range of blood concentrations from a few mM (usually achieved by the consumption of 10 g of pure alcohol, that is the quantity present in 100 ml of wine in a 80 Kg man) to 100 mM (4,6 g/l), causing anxiety, euphoria, diminished reaction times, lack of motor coordination and cognitive impairments, all of which can evolve to sedation, respiratory depression, coma and even death at higher concentrations (6) (the lethal concentration varies from 50 to 110 mM (2,3 to 5,1 g/l)).

Alcohol, similarly to other substances of abuse, strongly activates the brain reward system, thus resulting in strong positive feelings. However, the repeated exposure to these positive feelings causes a persistent suppression of the reward system to a point where the natural rewards lose the ability to properly activate it, thus leading the individual to a state of discord which can only be interrupted by potent activators of the reward system (7,8).

Alcohol addiction disease has been re-defined and renamed "Alcohol Use Disorder" in the 5th edition DSM (2013). AUD progresses through a two-component mechanism based on both positive and negative reinforcement. Primarily, the consumption of alcohol results in positive rewards for the consumer, thus augmenting the possibility of a further consumption. Then, as the brain adapts to the constant exposure of alcohol, the negative reinforcement kicks in, as alcohol consumption turns into the only strategy capable of avoiding withdrawal symptoms. Moreover, as time passes, people not only develop tolerance, but also crave more and more for alcohol, as a result of the two-component mechanism, which in turn results in a progressive increase in the amount consumed. Consequentially, the brain homeostasis is altered so profoundly that it simply cannot function normally without alcohol (1,2,8). This results in the

development of AUD, in which a subject is aware of the negative effects of alcohol in its health, but it is unable to cease its consumption, given that abstinence results in withdrawal symptoms.

AUD is defined in the Diagnostic and Statistical Manual for Mental Disorders, 5th edition, as a pattern of alcohol consumption, leading to problems associated with 2 or more of 11 potential symptoms of AUD (**Table** 2) (9). Unfortunately, current treatment options for AUD are limited to disulfiram, acamprosate, naltrexone and baclofen (which recently received the French "Autorisation de Mise sur le Marché" in April 2021) all of which have limited clinical efficacy, although their distinct mechanisms of action (**Table 1**). This lack of therapeutic options is essentially related to the complexity of alcohol addiction. Moreover, AUD can be related to other comorbidities such as liver diseases (10). For these reasons it is important to further investigate alcohol addiction physiopathology and its underlying mechanisms.

Table 1 Drugs to treat AUD and their mechanism of action

Drug	Mechanism of Action	Reference
Disulfiram	Inhibition of aldehyde dehydrogenase	(11,12)
Acamprosate	Antagonism of NMDAR	(12)
Naltrexone	Blockage of mu receptor	(12)
Baclofen	Agonist of $GABA_B$ receptor	(12)

Table 2 DSM V criteria (13)

Substance Use Disorder Will be Defined as a Clinically Significant Impairment or Distress as Manifested by 2 (or More) of the Following Occurring Within a 12-Month Period:
1. Failure to fulfil obligations at work/school or home
2. Use in dangerous situations (i.e., driving)
3. Continued use despite social or interpersonal problems due to the substance use
4. Tolerance (the need for increased amounts of substance to achieve the desired effect or a diminished effect if using the same amount)
5. Withdrawal (development of substance specific syndrome due to the cessation of use that can be serious and prolonged)
6. The substance is used more than was originally intended
7. Persistent desire or unsuccessful efforts to cut down on use
8. Significant time spent getting/using or recovering from substance

9. Decreased social, occupational, or recreational activities due to substance use
10. Continued use despite subsequent physical/psychological problems
11. Craving

Since ethanol only interacts with other molecules via hydrogen bridges and hydrophobic bounds, when alcohol addiction studies started, it was theorised that the effects of this drug were due to its actions in the membranes of neurons, thus rendering alcohol as a non-specific drug (6). However, it was demonstrated that neurons are relatively insensitive to ethanol (in structural terms), thus the effects of ethanol on neuroplasticity and toxicity should be attributed to another mechanism (14).

1.2. The reward system

Alcohol addiction is strongly related to alterations in the neurons constituting the so-called reward system. The reward system is the result of the evolution of species, since it is responsible for their inherent search for positive stimuli, thus rewarding activities that promote survival by producing a pleasurable effect, and the avoidance of negative stimuli in order to maximize the chances of survival. Moreover, organisms developed processes such as homeostasis sensory perception, associative and non/associative learning and decision making to refine an organism response to the environmental stimuli, namely through prediction of their positive or negative features (15–17).

The reward system is composed by several structures (18–20) (**Figure 1**):

- The ventral tegmental area (VTA), which is mainly composed of dopaminergic neurons that respond to glutamate in the presence of a reward-associated stimuli and thus release dopamine in other areas such as *nucleus accumbens* and pre-frontal cortex (7);
- The *nucleus accumbens* or *ventral striatum* which is related to the cognitive processing of rewards, the determination of the desirability of a stimuli and in the acquisition and eliciting of conditioned behaviours that enable future reward-seeking behaviours (7);
- The pre-frontal cortex, a region involved in the integration of the information that decides if a certain behaviour is elicited, it is also implicated in motivation, brain executive functions and the determination of the salience of a stimuli (7);
- The amygdala, which is determinant in conditioned learning and integration of surrounding cues with previous reward or aversion memories (7);

- The *hippocampus*, a structure that is crucial for learning and memory (7), namely in establishing association between drug consumption and the context related to it (21);
- The *dorsal striatum*, which is involved in motor behaviour (22) but also in the influence of habit or stimulus responses, in other words, if a behaviour is salient incentive directed or context directed (23).

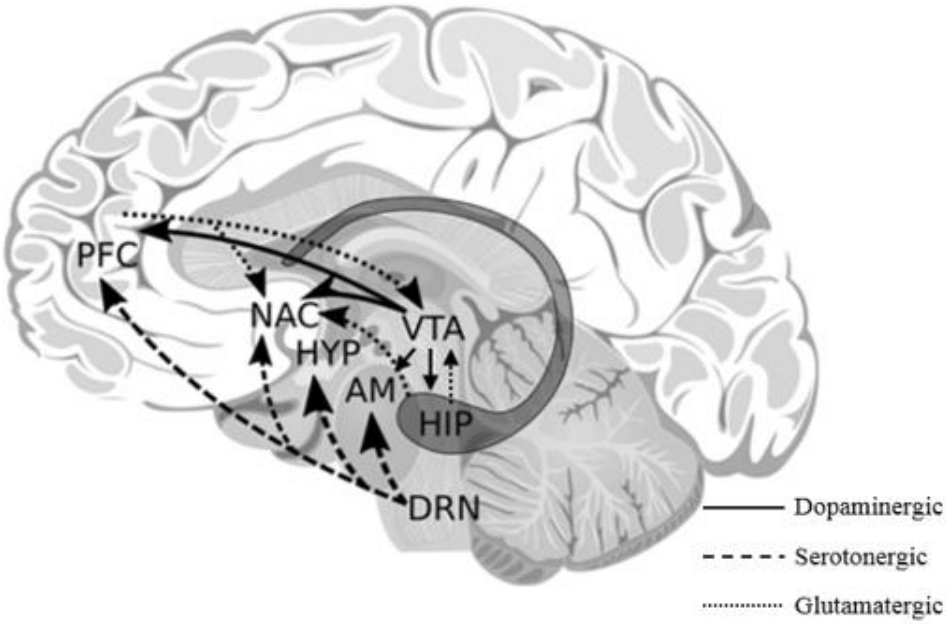

Figure 1- Structures of the reward system. This system is composed by important structures such as the VTA, the nucleus accumbens, the pre-frontal cortex and the dorsal striatum. All of which communicate through neurotransmitters. Figure adapted from (7).

The most famous neurotransmitter of this system is dopamine (24,25), whose increased activity is associated to the positive feelings that arise from the rewarding behaviour and decreased activity associated with the reward-seeking behaviour (26,27). However, dopamine is not the only neurotransmitter involved in the reward system with serotonin and glutamate also exerting their influence (28). Nevertheless, the process starts with a stimulated released of dopamine by the neurons of the VTA into the areas where they are projected, namely in the *nucleus accumbens* (29). The *nucleus accumbens* is a crucial structure of the reward circuit, being implicated as an important driver for goal-directed actions influenced by the estimated value of the associated goal (30). Moreover, as the consumption behaviour is repeated again and again, another set of dopaminergic neurons in a structure called *substantia nigra*, whose projections go to the *dorsal striatum*, are stimulated. Consequentially, the recurrent reward signals are transposed into regular actions, whose motivation to be repeated starts to reside in the action itself rather than in the reward. These reward-associated actions can evolve into a

habit (31) as the *dorsal striatum* surpasses the *ventral striatum*. In addition, the generation of these habits might also be associated with a reduction of pre-frontal cortex inputs in the *striatum* that will ultimately lead to a loss in the control of action selection (32). Finally, other set of dopaminergic neurons project from the VTA to the amygdala and the *hippocampus*, whose importance extends to emotional and memory associations, and to the pre-frontal cortex, which mediates salience attribution and self-regulation (33).

It is important to refer that the reward system also has other neurotransmitters than dopamine whose contribution should not be neglected, such as glutamate, GABA and serotonin. In fact, the dopaminergic stimulation produced by the dopaminergic neurons in the VTA can be explained by an alcohol-induced reduction in the inhibitory GABAergic influence in this region (8), probably through the potentiation of $GABA_A$ receptors (34) in the GABAergic neurons that project into the VTA. Interestingly, dopamine-deficient mice showed conditioned place preference for cocaine through a mechanism likely involving serotonin and glutamate (35). Moreover, repeated stimulation of the reward system results in frequent dopaminergic stimulation which in turn originates neuroadaptations in several neurotransmitter systems such as: the glutamatergic, a system responsible for neuronal excitability and neuroplasticity (36); the GABAergic, which is responsible for inhibiting action potential transmission (37); the opioid and endocannabinoid (38–40); and other neurotransmitters involved in arousal and mood such as: cholinergic (41,42); serotoninergic (43,44) and noradrenergic (45) systems. One example is the glutamatergic projections from the *hippocampus* and pre-frontal cortex to the *nucleus accumbens*, thus modelling this structure's activity (46). Other important example is the serotonergic system: this system also plays a crucial role in mediating alcohol addiction. The majority of the ascendant serotonergic neurons are located in the *raphe nucleus*, a region associated with stress and coping, and have projections to areas such as the VTA and *nucleus accumbens* where they modulate the dopamine release and, consequentially the VTA activity in the reward system (47,48). Moreover, acute ethanol exposure not only enhances serotonin receptor $5\text{-}HT_3$ (49,50), but also increases serotonin levels (51). These findings are fundamental since increased $5\text{-}HT_3$ receptor expression leads to higher activity of the neurons in areas that receive serotonergic input. Interestingly, chronic ethanol exposition results in lower serotonin levels (52), probably an adaptation that results from the acute effects of alcohol. In addition, the serotoninergic system also enhances GABA activity in the *hippocampus* through $5\text{-}HT_3$ activation (53,54).

1.3. Glutamate

One of the most interesting adaptations during alcohol consumption is the NMDA receptor plasticity, as the number of the NMDAR are altered in response to alcohol exposure. NMDAR are a type of ionotropic glutamate receptor which is a ligand-gated ion channel that allows the passage of several cations such as Na, K and Ca, thus affecting several metabolic pathways (55). They are multimeric four subunit complexes, whose name comes after the agonist that activates it (in this case N-methyl-D-aspartate) (55). They are formed by two identical NR1 subunits whose presence is required to the formation of functional channels, and two NR2 (either NR2A, NR2B, NR2C or NR2D) or NR3 subunits, whose alterations in their expression affect the pharmacological and biophysical properties of the channel (56,57).

Alcohol withdrawal symptoms are partially associated with an imbalance between excitatory (glutamate) and inhibitory (GABA) neurotransmitter systems. Acute alcohol administration both *in vitro* and *in vivo* models inhibits NMDAR function and expression (4,58–66), in the *hippocampus* (67) and frontal cortex (63), while also promoting a decrease in the extracellular glutamate levels in regions such as *hippocampus* and *nucleus accumbens* (68,69), a mechanism that is partially responsible for the acute effects of alcohol. Interestingly, chronic alcohol consumption seems to increase NMDAR function and expression (4,62,78–87,70,88–91,71–77), which can be a mechanism to compensate the initial inhibition (92). These alterations produce several consequences when alcohol consumption is held, namely an increase in seizures risk (70,72), mortality (72) and an hyper-excitatory state in withdrawal and protracted abstinence (70,82), as a result of both the higher glutamate levels observed in the *striatum* (93), *hippocampus* (94), *nucleus accumbens* (95,96) and the higher NMDAR levels, thus leading to a hyperglutamatergic state associated with *delirium tremens* (97). Moreover, alcohol addiction generally translates into repeated cycles of substance abused followed by short periods of withdrawal, a pattern that is associated with even higher glutamate levels after withdrawal in animals (92).

One of the mechanisms that explain the symptoms experienced not only during alcohol consumption, but also during withdrawal and abstinence, is the changes in NMDAR levels. Depending on the duration of the exposition and on the study design, NMDAR expression varies, as seen on a number of representative studies (**Table *3***, **Table *4*** and **Table *5***).

Table 3 Influence of sub chronic ethanol exposure on NMDAR expression without withdrawal in rats

Duration of ethanol exposure	BAL/Dose of ethanol	Technique	Rat strains	Result After Treatment	Area	Reference
84h	3-5 g/l	Binding assay with [3H] MK-801	Wistar	NMDAR =	CB	(98)
4 days	1,75-2,25 g/l	Western-Blot	C57BL/6 mice	NR1, NR2B ↑	PFC	(82)
5 days	2,25 g/l	Binding assay with [3H] MK-801	Sprague-Dawley	NMDAR ↑	FC, HIP	(89)
6 days	9-15 mg/Kg/day	Western-Blot	Sprague-Dawley	NR1, NR2A and NR2B ↑	CC, HIP	(79)
10-12 days	1,5 g/l	Cell patch-clamp	Sprague-Dawley	NMDAR ↑	AM	(56)
16 days	-	Western-Blot	Sprague-Dawley	NR1, NR2A, NR2B, NR2C =	HIP	(99)

CB: *Cerebrum*; HIP: *Hippocampus*; AM: Amygdala; CC: Central Cortex; PFC: Pre-Frontal Cortex; FC: Frontal Cortex.

Although an increase in the NMDAR or NMDAR subunit expression is not observed in all the studies, excluding the first one given its small exposure period, a trend of increased NMDAR expression clearly emerges. Taking into account that these are all sub chronic studies it can be concluded that prolonged alcohol exposure is not required to induce glutamate neuroplasticity and NMDAR up-regulation. Interestingly, even though there is a relation yet to be established between increased glutamatergic activity within the VTA and behavioural alterations, the learning of drug-associated clues has been related to higher NMDA receptor/mediated activity in this region (100).

Table 4 Influence of prolonged ethanol exposure on NMDAR expression without withdrawal in rats

Duration of ethanol exposure	BAL/Dose of ethanol	Technique	Rat strains	Result after treatment	Area	Reference
7 weeks	1,25-2,5 g/l	Western-Blot	Wistar	pNR2B, NR2B and NR2A ↑	HIP	(101)
7-10 weeks	1,25-2,5 g/l	Western-Blot	Wistar	NR2B ↑, pNR2B =	PFC	(90)
19 months	2±0,5 g/Kg/day	Western-Blot	Wistar	NR2B ↑ FC, = in all the other areas	FC, NAC, HIP, CDP	(102)

HIP: *Hippocampus*; PFC: Pre-frontal cortex; FC: Frontal-cortex; NAC: *Nucleus Accumbens*; CDP: *Caudate putamen*.

Prolonged exposure studies show that there is a general increase in the NMDAR subunits expression in areas such as the pre-frontal cortex and the *hippocampus*. The results are consistent with the previous ones from the sub chronic studies, since the increase in NMDAR is sustained. Interestingly, the 19 month study shows that the NMDAR levels remained unaltered in areas such as the *nucleus accumbens*, although that might be due to different ethanol exposure models. However, this finding does not discard a hyperglutamatergic state in this region, since prolonged ethanol exposure is related with higher glutamate levels (96,103), in a state that continues into protracted abstinence (104). Taking into account that the *nucleus accumbens* participates in behavioural responses to alcohol related cues (105,106) and that an increase in the glutamatergic transmission in the VTA is associated with the learning of drug associated cues (107,108), the augmented glutamatergic activity could lead to increased risk of cue induced relapse and higher salience of alcohol related cues (109). Furthermore, the increased glutamatergic activity received in the *nucleus accumbens* can influence the dopaminergic system of the VTA via the reciprocal VTA/*nucleus accumbens* connections. This will play a role in the long term effects of alcohol exposure, such as the increased reward threshold observed in reward and the altered homeostasis of the dopaminergic system (109).

Table 5 Influence of withdrawal from ethanol exposure on NMDAR expression

Duration of alcohol exposure	BAL /Dose of ethanol	Duration of withdrawal	Technique	Rat strains	Result	Area	Reference
84h	3-5 g/l	12 and 26 hours	Binding assay with [3H] MK-801	Wistar	12H WD: ↑ 26H WD: =	CB	(98)
4 days	1,75-2,25 g/l	2 days	Western-Blot	C57BL/ 6	NR1 and NR2B =	PFC	(82)
6 days	5 g/Kg in the 1 day, then 9/15 g/Kg/day	2 days	Western-Blot	Sprague-Dawley	NR1, NR2A and NR2B =	CrC, HIP	(79)
7 weeks	1,25-2,5 g/l	21 days	Western-Blot	Wistar	pNR2B, NR2B and NR2A =	HIP	(101)
7 weeks	1,25-2,5 g/l	21 days	Western-Blot	Wistar	pNR2B ↓, NR2B =	PFC	(110)
28 weeks	-	2 days	Binding assay with [3H] MK-801	Long Evans	NMDAR =	DG, HIP, SPT, HYP	(111)

WD: Withdrawal; HIP: *Hippocampus*; CB: *Cerebellum*; DG: *Dentate Gyrates*; C: cortex; SPT: *Septum*; HYP: Hypothalamus; PFC: Pre-frontal cortex; CrC: Cerebral Cortex.

Beside the duration of alcohol exposure, is crucial to assess the influence of abstinence in NMDAR expression. In the several studies reviewed here, whose durations range from a few days to several weeks, as well as different animal and exposure models, the majority of them shows that NMDAR expression seems to return to normal values after at least 48 hours of alcohol abstinence. These surprising results might lead one to the conclusion that the glutamatergic system rapidly recovers from alcohol consumption. Nevertheless, that might not be the case since a hyperglutamatergic state can be sustained into protracted abstinence through other mechanisms, namely increased NMDAR function and trafficking of the NMDAR without altering its expression levels. In fact, although a majority of symptoms of alcohol withdrawal cease within 5-7 days after withdrawal, craving and addiction-related symptoms remain during

long periods of time (112). These symptoms might be responsible for relapse (113–115) as well as perpetuating alcohol use (116–119). To corroborate this view, several studies have showed there is a compensatory trafficking of NMDAR as a result of chronic alcohol exposure (120,121). However, the neuroadaptations that lead to long-term psychological symptoms need further research and probably are not only resumed to the glutamatergic system.

1.4. Dopamine

Another crucial system for the development of alcohol addiction is the dopamine system, principally due to the crucial role it plays in the communication between the VTA and the *nucleus accumbens*. Dopamine has been viewed as one of the principal neurotransmitters in addiction. In the 50's Olds and Milner found that rats would repeatedly self-stimulate certain areas of the brain were dopaminergic neurons were present, later a series of large studies showed that the blockade of dopamine receptors prevented the reinforcing effects of stimulants in some animals, and finally in the 90's dopamine release in the human *striatum* was correlated with the euphoria (122) that comes with drug consumption. Interestingly, it was found that lower dopamine function was related to alcohol dependence, namely lower D2R availability (123–128) and dopamine release (125,128) were found in alcohol dependent subjects. Interestingly, a study found that higher levels of dopamine receptors exerted a protective effect against alcohol dependence (129). These findings, along with some studies referred in "1.2 The reward system" contradict the so called "dopamine addiction theory" since low availability of dopamine receptors is not only associated with higher risk of development of addiction but is also linked to more pleasurable effects from stimulants (130,131).

The influence of dopamine in the reward system is complex and it depends, among other factors, on the dopamine release but also on the availability of the dopamine receptors, namely the D1 and D2 receptors. It is theorized that the low-affinity D1R are associated with reward and goal directed behaviours, while the high-affinity D2R are associated with avoidance behaviour (132,133). This emerges from the basal ganglia direct and indirect pathways (**Figure 2**), where GABAergic neurons with dopamine receptors receive input from several brain regions (such as the amygdala, thalamus the SNc and the VTA). The resulting input is translated into either tonic or phasing fire of dopamine in the *striatum*. While tonic firing of dopaminergic neurons is responsible for the stimulation of the D2R, phasing fire of these neurons, which is associated with salient stimuli, results in higher levels of dopamine, thus leading to the stimulation of the D1R (132). When D1R of GABAergic neurons in the *striatum*

are stimulated, they inhibit GABAergic neurons at the SNr, thus leading to less inhibition of the thalamus, which is associated with motor actions. Moreover, the *striatum* has another population of GABAergic neurons that express D2R. During tonic dopaminergic firing, these receptors are stimulated, which leads to the inhibition of the *globus palidus*. Since this region exerts inhibitory effects on the SNr and STN (which has excitatory glutamatergic projections to the SNr), the SNr will be more activate, and consequentially, the thalamus will be more inhibited (134). These findings support the correlation between lower D2R density and higher risk to develop alcohol use disorder (127,135).

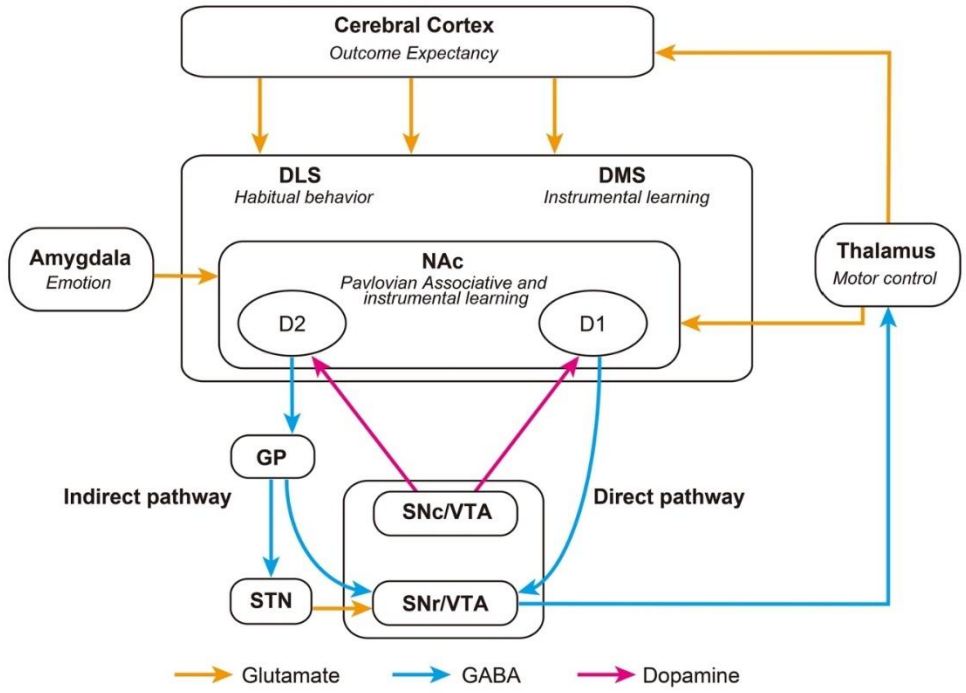

Figure 2- The direct and indirect mesolimbic pathway. They are both implied in certain behaviours such as goal directed and avoidance behaviours. Figure reproduced from (134).

Ethanol exerts its influence in the dopaminergic system by an increase in the dopamine release through induction of phase fire in dopaminergic neurons in the VTA (136), thus leading to dopamine release in the *nucleus accumbens* (137–140), among other regions, presumably conducting to the stimulation of D1 receptors in those regions. Interestingly, high doses of ethanol seem to lower dopamine release in *striatum* in a dose-dependent manner (141,142), with low doses of ethanol leading to increased dopamine release. Chronic and protracted abstinence effects of ethanol are sometimes contradictory, however, a number of studies in animal models were used to elaborate **Table 6** and **Figure 3** to better discuss these effects. It is worth mention the study by Hirth et al 2016, since it is the only one to assess the D1R, D2R

and dopamine levels during a 21-day abstinence. Moreover, it is a very robust study since it is a meta-analysis of 192 rats from 16 different studies exposed to ethanol for 7 weeks through PIE to ethanol vapour, the very same method that we used in our study and that will be further described.

Table 6 Effects of ethanol exposure on dopamine receptor 1 and 2 expression

Duration	BAL/Dose of ethanol	Duration of withdrawal	Technique	Rat strains	Results	Area	Reference
1 day	1 dose of 3 g/Kg	none	[3H] Spiroperidol binding assay	Sprague-Dawley	AT: D1 and D2 =	*Striata*	(143)
21 days	4,9 g/Kg/day	none	[3H] Spiperone binding assay	Wistar	AT: D2 =	*Striata*	(144)
25 days	2-3 g/Kg/day	none	[3H] Spiperone and SCH 23390 binding assay	Sprague-Dawley	AT: D1 and D2↓	*Striata*	(145)
2 weeks	2,5-3 g/l	7 days	[3H] raclopride binding assay	Long-Evans	1 week WD: D2 =	PFC	(146)
8 weeks	4 g/Kg/day	none	[3H] Spiperone and SCH 23390 binding assay	Wistar	AT: D1↑□, D2 =	*Striata*	(147)
AT: After treatment; WD: Withdrawal; PFC: Pre-frontal cortex.							

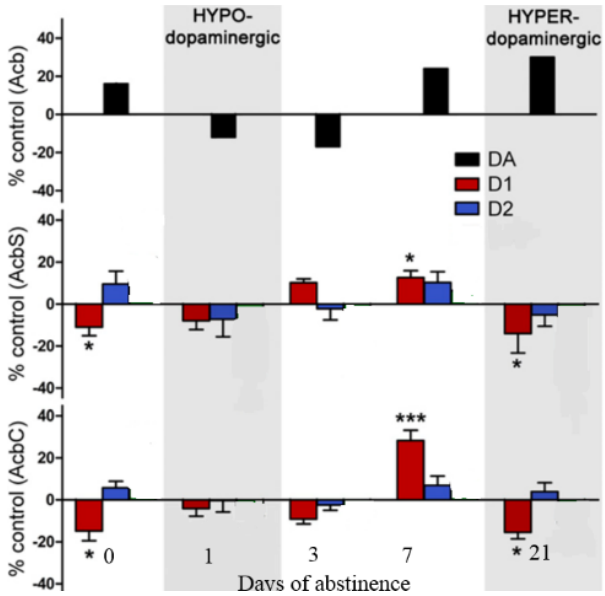

Figure 3- Influence of prolonged exposition to ethanol in D1R and D2R in the nucleus accumbens. Figure reproduced from (148).

The levels of dopamine receptors are not altered after acute exposition to ethanol (1 day). Interestingly, either in the post-treatment time or at the term of the abstinence period, a constant trend emerges: the D2 receptor levels do not seem to be altered as a result of prolonged ethanol exposure. This trend is observed not only in different rat strains, but also in different models of ethanol exposure.

D1 receptor expression results are not as straightforward, since after treatment, studies often present contradictory results, even for the same brain region. This variability might be due to different exposure models or to interstrain variability. Nevertheless, the study by Hirth et al 2016 presents itself as the most complete one since it is based on PIE exposure, a model that is proven to effectively replicate alcohol dependence (149). Moreover, it tracks dopamine and its receptors levels along the abstinence period. This meta-analysis reports that the D2R levels remained unchanged after prolonged alcohol exposure and during abstinence. Concerning D1R, results lay ground for a new approach to the influence of dopamine in alcohol dependence: D1R levels are decreased after alcohol exposition, which can be a down-regulation following prolonged high levels of dopamine that result from alcohol consumption. This hypodopaminergic state associated with short periods of abstinence represents a higher risk for relapse since a D1R down regulation is related to reward deficit (148). This finding can also explain the dose escalation observed in chronic alcohol abuse since high doses of alcohol result in higher dopamine levels, which in turn promote D1R down-regulation, which ultimately causes reward deficit. This means that the consumer has to take more quantity of alcohol to reach a reward. Furthermore, as the abstinence progresses, the D1R levels increase as an up-regulation to the lower levels of dopamine (that result from the abstinence itself). When a hyperdopaminergic state is observed in protracted abstinence (21 days), the D1R expression is down-regulated. This hyperdopaminergic state manifests through increased motor activity and alcohol seeking behaviour and use (148), which means that there is also an increased risk for relapse in this stage. Both the hypo and the hyperdopaminergic states are related with relapse, so a possible treatment has to reinstate the dopamine's homeostasis either through an initial increase in the dopamine levels, to counteract the hypodopaminergic state, or through a decrease in the dopamine levels to control the late hyperdopaminergic state.

1.5. Intermittent ethanol vapour exposure in rats: a behavioural model of alcohol addiction

The aim of this project is to study the prolonged alcohol exposure effects on NMDAR, D1R and D2R expression in several brain regions of Wistar rats. The ethanol exposure model chosen was the intermittent ethanol vapour exposure in rats (150–152).

With the purpose of exploring abstinence-related neuroadaptations, we selected a model where rats are exposed to high blood alcohol levels for several weeks (PIE). This model has been studied for over 15 years in the GRAP laboratory. This type of prolonged alcohol exposure induces an increase in voluntary ethanol consumption in rats and the display of withdrawal signs. This last condition is extremely important because a number of inaccurate models were based in a continuous exposure to ethanol, and since that is not what happens in humans, they failed to reproduce ethanol provoked neuroadaptations and behaviours. The alcohol consumption in humans happens in a cyclic way, with repeated cycles of increasingly alcohol abuse followed by periods of withdrawal. Thus, to induce chronic alcohol use effects, the PIE model was proposed, in which the animals are constantly exposed to high quantities of alcohol, followed by withdrawal periods, over weeks to months, hence yielding a neurobiological model of chronic alcohol consumption. This way, the animals enter in the so called "post dependent state", where they despise the typical behaviours and neuroadaptations seen in alcohol addiction in humans (149). For these reasons, the PIE was selected as a model of ethanol exposure.

1.6. Principles of radioligand binding techniques

Other important aspect of this study, was the technique used to measure the density of D1R, D2R and NMDAR. This determination was made through a method known as radioligand binding technique on brain membranes. This methodology is widely used given its simplicity to establish K_D and B_{max} pharmacokinetic values of ligand/receptor binding. It is based on the binding of a radioligand to its receptor of interest and the consequent determination of the radioactivity of the sample after an incubation period. This technique has three variations that depend on the objective of the study: saturation, kinetic and inhibition. For the purposes of this project, the only type of experiments made were saturation binding. This methodology is based on keeping the quantity of the receptor constant while varying the radioligand concentration. This allows the determination of two parameters: the K_D constant, which is a measure of the affinity between the radioligand and the receptor, since it is the concentration of radioligand

where half of B_{max} is reached, and the B_{max}, which is the maximum binding between the radioligand and the receptor. Another important aspect of this technique is the selection of either an agonist or an antagonist to be used as a radioligand. In this project, antagonists were used to determine the densities of all the studied receptors: [3H] MK-801 for NMDAR, [3H] SCH-23390 for D1R and [3H] Raclopride for D2R. The use of antagonists has several advantages, namely they label all the available receptors while the agonist radioligands may only label a portion of the available receptors, namely the G-coupled portion of them (153).

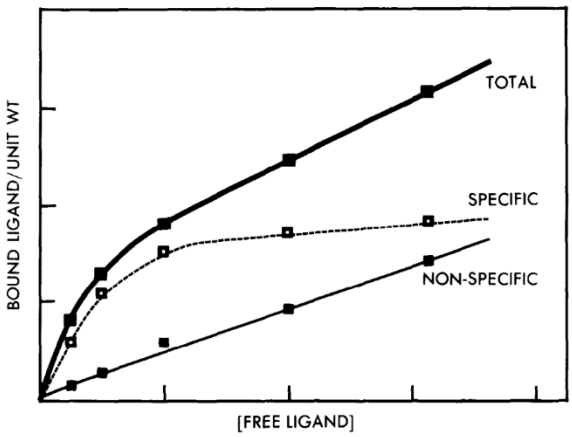

Figure 4- Total, specific and non-specific binding in a radioligand binding assay. Typical curve from a radioligand binding assay. Notice the graphical representations of total binding and non-specific binding. The specific binding is obtained by the subtraction of non-specific binding from total binding. Figure reproduced from (154).

In a theorical radioligand saturation binding assay, as the concentration of the radioligand increases, its binding to the receptor also increases until saturation of the receptor sites is achieved. This portion of the binding corresponds to the specific binding. However, experimentally, we cannot obtain a pure specific binding. What happens in reality during the experiment, is a continuous increase in the radioactive binding, achieving what is named "total binding". This is due to a non-specific binding, which is directly proportional to the concentration of radioligand, and results from a low-affinity, non-specific binding of the radioligand to non-specific interacting sites on the brain membranes (proteins, lipids,…). In order to determine the specific binding, which is the binding of the radioligand to the receptor of interest, each sample has to undergo two different assays: one to determine the total binding and the other one to determine the non-specific binding. The first one is conducted, as explained above, with the addition of a radioligand to the sample and the consequent measurement of the radioactivity after an incubation period. The second one is similar in almost everything to the

first with the exception of one factor: the addition of a large quantity of unlabelled ligand (a ligand specific for the same receptor than the radioligand, but devoid of radioactive labelling). Since the unlabelled ligand is in great quantity it is going to occupy the receptors of interest, while not doing the same in relation to its non-specific binding since this binding is a result of non-specific interactions with several membrane molecules. Thus, with the realization of these two assays the specific binding is calculated as the difference between total binding and non-specific binding (**Figure 4** and **Figure 5**) (153,154).

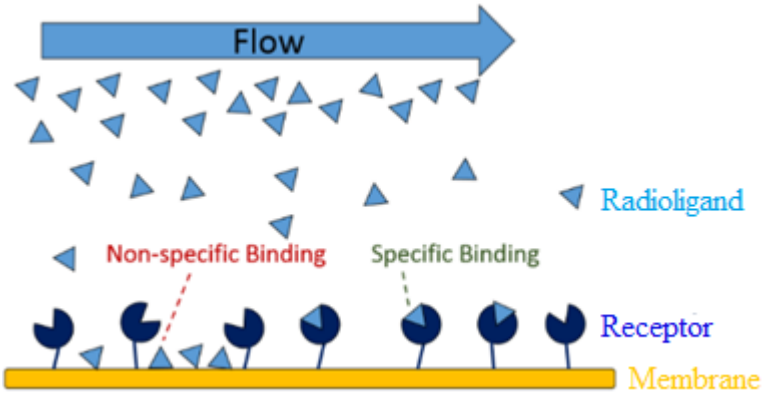

Figure 5- Representation of specific and non-specific binding. Figure reproduced from (155).

1.6.1. Evaluation of [3H] MK-801 specific binding sites density for NMDAR, namely B (fmol/mg protein)

The binding of the radioactive ligand at a working concentration of 1.5 nM of [3H] MK-801 is going to be the comparison metric between control and experimental group for the NMDAR binding assays.

Equation (4) (see below) cannot be applied here to determine B_{max} because that approach assumes a constant K_D, which is not verified in NMDAR assays, since their subunit expression varies with alcohol exposition to a degree where K_D is not constant.

1.6.2. Evaluation of Bmax for [3H] Raclopride binding sites density for D2R, and for [3H] SCH 23390 to D1R, namely B max (fmol/mg protein)

Our aim was to calculate the ratio between the exact number of D1R and D2R. This ratio cannot be calculated from the bound B (fmol/mg protein), as this number gives the "binding sites density", a data that depends both on real receptors quantity and on affinity of this receptor for the radioligand.

According to previous work we assumed the K_D of [3H] Raclopride binding to D2R (156) and of [3H] SCH 23390 binding to D1R (145) was unaltered following ethanol exposure. In this case of a constant K_D, we can use the Michaelis-Menten equation to calculate B_{max} (157). In accordance with the Michaelis-Menten kinetics, the law of mass action, a reaction between ligand and a receptor can be represented by the following equation:

$$R + L \rightleftarrows R.L \qquad (1)$$

$$Kd = \frac{[R].[L]}{[RL]} \qquad (2)$$

R=amount of free receptor; L= the amount of free ligand.

Moreover, since [R] = [Rt] - [RL], the equation (2) can also be written:

$$[RL] = \frac{[\mathrm{Rt}].[\mathrm{L}]}{Kd + [\mathrm{L}]} \Leftrightarrow \frac{[\mathrm{RL}]}{[\mathrm{Rt}]} = \frac{[\mathrm{L}]}{\mathrm{Kd} + [\mathrm{L}]} \qquad (3)$$

[Rt] = concentration of the total number of receptors in the membrane preparation.

[RL] is the concentration of receptors R bound to ligand L.

In this last equation, the concentration of receptor–ligand complex it is equivalent to a certain binding (designated B for "bound"), and the total concentration of receptors corresponds to "maximum bound" (B_{max}), which applied to equation (3):

$$\frac{B}{\mathrm{Bmax}} = \frac{[\mathrm{L}]}{\mathrm{Kd} + [\mathrm{L}]} \qquad (4)$$

Since the K_D and the concentration of ligand is known, B_{max} can be determined through equation (4) from a certain binding at a given ligand concentration.

2.Methods

2.1. Animals

Twenty male Wistar rats that weighted between 260-280g at the beginning of the experiments were obtained from Janvier labs (Le Genest Saint Isle, France). Each rat was housed in an individual cage in a controlled environment under a 12h light/dark cycle (lights at 8 am) with food and water available *at libitum*. All experiments were performed in conformity with the European Community guiding principles for the care and use of animals (2010/63/UE, CE Off. J. 20 October 2010), the French decree n° 2013–118 (French Republic Off. J., 2013) and approved by the local ethics committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Picardie (CREMEAP), University of Picardie Jules Verne).

2.2. Alcohol exposure

Alcohol exposure was based on a prolonged exposition model. After a week of acclimation in the animal care unit of the laboratory, the rats were exposed to a prolonged and intermittent flow of ethanol vapours, individually, in pressured and ventilated inhalation chambers for 14h/day (from 05:00 pm to 07:00 am). With this protocol, the rats display blood ethanol concentrations between 150 and 250 mg/dl (1.5 to 2.5 g/l). Ethanol vapours were generated through the introduction of pressurized air into a reservoir with 96% ethanol solution (60-80% hygrometry). The ethanol concentration was progressively increased during 2 weeks. This protocol was followed for 8 weeks and produced, in rats, a robust and well described post-dependent state. N=9 dependent rats and N=9 control, non-dependent rats were used in this work.

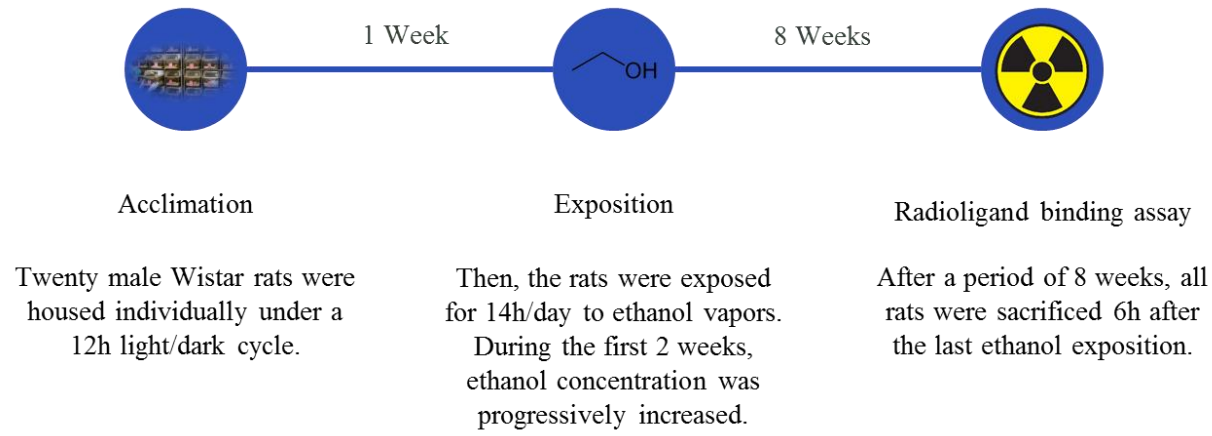

Figure 6- Study design. Firstly the rats were housed for 1 week under a 12h light/dark cycle for acclimation. Then they were exposed to ethanol vapours for 14h/day during 8 weeks, and lastly they were sacrificed 6h after the last ethanol exposition.

2.3. Protein extraction

Rats were sacrificed 6 hours after the last alcohol exposition, a time point that corresponds to peak withdrawal. The two *hippocampi* were dissected out for [3H] MK-801 binding assays, and the *dorsal striatum* and "cortex" regions were dissected out for [3H] Raclopride and [3H]-SCH 23390 binding assays. Those regions were removed rapidly after death, put on ice and homogenized in an Elvejhem-type potter at 1000 rpm in 4 ml of a sucrose buffer (Tris-HCl 50 mM; 0.32 M sucrose). Then, the extraction of the membrane fraction began. The samples were centrifuged for 10 minutes at 3000 *g* at 4ºC, the supernatant was centrifuged again for 20 minutes at 2000 *g* at 4ºC. Next, the pellet was kept and rinsed in 2 ml of ice-cold Tris-HCl buffer (Tris 50 mM, pH 7.4, 20ºC), then centrifuged for 20 minutes at 20 000 g at 4ºC. The pellet was kept and rinced 3 times. The pellet was kept and resuspendend in 2 ml of distilled water. It was centrifuged again for 20 minutes at 20 000 g at 4ºC. The pellet was kept again and it was resuspended in 500 µl of Tris-HCl 10 nMand frozen at -80ºC, with the exception of a 10 µl aliquot for protein quantification through the Lowry method (158).

2.4. [3H]MK-801 binding assay

[3H] MK-801 binding assays was performed on the *hippocampus* membrane. For total binding, 75 µg of protein was incubated in 0.5 ml of 10 mM Tris-HCL (pH 7.4, 20°C) containing 1.5 nM of [3H] MK-801 (Perkin Elmer, specific activity 25.2 Ci/mmol), 100 µM glutamate and 30 µM glycine at room temperature for 2 hours. Non-specific binding was defined in the same condition using non-labelled MK-801 100 µM. Afterwards, the membranes were rapidly filtered on Whatman glass fibre filters (GF/B, 25 µm pore diameter, Cat No 1821-025) and rinsed two times with 5 ml of ice-cold Tris-HCl 10 mM buffer, pH 7.4 (20ºC). Radioactivity on filters was measured through the addition of 5 mL of ACS scintillation fluid (Optisant Hisafe, Perkin Elmer, ref 1200-436) and counted in a Wallac 1414 Winspectral liquid scintillation counter (Perkin Elmer, Courtaboeuf, France, around 30% efficiency for [3H]). The CPM data was determined through MultiCalc Software (Perkin Elmer).

2.5. [3H]Raclopride binding assay

[3H] Raclopride binding assays was performed in two sets of membranes, from *dorsal striatum* and "cortex". For total binding, 50 µg of protein was incubated in 0.5 ml of Tris HCl buffer (50 mM Ph 7.4; MgCl2 5 mM; CaCl2 120 mM; 120 mM NaCl2, 0.1% ascorbate, 5mM KCl

50 mM) containing [3H]Raclopride 1.5 nM (Perkin Elmer, specific activity 80.8 Ci/mmol). Non-specific binding was defined in the same condition using non-labelled Raclopride 0.4 mM. Then, the samples were incubated for 1:30 hours at 25ºC. Afterwards, the membranes were rapidly filtered on Whatman glass fibre filters (GF/B, 25 µm pore diameter, Cat No 1821-025) and rinsed two times with 5 ml ice-cold Tris HCl 50 mM Ph 7.4; $MgCl_2$ 5 mM; $CaCl_2$ 120 mM and 120 mM $NaCl_2$, 0.1% ascorbate, 5mM KCl at 4ºC. Radioactivity on filters was measured through the addition of 5 mL of ACS scintillation fluid (Optisant Hisafe, Perkin Elmer, ref 1200-436) and counted in a Wallac 1414 Winspectral liquid scintillation counter (Perkin Elmer, Courtaboeuf, France, around 30% efficiency for [3H]). The CPM data were determined through MultiCalc Software (Perkin Elmer).

2.6. [3H]SCH-23390 binding assay

[3H] SCH 23390 binding assays was performed in two sets of membranes, from *dorsal striatum* and "cortex". For total binding, 70 µg of protein was incubated in 0.5 ml of Tris HCl 50 mM pH 7.4; 4mM $MgCl_2$ containing [3H] SCH 23390 1.5 nM (Perkin Elmer, specific activity 83.6 Ci/mmol). Samples were incubated for 1:30 hours at 25ºC. The non-specific binding was defined in the same condition using non-labelled SCH-23390 0.4 mM. Afterwards, the membranes were rapidly filtered on Whatman glass fibre filters (GF/B, 25 µm pore diameter, Cat No 1821-025) and rinsed two times with 5 ml of ice-cold buffer Tris HCl 50 mM Ph 7.4; 4mM $MgCl_2$. Radioactivity on the filters was measured through the addition of 5 mL of ACS scintillation fluid (Optisant Hisafe, Perkin Elmer, ref 1200-436) and counted in a Wallac 1414 Winspectral liquid scintillation counter (Perkin Elmer, Courtaboeuf, France, around 30% efficiency for [3H]). The CPM data were determined through MultiCalc Software (Perkin Elmer).

3.Results

3.1. [3H]-MK-801 binding assay

Membranes from *hippocampus* of two groups of rats were submitted to a [3H]-MK-801 binding assay to determine the NMDAR binding site density (**Figure 7**). In the *hippocampus*, the [3H]-MK-801 binding sites density was not changed between controls (B (fmol/mg protein = X) and Post-dependant rat (B (fmol/mg protein = X), as t-Sudent analysis revealed no statistical changes (p=0.14).

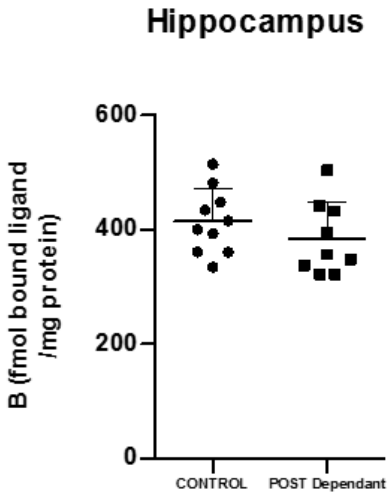

Figure 7- [3H]-MK-801 binding site density in hippocampus. Result are mean +/- SEM. T-student analysis revealed that no statistical difference was observed (P=0.14). Control (n=10) and POS-dependent (n=9).

3.2. [3H]-Raclopride binding assay

Membranes from "cortex" and *dorsal striatum* of two groups rats were submitted to a [3H]-Raclopride binding assay to determine the D2R binding site density. Then, the maximum binding site density B_{max} was calculated using a K_D data previously obtained in rat *striatum* K_D= 2 nM (159).

In the "cortex" (**Figure 8**), [3H]-Raclopride binding sites density was decreased of 46% in Post-dependant rat (B_{max} (fmol/mg protein) = X+/-) compared to controls (B_{max} (fmol/mg protein) = X+/- X) and Student t-test analysis revealed statistical changes (p=0.037).

In the *dorsal striatum* (**Figure 8**), [3H]-Raclopride binding sites density was not decreased in Post-dependant rat (B_{max} (fmol/mg protein) = X+/-) compared to controls (B_{max} (fmol/mg protein) = X+/- X) and Student t-test analysis revealed no statistical changes, although the statistical significant level is close (p=0.068).

This means that the D2R receptor expression in "cortex" was decreased in 6h-withdrawal, PIE rats, while it was not changed in *dorsal striatum*.

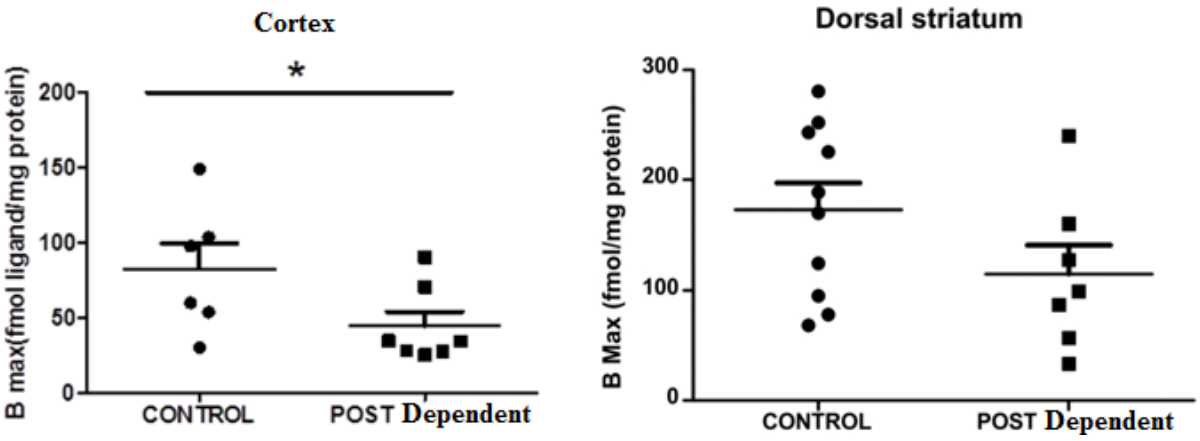

Figure 8- [3H]-Raclopride maximum binding site density in cortex and dorsal striatum respectively. Results in the cortex are mean +/- SEM *: statistical difference according to t-student (P=0.037). Control (n=6) and POST-dependent (n=7). Results in the dorsal striatum are mean +/- SEM. No statistical difference according to t-student (P=0.068) Control (n=10); POST-dependent (n=8).

3.3. [3H]-SCH23390 binding assay

Membranes from "cortex" and *dorsal striatum* of two groups rats were submitted to a [3H]-SCH 23390 binding assay to determine the D1R binding site density. Then, the maximum binding site density B_{max} was calculated using a K_D data previously obtained in rat *striatum* K_D= 0.53 nM (160).

In the "cortex" (**Figure 9**), [3H]-SCH 23390 binding sites density was not increased in Post-dependant rat (B_{max} (fmol/mg protein) = X+/-) compared to controls (B_{max} (fmol/mg protein) = X+/- X) and Student t-test analysis revealed no statistical changes (p=0.15).

In the *dorsal striatum* (**Figure 9**), [3H]-SCH 23390 binding sites density was not decreased in Post-dependant rat (B_{max} (fmol/mg protein) = X+/-) compared to controls (B_{max} (fmol/mg protein) = X+/- X) and Student t-test analysis revealed no statistical changes (p=0.14).

This means that the D1R receptor expression in "cortex" and in *dorsal striatum* was not changed in 6h-withdrawal of PIE rats.

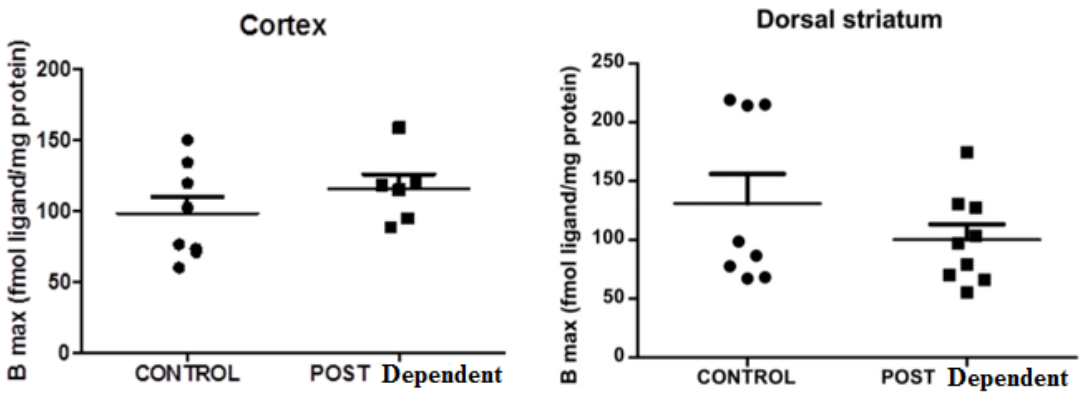

Figure 9- [3H]-SCH23390 Raclopride maximum binding site density in cortex and dorsal striatum respectively. Results in the "cortex" are mean +/- SEM. No statistical difference according to t-student (P=0.15). Control (n=9) POST-dependent (n=7). Results in the dorsal striatum are mean +/- SEM. No statistical difference according to t-student (P=0.14). Control (n=8) POST-dependent (n=9).

3.4. Correlation between D1R and D2R

The results in **Figure 10** show that there is a significant increase in the D1R:D2R ratio in the "cortex" region. This change is due to a significant decrease in the D2R density in this region, along with a slight increase in the D1R density, as previously depicted.

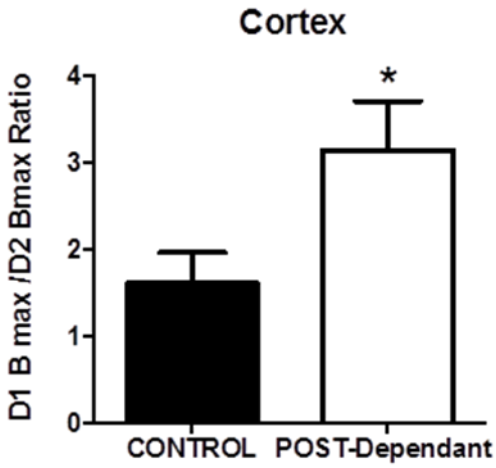

Figure 10- D1R:D2R ratio in the "Cortex". Result are mean +/- SEM. Statistical difference according to t-student (P=0.02). Control (n=6), POST - dependent (n=6).

In the *dorsal striatum* region, the D1R:D2R ratio is slightly increased. The binding assays results show (**Figure 11**) that, in this region, both the D1R and the D2R density decrease after alcohol exposure. Still, an increase in the ratio is observed, which can be explained by a bigger decrease in D2R when compared to D1R.

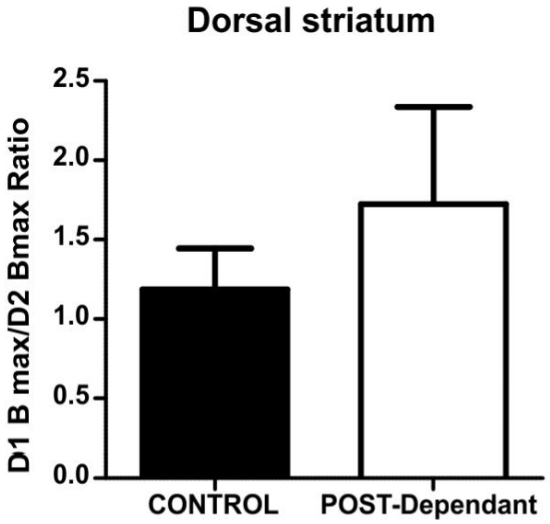

Figure 11- D1R:D2R ratio in the Dorsal Striatum. Result are mean +/- SEM. Statistical difference according to t-student (P=0.2). Control (n=8), POST-dependent (n=7).

4.Discussion

The reward system is composed of many structures that interact with each other through various neurotransmitters systems. Two of the most important ones are dopamine and glutamate: the first one plays a crucial role in the positive reinforcing effects of the reward, and the second one is responsible for important symptoms observed in the withdrawal such as seizures and a hyperexcitatory state. Thus, the study and comprehension of these systems is crucial to the development of novel treatments to treat addiction diseases like AUD.

In this project, the influence of prolonged alcohol intoxication with the PIE model in the expression of NMDAR was investigated in *hippocampus* and in the D1R, D2R expression was investigated in *dorsal striatum* and "cortex". It is worth of note that in this study, rats were not self-administrating alcohol. They were submitted to daily alcohol vapour exposure in order to

submit their body to a daily 1.5 to 2.5 g/l, a procedure that led us to experiment repeated daily withdrawal session. These repeated withdrawal sessions are thought to play a role in the development of addiction-facilitating neuroadaptations.

4.1. Prolonged ethanol effect on NMDAR expression in *hippocampus*

The *hippocampus* plays a crucial role in memory formation and consolidation (161). It belongs to the limbic system and is essential in emotions elaboration and memorisation. Moreover, dopaminergic neurons also project from the VTA to this region, thus making memory associations between elements such as visual cues and context to a certain reward (132). For these reasons, the *hippocampus* can be conceptualized as a hub between memory, emotion and reward.

As depicted in section "1.3 Glutamate", accordingly to literature, acute ethanol exposition inhibits NMDAR function, consequentially, chronic ethanol exposition results in increased NMDAR expression to compensate the alcohol-induced inhibition. This mechanism is responsible for some of the symptoms during withdrawal. Our results show that NMDAR expression was not affected at the term of a 6h-withdrawal in the PIE model in *hippocampus*. These results contradict observations in other brain regions (**Table 5**), however they concur to a 19 month exposition study (102). Moreover, during this study, there were evidences that supported an altered glutamatergic function, since during PIE, the rats still displayed withdrawal signs associated with these alterations. For these reasons, although the NMDAR expression remained the same, the effect of ethanol in glutamatergic transmission in the *hippocampus* cannot be discharged since there are other mechanisms by which it can be influenced. One possibility is higher levels of glutamate that will lead to an excitatory state that is usually observed after withdrawal. Other possibility is altered function of NMDAR receptors. In fact, NMDAR receptors are characteristically heterogenic, with their subunit composition highly variable and sensible to external stimuli. NMDAR receptor have three different subunits: NR1, NR2 and NR3. Functional receptors must have two NR1 subunits and either two NR2 or NR3 subunits (57). Some results showed that the expression of some sub-units of the NMDAR, the NR2B, and NR2A sub-units, was increased in *hippocampus* at 3h-withdrawal from PIE (101). For these reasons, it would be interesting to include the determination of the 6h-withdrawal PIE-influence effects of prolonged ethanol exposure on the

NMDAR subunit density, under the same circumstances as this study in order to evaluate the hypothesis of a hyper excitatory state as a result of altered NMDAR subunit composition.

4.2. Prolonged ethanol effect on D1R and D2R expression in *dorsal striatum* and "cortex"

The *dorsal striatum* had long been set aside in addiction studies as it was thought to be implicated in motor regulation (through the basal ganglia loop) but not in addiction-related processes. This view has been abandoned, as we know now that the *dorsal striatum* plays a key role in the development of addiction, namely in the move from goal- or reward-directed behaviour into a repetitive, habitual behaviour. So we evaluated D1R and D2R expression in *dorsal striatum* and finally, we evaluated all the regions above the red line in the **Figure 12** as a "cortex" region, at first considered as a control region.

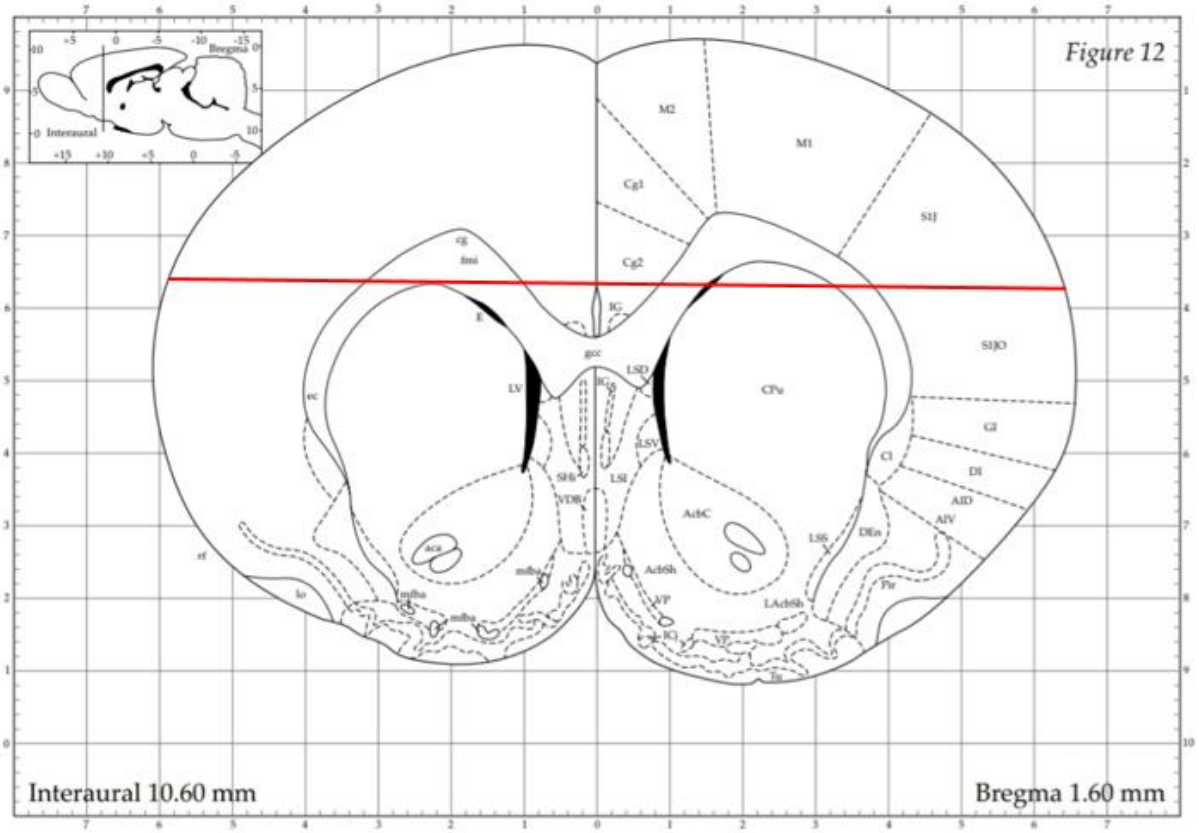

Figure 12- Brain section. The brain regions above the red line were analysed together as a control analysis. Figure reproduced from (162).

Among the "cortex" regions analysed, was the somatosensory cortex, a region that is responsible for the monitoring of the surrounding environment of an individual (163), the

cingulate cortex, an emotional region that links reward and behaviour (164) and the motor cortex, which controls motor neurons.

When a positive stimuli and its consequent reward are experienced by an individual, the *nucleus accumbens* is stimulated by dopaminergic neurons from the VTA, thus making it responsible for the subsequent motivation in repeating the same behaviour based on the current value of the reward. However, although this process happens when the stimuli is introduced and the reward is unexpected, when there is repeated presentation of the stimuli, the reward passes from unexpected to expected, and the firing of dopaminergic neurons starts to be due to predictive cues, rather than the stimuli itself. This is accompanied by reduced firing of dopaminergic neurons after exposition to the stimuli in a process called "reward prediction error". Simultaneously, there is dopaminergic stimulation of *dorsal striatum* from dopaminergic projections from *substantia nigra*, in a process that ultimately shifts the motivation to try a certain stimuli from the reward to the exposition itself (the action that leads to the stimuli exposure). Moreover, since the reward obtained from the stimuli is often lower than expected, the subject tends to aggravate its substance abuse as an attempt to achieve higher reward (132).

To better understand this process, membranes from the *dorsal striatum* region in Wistar rats with prolonged exposition to ethanol were evaluated for the effect of alcohol in D1R and D2R density. The results show that D1R and D2R density remains the same, however, it is important to note that D2R density depicts a clear tendency to diminish after prolonged exposition to ethanol, since the results are almost in the limit of statistical difference (**Figure 8**). This finding is of extreme importance given the excitatory and inhibitory nature of both D1R and D2R respectively. Moreover, the D1R:D2R ratio slightly increases after ethanol exposition, a finding that supports the hypothesis that alcohol exposition decreases the inhibition of *dorsal striatum*. Interestingly, an already mentioned study (148) showed that prolonged ethanol exposition in rats leads to lower D1R density and does not produce any effect on D2R density in the *nucleus accumbens* region. Upon these results, it can be hypothesised that, as the addiction progresses, the *nucleus accumbens* stimulation decreases simultaneously with a decrease in the inhibition in the *dorsal striatum*. These findings suggest that in fact, during the development of the addiction process, the *dorsal striatum* overthrows the *nucleus accumbens*, and thus the driving of the addiction shifts towards motor behaviour, which often leads to prediction error and consequentially to higher substance abuse in order to compensate the lack of reward that the stimuli (substance) provides. Consequentially, since the drive is now stronger

on the *dorsal striatum*-driven motor behaviour, the reward obtained from the stimuli is never going to match the expected reward.

Still, further research is necessary to better comprehend this hypothesis, namely, it would be interesting to study the influence of self-administration in a protocol similar to ours, to see if the addition of a behavioural component would push the results in the *dorsal striatum* towards statistical difference. Moreover, it would be important to understand the influence of each region of the *dorsal striatum* (*dorsolateral striatum* and *dorsomedial striatum*) in the changes observed, since the region more associated with habit formation is the DLS (165).

In addition, there are some differences between this study and the one by Hirth et al 2016, namely the rats in our study were sacrificed 6h after the last ethanol exposition and the rats in Hirth et al 2016 were sacrificed immediately after the last ethanol exposition. This 6h difference might not seem significant, but it might play a role in the results since our rats were analysed while on abstinence (during craving) and the one in Hirth et al 2016 were considered to be analysed while on ethanol exposition.

Although the set of the *cingulate*, motor and somatosensory cortices were analysed as control regions named "cortex", our results show that alcohol produces an unexpected effect. One of the regions that might be responsible for these effects is the somatosensory cortex, a region associated with the processing of stimuli from the surrounding environment. Despite its importance, current literature on the effects of ethanol in this region is very scarce. The results obtained show that PIE induced a slight, although not statistically significant D1R increase, and D2R density significantly decreased. If these findings are due to alterations in the somatosensory cortex, they might suggest that chronic ethanol exposition results in a neuroplasticity process that turns this region prone to activation, in other words, it decreases inhibition in the somatosensory cortex. This adaptive response might be due to ethanol-mediated over stimulation of D2R and under stimulation of D1R, which might explain some symptoms associated with acute alcohol consumption, such as numbness. Moreover, since these results might also be due to changes in the motor cortex, these results are very interesting because the motor cortex is connected to the *striatum* through the basal ganglia loop. Finally, the changes observed might be due to alterations in the *cingulate* cortex, which can reflect an influence of alcohol in emotion processing. Nevertheless, more research is necessary to establish the effects of long ethanol exposure on D1R and D2R both during the consumption but also during withdrawal in all of these regions.

5.Conclusion

During this study, a number of male Wistar rats were exposed to ethanol through a model designated prolonged intermittent exposure. This model reproduces several traits associated with alcohol addiction, such as increased motivation to self-administer alcohol, withdrawal symptoms and addiction-associated neuroplasticity. The rats were submitted to ethanol exposition for a period of 8 weeks, in order to develop alcohol dependence. After the 8-week period, rats were sacrificed 6h after the last ethanol exposition, a time period that corresponds to high peak in craving. Then, membranes from *hippocampus*, *dorsal striatum* and "cortex" regions were evaluated regarding the binding sites density of 3H-MK801 to NMDAR, 3H-Raclopride to D2R and 3H-Scherring 23290 to D1R. In the *hippocampus*, ethanol exposure did not produce any significant changes in the density of NMDAR. In the *dorsal striatum*, alcohol exposition did not significantly alter D1R and D2R density, however, D2R density was almost on the verge of statistical difference, which certainly translates a tendency towards an alcohol-induced decrease in this receptor's density. Finally, in the set of somatosensory, *cingulate* and motor cortices, there was a statistically significant alcohol-induced decrease in the D2R density. The D1R density was not statistically different from the control group.

These results, showed that prolonged ethanol exposure did not significantly affect NMDAR density in the *hippocampus*, this was surprising as withdrawal symptoms associated with excessive glutamatergic activation were still observed in the rats. With these results, it can be concluded that prolonged ethanol exposition might result in alterations in the glutamatergic system by other mechanisms than altered NMDAR density, namely alterations in NMDA sub-units composition.

Prolonged ethanol exposure did not altered D1R density in *dorsal striatum* and "cortex". On the other hand, D2R density was significantly decreased in "cortex" and the decrease observed in the *dorsal striatum* almost reached statistical difference. This was reflected in the D1/D2 ratio, demonstrating that the balance of dopamine-induced activity was pushed toward less inhibition process in "cortex", and a tendency towards less inhibition process in *dorsal striatum*. The alteration of the dopaminergic tonus in cortical region during a long-term alcohol exposure is new, so we will have to explore which specific region of the "cortex" is concerned, the motor cortex, the *cingulate* cortex (yet a so limbic cortex), or the somatosensory cortex.

6.Bibliography

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Annexes

A.1 MK-801 data

Control group						
Male number	Total binding assay 1 (cpm)	Total binding assay 2 (cpm)	Average total binding (cpm)	Non-specific binding (cpm)	Specific Binding (cpm)	Specific binding (fmol/mg protein)
1	870.30	942.50	906.40	187.80	718.60	434.23
2	864.50	831.50	848.00	185.70	662.30	400.21
3	933.30	902.70	918.00	229.90	688.10	415.80
4	885.20	790.30	837.75	238.70	599.05	361.99
5	749.00	748.50	748.75	194.80	553.95	334.74
6	810.10	778.60	794.35	196.80	597.55	361.08
7	949.20	859.20	904.20	253.40	650.80	393.26
8	906.70	1175.30	1041.00	189.30	851.70	514.66
9	1037.30	962.10	999.70	258.00	741.70	448.19
Average						414.58
Standard deviation						17.86
Experimental group						
10	796.80	818.90	807.85	149.90	657.95	441.76
11	815.30	946.90	881.10	164.80	716.30	432.84
12	781.50	842.40	811.95	277.80	534.15	322.77
13	802.80	896.80	849.80	273.10	576.70	348.48
14	695.40	883.00	789.20	229.40	559.80	338.27
15	773.30	932.50	852.90	262.20	590.70	356.94
16	980.40	1175.80	1078.10	243.90	834.20	504.08
17	781.00	1007.10	894.05	241.10	652.95	394.56
18	727.60	780.90	754.25	219.90	534.35	322.89
Average						384.73
Standard deviation						21.02
t-student analysis						0.146

A.2 Raclopride data in *dorsal striatum*

Control group					
Male number	Total binding (cpm)	Non-specific binding (cpm)	Specific Binding (cpm)	Specific binding (fmol/mg protein)	Maximum specific binding (fmol/mg/protein)
1	465.75	246.00	219.75	80.80	188.50
2	316.45	225.30	91.15	33.51	78.20
3	348.25	237.30	110.95	40.79	95.20
4	316.15	236.50	79.65	29.29	68.30
5	435.00	289.80	145.20	53.39	124.60
6	503.15	240.40	262.75	96.61	225.40
7	411.70	214.19	197.60	72.65	169.50
8	580.55	253.50	327.05	120.25	280.60
9	524.80	230.80	294.00	108.10	252.20
10	501.80	218.30	283.50	104.24	243.20
Average				73.96	172.58
Standard deviation				10.51	24.52
Experimental group					
11	375.20	226.50	148.70	54.67	127.60
12	284.85	218.50	66.35	24.40	56.90
13	554.65	275.00	279.65	102.82	239.90
14	351.35	250.20	101.15	37.19	86.80
15	439.40	400.60	38.80	14.27	33.30
16	964.65	239.10	116.00	42.47	99.10
17	414.15	227.60	186.55	68.59	160.00
Average				49.20	114.80
Standard deviation				11.24	26.23
t-student analysis					0.068

A.3 Raclopride data in the “cortex” region

Control group					
Male number	Total binding assay (cpm)	Non-specific binding (cpm)	Specific binding (cpm)	Specific binding (fmol/mg protein)	Maximum specific binding (fmol/mg/protein)
1	583.10	353.40	229.70	23.07	53.80
2	442.40	314.30	128.10	12.87	30.00
3	538.50	282.70	255.80	25.70	60.00
4	938.20	495.70	442.50	44.45	103.70
5	990.40	353.60	636.80	63.97	149.30
6	802.60	385.70	416.90	41.88	97.70
Average				35.32	82.42
Standard deviation				7.51	17.53
Experimental group					
7	612.70	226.90	385.80	38.76	90.43
8	700.50	399.40	301.10	30.25	70.58
9	576.30	460.10	116.20	11.67	27.24
10	550.50	402.80	147.70	14.84	34.62
11	537.80	418.60	119.20	11.97	27.94
12	545.30	398.60	146.70	14.74	34.38
13	469.90	362.10	107.80	10.83	25.27
Average				19.01	44.35
Standard deviation				4.15	9.68
t-student analysis				0.037	

A.4 SCH-23390 data in *dorsal striatum*

Control group							
Male number	Total binding assay 1 (cpm)	Total binding assay 2 (cpm)	Average total binding (cpm)	Non-specific binding (cpm)	Specific Binding (cpm)	Specific binding (fmol/mg protein)	Maximum specific binding (fmol/mg/protein)
1	880.30	-	880.30	442.40	437.90	215.08	291.07
2	560.80	555.90	558.35	382.20	176.15	86.52	117.09
3	557.90	623.40	590.65	432.70	157.95	77.58	104.99
4	760.60	831.10	795.85	349.90	445.95	219.03	296.42
5	416.00	587.90	501.95	362.90	139.05	68.30	92.43
6	830.00	757.60	793.80	357.50	436.30	214.29	290.01
7	531.90	618.80	575.35	438.50	136.85	67.22	90.96
8	570.90	360.10	465.50	264.60	200.90	98.67	133.54
Average						130.84	177.06
Standard deviation						25.23	34.14
Experimental group							
9	584.30	523.40	553.85	411.10	142.75	70.11	94.89
10	513.20	423.30	468.25	271.00	197.25	96.88	131.11
11	710.10	680.00	695.05	340.00	355.05	174.39	236.00
12	351.40	429.20	390.30	255.50	134.80	66.21	89.60
13	341.00	397.60	369.30	208.00	161.13	79.14	107.10
14	548.90	583.20	566.05	300.10	265.95	130.62	176.78
15	664.90	612.50	638.70	379.40	259.30	127.36	172.36
16	669.10	653.10	661.10	548.30	112.80	55.40	74.98
17	612.90	521.80	567.35	356.70	210.65	103.46	140.01
Average						100.40	135.87
Standard deviation						12.09	17.25
t-student analysis						0.138	

A.5 SCH-23390 data in the “cortex” region

Control group					
Male number	Total binding assay (cpm)	Non-specific binding (cpm)	Specific binding (cpm)	Specific binding (fmol/mg protein)	Maximum specific binding (fmol/mg/protein)
1	1094.30	760.00	334.30	44.39	60.01
2	1460.10	825.50	634.60	75.83	102.63
3	1564.00	824.80	739.20	88.33	119.54
4	1291.50	818.50	473.00	56.52	76.49
5	1512.20	683.30	828.90	99.05	134.05
6	1804.60	875.80	928.80	110.99	150.21
7	1475.10	1021.50	453.60	54.20	73.36
8	1533.70	1094.90	438.80	52.44	70.96
Average				72.72	98.41
Standard deviation				8.69	11.76
Experimental group					
9	1431.30	772.20	659.10	87.51	118.43
10	1545.10	834.00	711.10	84.97	114.99
11	1356.60	810.30	546.30	65.28	88.35
12	1683.20	937.80	745.40	89.07	120.55
13	1817.40	835.00	982.40	117.39	158.87
14	1485.60	899.10	586.50	70.09	94.85
Average				85.72	116.01
Standard deviation				7.48	10.13
t-student analysis				0.15	