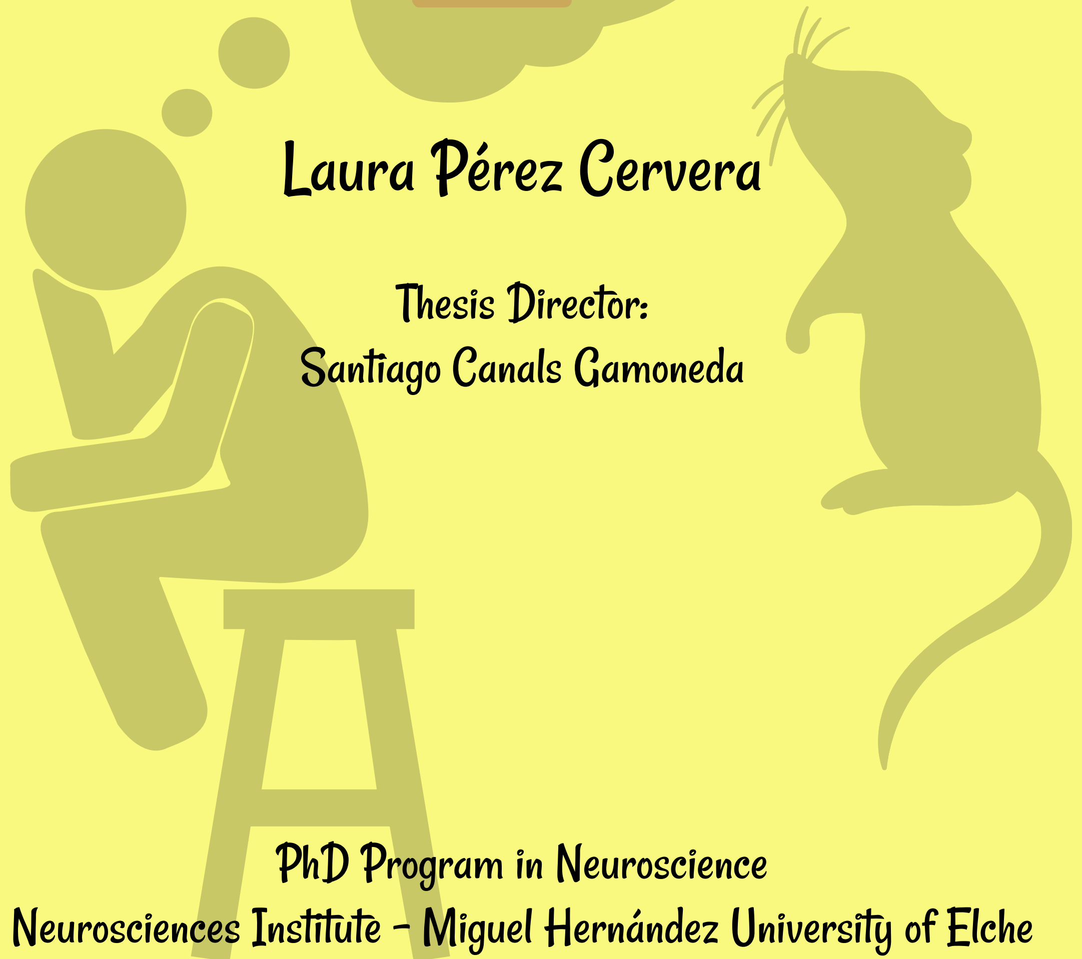
TRANSLATIONAL INVESTIGATION IN HUMANS AND ANIMAL MODELS OF ALCOHOL USE DISORDERS



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ABBREVIATIONS

ACTH = Adrenocorticotropic Hormone

AMY = Amygdala

AUD = Alcohol Use Disorder

BAC = Blood Alcohol Concentrations

CIE = Chronic Intermittent Ethanol

CRF = Corticotropin-Releasing Factor

CSF = CerebroSpinal Fluid

CTF = Corrected Total Fluorescence

DA = Dopamine

DG = Dentate Gyrus

dHC = dorsal Hippocampus

DS = Dorsal Striatum

DSM = Diagnostic and Statistical Manual of Mental Disorders

dw-MRI = diffusion-weighted Magnetic Resonance Imaging

EC = Entorhinal Cortex

eLFP = evoked Local Field Potential

FA = Fractional Anisotropy

GABA = Gamma-Aminobutyric Acid

GC = Granule Cells

GP = Globus Pallidus

HC = Hippocampus

HP = Healthy Patients

HPA = Hypothalamic-Pituitary-Adrenal axis

IC = Insular Cortex

LFP = Local Field Potential

LTD = Long Term Depression

LTP = Long-Term Potentiation

MBP = Myelin Basic Protein

MD = Mean Diffusivity

MF = Myelin Fraction

MRI = Magnetic Resonance Imaging

msP = Marchigian Sardinian rats

NAcc = Nucleus Accumbens

NMDAr = N-Methyl-D-Aspartate receptor

PBS = Phosphate-Buffered Saline

PFA = Paraformaldehyde

PFC = Prefrontal Cortex

PP = Perforant Pathway

ROI = Region Of Interest

SNc = Substantia Nigra pars compacta

Sub = Subiculum

TBSS = Tract-Based Spatial Statistics

VTA = Ventral Tegmental Area

WM = White Matter

ABSTRACT

Alcohol addiction is a health problem that causes millions of deaths worldwide every year. However, the understanding of how alcohol-induced brain alterations lead to addiction remains limited and thus, effective targets for treatment are elusive. The diversity of damage it can cause to the brain has been studied in depth, but is very heterogeneous between individuals. Vulnerability to relapse, which is maximal in the early stages of the disease, remains an unsolved enigma. Typical characteristics of alcoholic patients, such as lack of cognitive control or behavioral inflexibility, could trigger relapse, but little is known about the possible mechanisms underlying them.

A significant problem in clinical studies on AUD is the high variability of the results obtained. This variability is partly due to the variety of personal trajectories in alcohol use and abuse and genetic factors, but also to the high number of possible comorbidities associated with alcoholism. These include poly-substance use (tobacco, cannabis, cocaine, etc.) and pharmacological treatments to alleviate the multiple associated symptoms. In this thesis work, I have taken advantage of well-controlled animal models to study the transformations that occur from a control state, naive to alcohol consumption, to excessive consumption and subsequent abstinence. At the same time, several cohorts of humans have been studied, using at all times the same magnetic resonance imaging (MRI) modalities for the study of brain alterations. This design allows to establish causal relationships with alcohol consumption in the animal models, avoiding comorbidities and other confounding factors, without moving away from translation to the clinic (thanks to the convergent set of brain imaging tools used).

The first objective was to study the evolution of brain structure during the early phases of abstinence. We used a rat model with a genetic predisposition to consumption, and two cohorts of AUD patients at different times of early abstinence. Brain alterations were studied using diffusion tensor magnetic resonance imaging (DTI), a non-invasive

technique with diagnostic value, which informs about water diffusion in tissues. From this information we can infer microstructural alterations. We found that both rats and humans showed comparable microstructural alterations in the white matter (WM) in the early stages of withdrawal. Unexpectedly, we were able to demonstrate that these alterations progressed throughout the period of abstinence studied. Taken together, these findings suggested the existence of an underlying biological process that triggers the progression of WM alterations in the absence of alcohol.

In a follow-up study, using the same cohorts of patients, we made a detailed study of the effect size of the alterations found, distinguishing between brain tracts. This study revealed that the most vulnerable tract in alcoholic patients was the fimbria/fornix, which communicates the hippocampus with the prefrontal cortex. Considering that this communication is fundamental for both learning and memory functions, as well as for emotional regulation (together with the amygdala) and cognitive flexibility, we argue that fimbria/fornix dysfunction in the early phase of abstinence could contribute to some key symptoms commonly observed in patients. Consequently, we decided to study in more detail and using again an animal model, the structure and functionality of the fimbria/fornix after alcohol withdrawal. On this occasion, we used a rat model that has demonstrated alcohol dependence, based on wild type animals, known as the postdependent or chronic intermittent exposure model. In addition to diffusion MRI, we used immunohistological and electrophysiological techniques. The results indicated a microstructural alteration in the tract, measured both as a decrease in myelin fraction quantified with MRI, and a decrease in myelin basic protein in postmortem histological examinations. In addition, simultaneous electrophysiological recordings demonstrated reduced efficiency in the directed connectivity from the hippocampus to the prefrontal cortex, together with an increased excitation/inhibition balance in the hippocampus.

Taken together, these results suggest that an alcohol-driven mechanism could be damaging the brain microstructure even in the absence of alcohol. Although the underlying mechanism has not been studied, in the case of fimbria/fornix it corresponds to a demyelinating process. The functional consequence of the latter is the partial

disconnection of the hippocampus and the prefrontal cortex. We propose that this alteration could be triggering some of the symptoms that characterize alcoholic patients, such as memory deficits and reduced cognitive flexibility, which condemns patients to compulsively repeat behaviors associated with alcohol consumption.

RESUMEN

La adicción al alcohol es una enfermedad peligrosa que causa millones de muertes al año en el mundo. Sin embargo, la ciencia todavía no ha conseguido dar con la clave para erradicarla al completo. Se han estudiado en profundidad la diversidad de daños que ésta puede causar en el cerebro, pero son muy heterogéneos entre individuos. La vulnerabilidad a la recaída sigue siendo un enigma sin resolver, la cual se ve aumentada en la fase más aguda de la abstinencia. Características típicas de pacientes alcohólicos como la falta de control cognitivo o la inflexibilidad ante cambios rutinarios podrían propiciar dicha recaída, pero poco se ha estudiado sobre los posibles mecanismos que los subyacen.

Uno de los grandes problemas de los estudios clínicos en trastornos por consumo de alcohol (TCA) es la gran variabilidad de los resultados obtenidos. Esta variabilidad se debe, en parte, a la variedad en las trayectorias individuales en el uso y abuso del alcohol, así como a factores genéticos. También hay una gran variedad de comorbilidades asociadas al alcoholismo, como por ejemplo el policonsumo de sustancias (tabaco, cannabis, cocaína, etc.), y los tratamientos farmacológicos para aliviar los múltiples síntomas asociados. En este trabajo de tesis, he utilizado modelos animales bien establecidos para estudiar los cambios que se producen desde un estado "control", ajeno al consumo de alcohol, hasta el consumo excesivo y la posterior abstinencia. Al mismo tiempo, se han estudiado varias cohortes de humanos, utilizando en todo momento las mismas modalidades de resonancia magnética (RM), para el estudio de las alteraciones cerebrales. Este diseño permite establecer relaciones causales con el consumo de alcohol en los modelos animales, evitando comorbilidades y otros factores de confusión, sin alejarse de la traslación a la clínica (gracias al conjunto de herramientas de imagen cerebral utilizadas).

Por un lado, el primero objetivo fue estudiar la evolución de la estructura cerebral durante la fase aguda de la abstinencia. Empleamos un modelo de ratas con predisposición genética a beber, y dos cohortes de pacientes alcohólicos, y adquirimos datos a distintos puntos de la abstinencia. Las alteraciones en el cerebro las estudiamos utilizando imagen de resonancia magnética por tensor de difusión (IRM-TD), una técnica no invasiva con valor diagnóstico. Ésta nos da información sobre la difusión del agua en los diferentes tejidos del cerebro, a partir de la cual se pueden inferir alteraciones microestructurales. El hallazgo principal fue que ambas especies presentaron alteraciones comparables en la sustancia blanca en las primeras fases de la abstinencia. Sorprendentemente, dichas alteraciones progresaban a lo largo del tiempo de abstinencia estudiado. Este hallazgo sugiere que existe algún proceso biológico que desencadena esta progresión de los daños en ausencia del alcohol, pudiendo ser una desmielinización o una activación glial.

Además de esto, utilizando las mismas dos cohortes de pacientes, realizamos un estudio detallado del tamaño del efecto de las alteraciones encontradas, distinguiendo entre tractos cerebrales. Éste reveló que el tracto más vulnerable en los pacientes alcohólicos era la fimbria/fórnix, el cual comunica el hipocampo con el córtex prefrontal. Considerando que esta comunicación es vital para llevar a cabo funciones de memoria y aprendizaje, además de gestión emocional (junto con la amígdala) y flexibilidad cognitiva, hipotetizamos que dicha disfunción en los primeros estadios de la abstinencia podría ser responsable de algunos de los síntomas típicos de los pacientes alcohólicos.

Por otro lado, motivados por este primer hallazgo, decidimos estudiar más en profundidad y de nuevo usando un modelo animal la estructura y funcionalidad de la fimbria/fórnix en la abstinencia. Para esto empleamos un modelo de ratas de dependencia al alcohol, basado en ratas wild-type, conocido como modelo postdependiente o de exposición crónica intermitente. Además, utilizamos de nuevo la resonancia magnética (IRM-TD), combinada con la electrofisiología o la inmunohistología. Los resultados indicaron una alteración microestructural de la fimbria/fórnix, medida como una disminución en la fracción de mielina cuantificada con IRM-TD, y una disminución de la proteína básica

de la mielina en el tejido postmortem. Además, la electrofisiología mostró una eficiencia reducida en la conectividad entre hipocampo y corteza prefrontal, junto con un incremento en el balance excitación/inhibición del hipocampo.

En su conjunto, estos resultados sugieren que un mecanismo desencadenado por el alcohol podría estar dañando la microestructura del cerebro incluso en ausencia del mismo. Aunque no hemos estudiado el mecanismo subyacente, en el caso de la fimbria/fórnix corresponde a un proceso desmielinizante, cuya consecuencia funcional es la desconexión parcial del hipocampo y el córtex prefrontal. Proponemos que esta alteración podría estar desencadenando algunos de los síntomas que caracterizan a los pacientes alcohólicos, como los déficits de memoria y la reducción de la flexibilidad cognitiva, que condena a los pacientes a repetir compulsivamente las conductas asociadas al consumo de alcohol.

1. INTRODUCTION

1.1. Alcohol Use Disorder: context, diagnosis and epidemiology

Context of the disease

Alcohol has been consumed worldwide by humans since the beginning of its existence, as evident both in literature (*Los Paraísos Artificiales,* 1860) and art (el-Guebaly N, el-Guebaly A, 1981, *Int J Addict.*, 16:1207-21). In the 17th century, alcohol was commonly employed in hospitals, as anesthetic and/or digestive. It was not until the end of the 18th century that its excessive consumption began to be diagnosed as a disease, Alcoholism, becoming a serious burden at the end of the 19th century.

At the beginning of the last century, any drug addiction was considered a moral problem attributed to a lack of willpower. However, around 1940, with more and more research focusing on this problem, it was suggested that a mental disorder could be underlying. Actually, alcohol addiction was included in the first edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-I, 1952). This changed the paradigm of the disease, and opened new ways toward prevention and treatment.

Diagnosis

Nowadays, alcohol addiction is included in the last edition of the DSM (DSM-5, 2013) under the umbrella of the Alcohol Use Disorder (AUD). DSM-5 defines any Substance Use Disorder by a group of cognitive, behavioral and physiological symptoms promoting continuous use of the substance by the individual, despite significant substance-related negative consequences. Interestingly, the previous DSM edition differentiated two independent disorders depending on how many criteria meet a subject: alcohol abuse and alcohol dependence (table 1; DSM-IV, 1994). Currently, AUD is a unified disorder, defined by several criteria (table 2; DSM-V, 2013). As alcohol use is not necessarily related neither with abuse nor intoxication, a range of severity is defined based on the

number of criteria that people present: mild (2-3 criteria), moderate (4-5 criteria) or severe (6 or more criteria).

DSM-IV	
In the past year, have you:	
Any 1 = ALCOHOL ABUSE	Found that drinking—or being sick from drinking—often interfered with taking care of your home or family? Or caused job troubles? Or school problems?
	More than once gotten into situations while or after drinking that increased your chances of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex)?
	More than once gotten arrested, been held at a police station, or had other legal problems because of your drinking? ****This is not included in DSM–5****
	Continued to drink even though it was causing trouble with your family or friends?
Any 3 = ALCOHOL DEPENDENCE	Had to drink much more than you once did to get the effect you want? Or found that your usual number of drinks had much less effect than before?
	Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure? Or sensed things that were not there?
	Had times when you ended up drinking more, or longer, than you intended?
	More than once wanted to cut down or stop drinking, or tried to, but couldn't?
	Spent a lot of time drinking? Or being sick or getting over other aftereffects?
	Given up or cut back on activities that were important or interesting to you, or gave you pleasure, in order to drink?
	Continued to drink even though it was making you feel depressed or anxious or adding to another health problem? Or after having had a memory blackout?

Table 1. Diagnostic criteria for AUD under the fourth edition of the DSM-IV in 1994.

<table>
<tr><th colspan="2">DSM-5</th></tr>
<tr><th colspan="2">In the past year, have you:</th></tr>
<tr><td rowspan="11">The presence of at least 2 of these symptoms indicates Alcohol Use Disorder (AUD).
The severity of the AUD is defined as:

Mild: the presence of 2 to 3 symptoms.

Moderate: the presence of 4 to 5 symptoms.

Severe: the presence of 6 or more symptoms.</td><td>Had times when you ended up drinking more, or longer, than you intended?</td></tr>
<tr><td>More than once wanted to cut down or stop drinking, or tried to, but couldn't?</td></tr>
<tr><td>Spent a lot of time drinking? Or being sick or getting over other aftereffects?</td></tr>
<tr><td>Wanted a drink so badly you couldn't think of anything else?
This is new to DSM–5</td></tr>
<tr><td>Found that drinking – or being sick from drinking- often interfered with taking care of your home or family? Or caused jobs troubles? Or school problems?</td></tr>
<tr><td>Continued to drink even though it was causing trouble with your family or friends?</td></tr>
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<tr><td>Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure? Or sensed things that were not there?</td></tr>
</table>

Table 2. Diagnostic criteria for AUD under the fifth edition of the DSM-5 in 2013.

Epidemiology and prevalence

The global report on alcohol and health from the World Health Organization (WHO, 2018) confirms that worldwide, 3 million deaths every year result from the harmful use of alcohol. In Spain, the last annual survey from the Government about alcohol, drugs and other addictions, called EDADES, shows a stable and high alcohol consumption along time. It also reveals that the risk perception of alcohol is the lowest in comparison with other drugs, which is consistent with the fact that it is the most widely consumed psychoactive drug.

It is important to discuss the comorbidity of this disease. AUD is related with more than 60 pathologies, such as cirrhosis, cardiovascular problems, cancer, hypertension, or mental alterations such as anxiety and depression (WHO. Global status report on alcohol and health, 2011). Indeed, around 44% of people suffering from AUD present another psychiatric disorder (Pascual Pastor F, et al., 2013). As a consequence, both diagnosis and treatment become more difficult. Additionally, alcohol is usually consumed along with other drugs, for instance nicotine or cannabis. All together, the evidence suggests that, in humans, it is difficult to isolate the mechanisms underlying alcohol addiction *per se*.

1.2. Neurocircuitry of addiction

When ethanol is orally consumed, it is directly absorbed by the blood capillaries both in the stomach (around 10-20% of the total amount) and gut. Once into the bloodstream, it is transported to all organs in the body, in which it is absorbed proportionally to their water content (more water, more alcohol absorption). The brain is one of the main targets for the ethanol, since its lipophilic nature easies the crossing of the blood-brain-barrier. Alcohol affects the brain at multiple levels, from the molecular, cellular and systems level, causing maladaptations if consumption is persistent. These changes, called neuroadaptations, are responsible for the individual losing the ability to control his

behavior and falling into compulsive habits, gradually approaching addiction (see Ron & Barak, 2016 for a review about the alcohol effects on intracellular signaling cascades).

Drug addiction is a chronically relapsing disorder characterized by a compulsion to consume the drug, the loss of control in limiting intake and the emergence of negative emotional state during the abstinence (G. Koob et al., 2014). There are some differences in the mechanisms of action of each drug, for example, cocaine inhibits dopamine reuptake from the synaptic cleft, whereas alcohol acts on inhibitory GABAergic channels. However, it has been proposed that addiction to any substance develops as a three-stage cycle (Koob, 1997):

I. Binge/intoxication

This stage corresponds to the time in which the drug is in the biological system. Alcohol enhances the activity of mesolimbic system, leading to an increase of dopamine (DA) levels in nucleus accumbens (NAcc; Gonzales, Job, & Doyon, 2004). DA gives rise to conditioned reinforcement, based on the association of neutral stimuli with a reinforcer. In the case of alcohol, high doses are considered necessary to develop dependence in humans due to the weakly reinforcing effects that it causes. As an example, in monkeys, differently to cocaine, alcohol self-administration is interrupted even after periods of daily intake (Deneau et al., 1969). For this reason, it is considered that environmental (e.g. context) or individual factors (e.g. genetics or temperament) are especially relevant to generate alcohol addiction.

The regions involved in this stage, additionally to NAcc, is dorsal striatum (DS), necessary both for habits formation, and development of compulsive drug-seeking (Everitt et al., 2008). The connectivity between NAcc and globus pallidus (GP) enables the initiation of the locomotion, relating the pleasure of the drug consumption with specific actions (Pycock and Horton (1976). Thalamus is a central region mediating the information transfer between basal ganglia and cortical structures (Haber 2009), relevant for the reinstatement of drug-seeking behaviors in rodents (Wedzony 2003; Haack 2014), and salience attribution and cognitive control in humans (Huang 2018). Ventral

Tegmental Area (VTA) and Substantia Nigra pars compacta (SNc) are also responsible for DA rewarding effects of the drug, and the generation of habits through their dopaminergic connections to the DS.

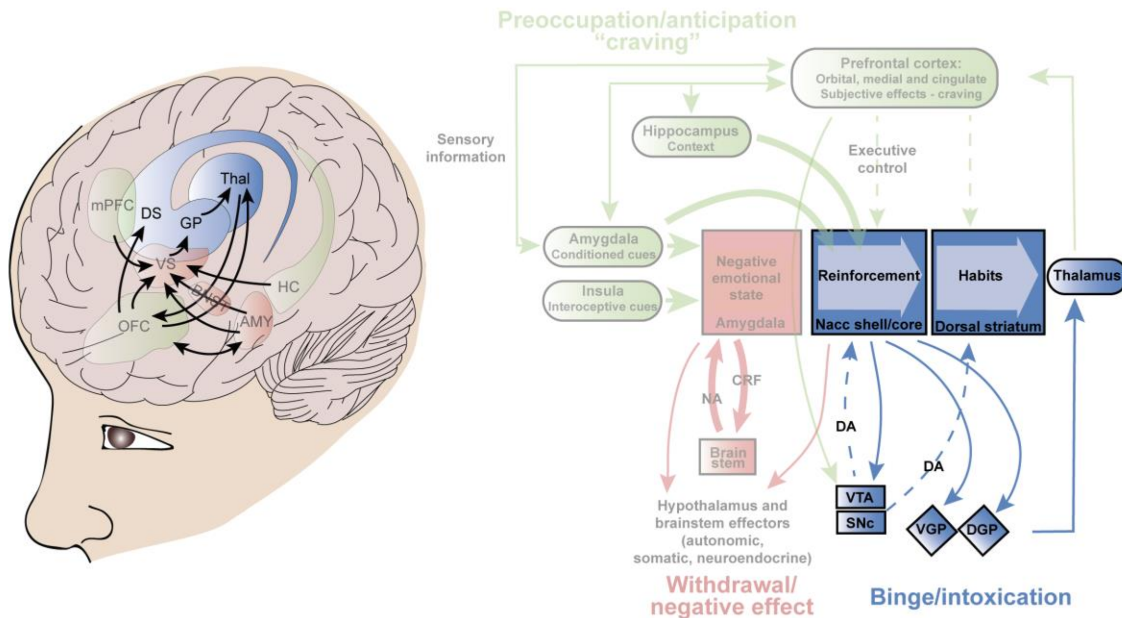


Figure 1.1. Neurocircuitry underlying the binge/intoxication stage. On the left, a scheme of the regions principally involved in this stage is illustrated. On the right, a model is shown of the manner in which regions and neurotransmitters interact in this stage. GP: globus pallidus, DS: dorsal striatum, Thal: thalamus, NAcc: nucleus accumbens shell and core, DGP: dorsal globus pallidus, VGP: ventral globus pallidus, VTA: ventral tegmental area, SNc: substantia nigra pars compacta; DA: dopamine. Adapted from G. Koob et al., 2014.

II. Withdrawal/negative affect

Withdrawal occurs when the drug is not in the biological system. Each drug generates its characteristic symptoms, for instance the opioids withdrawal syndrome has been defined as a "flu-like state" while psychostimulants include fatigue or suppressed heart rate (Jaffe, JH., 1990). Ethanol withdrawal, in turn, is characterized by tremors, hyperthermia, and even hallucinations and delirium (Jaffe, JH., 1990). However, a common symptomatology is the form of negative emotional states, such as dysphoria, anxiety, sleep disturbances or irritability.

Changes in the extended amygdala are thought to underlay this symptomatology, including in the bed nucleus of the stria terminalis, central nucleus of the amygdala, and

a transition zone in the nucleus accumbens (fig. 1.2; Koob and Volkow, 2010). When the individual feels these emotions, the negative reinforcement appears. Differently to the positive reinforcement mentioned in the previous stage, the motivation of the individual now to take the drug is the avoidance of this anxiety-like mood. This is the result of the decrease of the mesolimbic activity (Wise and Koob, 2014), contrarily to what occurs during intoxication.

It is well-known that a stressful context increases the risk of drug abuse and relapse (Sinha 2007). Pioneer experiments made in rats demonstrated that anxiety and alcohol craving are associated with stress-related corticotropin-releasing factor (CRF) and noradrenaline mechanisms (Knapp et al., 2004; Lê et al., 2005; Liu & Weiss, 2002; Valdez et al., 2003). Actually, stress exposure reinstates alcohol self-administration in rats, whereas CRF receptors antagonism is sufficient to block this effect (Funk et al., 2007). In healthy conditions, CRF, adrenocorticotropic hormone (ACTH) and cortisol are released under stress conditions by the hypothalamic-pituitary-adrenal axis (HPA). However, this axis becomes dysregulated with chronic alcohol abuse both in animals (Lee and Rivier 1997; Richardson et al. 2008; Zhou et al. 2000) and humans (Adinoff et al. 1998, 2005; Wand and Dobs 1991), remaining altered during the first weeks of abstinence (Coiro et al., 2007; Sommer et al., 2008). Consequently, levels of CRF, ACTH and cortisol are increased, contributing to the dysphoria typical of alcohol withdrawal (Bruijnzeel et al., 2010) and to increases in the basal heart rate (Sinha 2008).

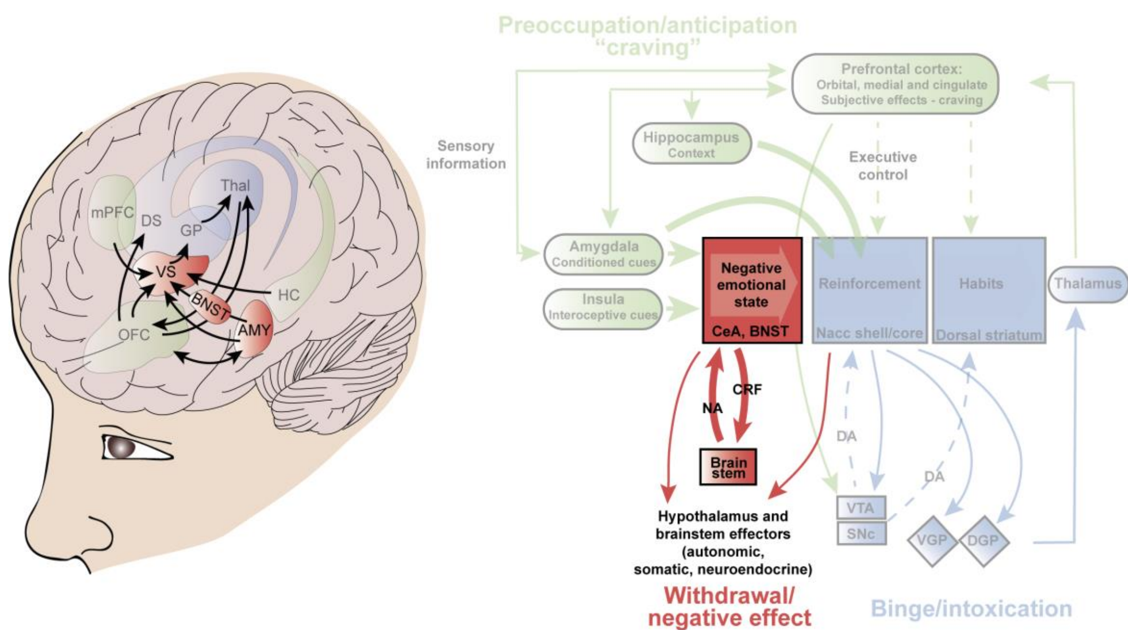


Figure 1.2. Neurocircuitry underlying the Withdrawal/negative affect stage. On the left, a scheme of the regions principally involved in this stage is reported. On the right, a model is shown of the manner in which regions and neurotransmitters interact in this stage. VS: ventral striatum, BNST: bed nucleus of the stria terminalis, AMY: amygdala, NA, noradrenaline, CRF: corticotropin-releasing factor. Adapted from G. Koob et al., 2014.

III. Preoccupation/anticipation (craving)

Some estimations show a high probability of relapse within the first years of abstinence (from 1 to 4 years; NIAAA, 1989, Kirshenbaum et al., 2009, Steckler et al, 2013). A clinical study showed that between 20 and 80 % of people receiving treatment and showing natural remission are prone to relapse (Moos and Moos, 2006). Craving is the desire for drug consumption (Drobes and Thomas, 1999), and it is considered key for relapse in humans, closing the three-cycle stage. However, the mechanisms supporting this state are not well-understood yet, and is difficult to measure clinically (Tiffany et al., 2000).

The principal region involved in this stage is the prefrontal cortex (PFC), a region responsible for the executive function. An impairment in this cortical region results key in the addiction perpetuation since it is responsible for the loss of control and,

subsequently, for the relapse. One of the key mechanisms for the self-control is an optimal inhibitory control, which is the ability to inhibit inappropriate behaviors. Animal studies have shown an impairment of this capacity after three intermittent cycles of alcohol intoxication (Irimia et al 2015), and addicted humans show alterations in the activity of cortical structures when performing inhibitory control-dependent tasks, which they perform worse than non-addicted individuals (Goldstein and Volkow, 2012). Additionally, PFC activity (along with other mesocorticolimbic areas) is higher in alcoholic patients when faced with an alcohol-related cue than with any other appetitive cue (i.e. juice; (Filbey et al., 2008), and in cocaine users when seeing a cocaine-related video (Volkow et al., 2010).

An important aspect of addiction is emotional functioning, which consists of the recognition and experiencing of emotions and interoception (i.e. somatic states associated with the hedonic experience of the drug). Addicted individuals make impulsive decisions driven by subjective thoughts directed toward drug intake, which fuel craving. The insular cortex (IC) is involved in the integration of interoceptive information (Critchley et al., 2004), processing decision-making-related uncertainty (Singer et al 2009) and impulsivity (Belin-Rauscent et al., 2016), and it is a key piece for drug craving (Droutman et al 2015, Garavan 2010).

The relapse can be triggered by exposure to the drug itself, drug-associated contexts and cues, or some stressors, and it is studied in animals by using the reinstatement model. The association of specific cues with drug intake is called reward learning, which is considered a form of associative long-term memory (Di Chiara 1998, Berke & Hyman 2000, Hyman & Malenka 2001, Everitt & Wolf 2002, Robbins & Everitt 2002, Chao & Nestler 2004, Hyman 2005). Both hippocampus (HC) and amygdala (AMY) are known to contribute to the reward learning but in different manners. The HC is critical for contextual learning (Jarrard, 1993; Holland and Bouton, 1999), and the formation of reward-associated contextual representations (Rezvani et al., 2010; Sjulson et al., 2018). Actually, dorsal HC (dHC) demonstrated to participate in the expression of conditioned

place preference to nicotine and cocaine (Meyers et al., 2006; Wang et al., 2018), and recently a causal role in context-induced reinstatement of alcohol-seeking in rats has been demonstrated (Felipe et al., 2021). On the other hand, lesions in the AMY were sufficient to block conditioned-cued reinstatement in rats (Meil and See, 1997; Kantak et al., 2002). Furthermore, intra-amygdala injections of tetrodotoxin, a voltage-dependent Na^+ channel blocker, prior to the associative learning blocked also the reinstatement, suggesting that this structure is central for the initial formation of cue-drug associations (Kruzich and See, 2001).

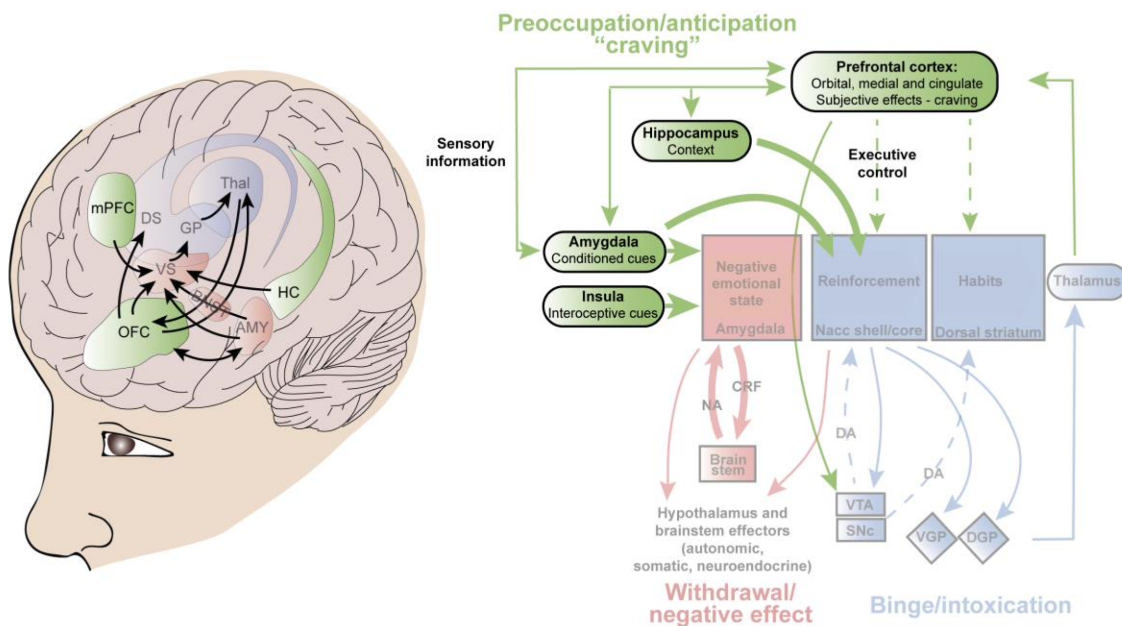


Figure 1.3. Neurocircuitry underlying the Preoccupation/anticipation (craving) stage. On the left, there is a scheme of the regions principally involved in this stage. On the right, a model is shown of the manner in which regions and neurotransmitters interact in this stage. mPFC: medial prefrontal cortex, OFC: orbitofrontal cortex, HC: hippocampus. Adapted from G. Koob et al., 2014.

In alcoholic individuals, abstinence is the most relevant target for developing treatments, especially during the first days when withdrawal symptoms are strongest, including negative affect and craving, which promote relapse (Heilig et al., 2010; Bach et al., 2020). However, the individual's vulnerability remains throughout the abstinence stage, and it is not known exactly why. The literature supports the idea that alcohol consumption

alters the microstructural integrity (Harper and Kril, 1985; Pfefferbaum et al., 2009, 2014; Alhassoon et al., 2012; Konrad et al., 2012; Sutherland et al., 2014; Monnig et al., 2015), but the evolution of these alterations along the abstinence period remains unclear. Classically, it has been thought that alterations start reverting as soon as alcohol drinking stops (Pfefferbaum et al., 2000). However, recent evidence points to a possible maintenance of the white matter (WM) impairment at the early abstinence stage (Alhassoon et al., 2012; Cosa et al., 2017; Zou et al., 2017). Further longitudinal studies with alcoholic patients are necessary to figure out how the brain responds in the absence of the drug, but this is not an easy task. On one hand, due to the comorbidity of this disease (mentioned above) a causal effect of alcohol-driven and abstinence-related effects to brain structure and function, is difficult to establish. On the other hand, the instability that characterizes these patients impacts on the permanence of the subjects in the study. For these reasons, translational research in animal models capable of representing salient features of human AUD is extremely important to understand this disease.

1.3. Hippocampus and fimbria/fornix pathway

Hippocampus

The HC is a region of the limbic system with a key role in several functions, such as spatial and contextual processing, episodic memory and emotional processing (Scoville and Milner, 1957; Morris et al., 1982; Squire, 1992; Buzsáki and Moser, 2013). It is divided in three main portions: dorsal, intermediate and ventral in rodents, and posterior, body and anterior respectively in humans (fig. 1.4A,B; Strange et al., 2014). The ventral portion has the strongest direct projections to PFC (Cenquizca and Swanson, 2007), and it is mainly involved in emotional and social processing (Bannerman et al., 2014). The dorsal one is more related to spatial memory, but finally they work together to generate a common output (Bannerman et al., 2014).

The HC in the longitudinal axis is divided similarly across species in different portions: subiculum (Sub), CA3, CA1 and dentate gyrus (DG; fig. 1.4.C,D). All these areas present a common cytoarchitecture: a layer containing the principal cells, another one containing their dendrites and a third layer with the axons (Fig. 1.5; Andersen, P. et al, 2007). Importantly, interneurons are present in every layer showing different morphologies and distributions (Freund & Buzsaki, 1996).

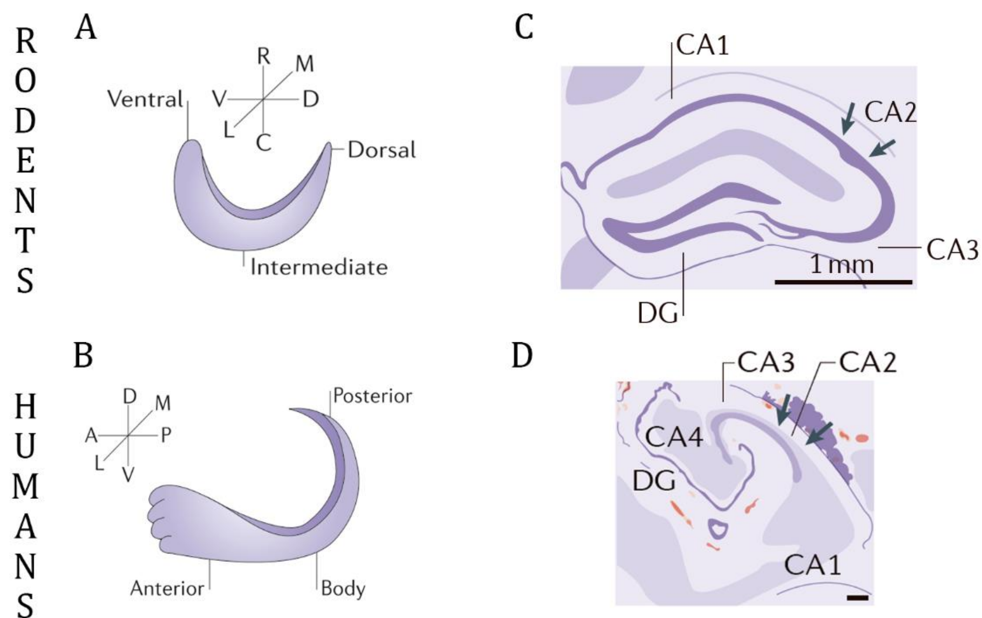


Figure 1.4. Anatomical comparison of the hippocampus between rodents and humans. The upper row corresponds to rodents, and the below one to humans. **A, B** shows the orientation of the hippocampal longitudinal axis in rats and humans. Note that it is ventrodorsal in rodents and anterioposterior in humans. **C, D.** Drawings of Nissl cross-sections of rodents' and humans' hippocampus. A, anterior; C, caudal; D, dorsal; DG, dentate gyrus; L, lateral; M, medial; P, posterior; R, rostral; V, ventral. Adapted from Strange et al., 2014.

The hippocampal areas compose three local circuits: trisynaptic (Ramón y Cajal, 1899), disynaptic (Tamamaki and Nojyo, 1993) and monosynaptic or temporoammonic pathway (Maccaferri and McBain, 1995; Ramón y Cajal 1911). The first one is unidirectional and comprises projections from the entorhinal cortex, via perforant pathway (PP), to the granule cells (GC) of the DG. Once this synaptic contact happens, GC fire an action

potential which travels through their axons called mossy fibers to the next neuronal population: the dendrites of the pyramidal CA3 neurons. Then, the axons of these neurons called Schaffer collaterals contact pyramidal CA1 neurons, activating the third synapse of the circuit. Finally, CA1 neurons contact either Sub or directly extra-hippocampal neurons. The disynaptic circuit comprises a direct projection from EC to CA3 neurons, bypassing DG neurons, while the temporoammonic circuit involves a monosynaptic projection from EC to CA1.

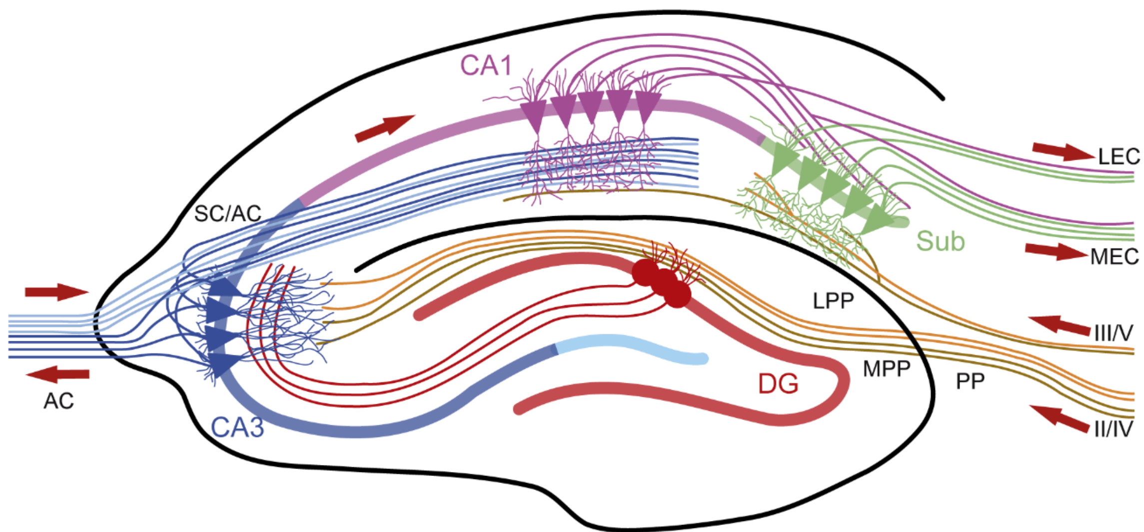


Figure 1.5. Trisynaptic circuit of the hippocampus. The red arrows represent the directionality of the flow information. CA1 and CA3: cornu ammonis regions 1 and 3, DG: dentate gyrus, Sub: subiculum, SC: Schaffer collateral, AC: anterior commissure, PP: perforant pathway, LPP lateral perforant pathway, MPP: medial perforant pathway, MEC: medial entorhinal cortex, LEC: lateral entorhinal cortex; II/IV and III/V: layers of entorhinal cortex. Adapted by Andrés Pérez-Segura from ://www.bristol.ac.uk/synaptic/pathways/

A relevant neuronal property carried non-exclusively out in the HC is the synaptic plasticity, defined as the modification of the efficacy of synaptic transmission after an event. This can be enhanced or depressed by activity, called potentiation or depression respectively. In turn, plasticity may act on a timescale of milliseconds or few minutes, known as short-term plasticity; or from minutes to hours, known as long-term plasticity.

The short-term plasticity plays a relevant role in adaptations to sensory inputs (Stevenson et al., 2010) and forms of memory which are short-lasting (Squire and Zola-Morgan, 1991; Goldman-Rakic, 1995). The presynaptic terminal has a finite pool of vesicles available to be released in response to a stimulus; if two stimuli arrive too close in the time, this pool is not enough to respond the second one and the response is depressed. Otherwise, if the neurotransmitter vesicles are enough, the residual calcium from the first action potential contributes to the second one, producing its facilitation.

The long-term plasticity comprises both long-term potentiation (LTP) and long-term depression (LTD), and both long-lasting changes in the synaptic weights are considered responsible for the integration of experiences into long-term memory (Kandel, 2001; Fusi et al., 2005). In 1973, LTP was discovered in the DG of rabbits after applying artificial stimulation at high frequencies (Bliss and Lømo, 1973), and later it was associated with learning (Morris et al., 1986; Giese et al., 1998; Whitlock et al., 2006). This high frequency stimulation protocol, typically referred to as a tetanus, is widely accepted to induce experimentally a memory, and the excitatory *N*-methyl-D-aspartate receptors (NMDAr) are mainly, but not only, underlying the mechanism (Lynch et al., 1983).

Relevance in AUD

Due to their implication in memory formation, alterations in both forms of plasticity might be underlying cognitive impairment, blackouts (Hermens and Lagopoulos 2018), memory deficits and executive dysfunction typical of alcoholic individuals (Bates et al. 2002; Pitel et al. 2007; Chen et al. 2018). Regarding the hippocampus, in animals there exist many evidences about the alcohol alterations on synaptic plasticity (Citri and Malenka, 2008; Avchalumov and Mandyam, 2020), neurogenesis (Morris et al., 2010; Geil et al., 2014), impact on hippocampal volume (Agartz et al., 1999; Lee et al., 2016; Wilson et al., 2017) and grey matter loss (Walker et al., 1980; Richardson et al., 2009; Dhanabalan et al., 2018).

Fimbria/fornix pathway

This white matter tract is a C-shape bundle of axons divided in several parts (figure 1.6). Arising from the HC, a group of myelinated fibers called alveus forms the fimbria of each HC, which finally is separated to form the crus of the fornix in each hemisphere. At the midline, these two crura are joined together through the hippocampal commissure (transverse fibers connecting both HC), forming the body of the fornix. This one travels anteriorly and, again, is divided into the anterior pillars of the fornix. At this point, fibers are subdivided in pre-commissural and post-commissural depending on where they project (Christiansen et al., 2016). Both in humans and rats, pre-commissural fornix provides the majority of axons connecting HC with PFC (Jay and Witter, 1991; Barbas and Blatt, 1995; Cenquizca and Swanson, 2007; Aggleton et al., 2015).

PFC is necessary to executive control functions (Stuss et al., 2001), as working memory (Lara and Wallis, 2015), decision making (Funahashi, 2017), temporal processing (Zhang et al., 2021) and goal-directed behavior (Valentin et al., 2007). Importantly, connectivity between PFC and HC is key for episodic memory processes (Simons and Spiers, 2003; Preston and Eichenbaum, 2013). Decades of research support the relevance of this fiber tract in HC-PFC communication both in primates (Rosene and van Hoesen, 1977; Goldman-Rakic et al., 1984; Barbas and Blatt, 1995), and rodents (Thierry et al., 2000).

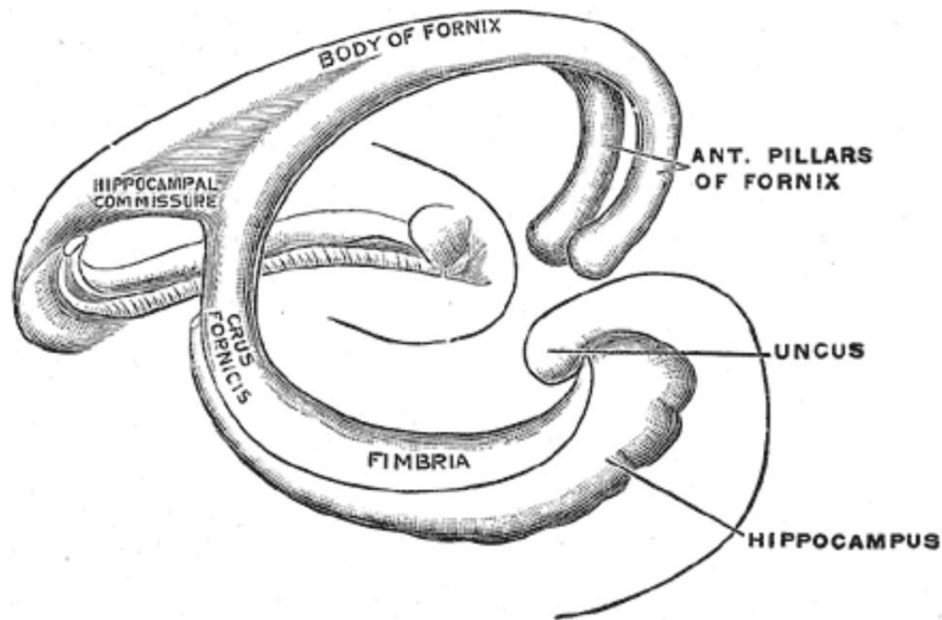


Figure 1.6. Representation of the fimbria/fornix pathway. It is shown the hippocampus and the uncus (from humans), the fimbria, the different parts of the fornix: crus, body and anterior pillars/columns; and the hippocampal commissure. Drawing from the book Anatomy of the Human Body (Gray, 1918).

Relevance in AUD

In the context of the alcohol disease, impairments in the fimbria/fornix microstructure have been reported (Pfefferbaum et al., 2009; Yeh et al., 2009) whose consequences are compatible with the physiopathology of alcoholic patients, such as memory deficits or poor cognitive flexibility. Other psychiatric illnesses related to alcoholism (Smith and Cottler, 2018; Archibald et al., 2019) present aberrant functional coupling between HC-PFC during rest (Zhou et al., 2008; Goveas et al., 2011), abnormalities in fornix (Kubicki et al., 2005) or cognitive impairment (Andrews and Thomson, 2009). However, though these results point to an impairment in the corticolimbic system in alcoholism, how this drug affects the communication between these structures remains poorly understood.

Although the HC has not traditionally received excessive attention in the addiction cycle introduced above, limiting its participation to the establishment of cue-reward associations that contribute in the final phases to cue-reactivation, there is literature demonstrating the deterioration of this structure with alcohol consumption (see the

previous section). Considering the hippocampal functions and the alcohol-induced deterioration, we will hypothesize in this work a greater relevance of this brain region in the pathophysiology of AUD, as we will see below.

1.4. Animal models

The symptomatology of this disease is wide and heterogeneous, since it involves a large number of factors. In order to cover different aspects of the addiction process, we have employed two rat models. On the one hand, we have used a model of genetic preference to drink, in animals that presents a voluntary predisposition to consume alcohol. On the other hand, we use a rat model in which alcohol dependence is induced by intermittent exposure to alcohol vapor. In this way, we can study the effect of alcohol drinking in wild type animals.

Voluntary intake: Marchigian Sardinian model (msP rats)

For years, researchers have been selectively breeding rats which showed preference for alcohol. In this manner, a number of alcohol-preferring animal models were developed (Spanagel, 2003). For instance, the Finnish model, called Alko Alcohol, characterized by high impulsivity and risk-taking behaviors, together with high voluntary ethanol consumption (Eriksson K., 1968). Another example is the Marchigian Sardinian (msP) rat model (Ciccocioppo et al., 2006), one of the models employed in this thesis work, which display anxiety-like behaviors and is susceptible to stress. Both models perfectly illustrate the fact that excessive alcohol consumption can be accompanied, or maybe even caused, by very different behavioral traits, e.g., impulsivity vs. stress (Pallarés, et al 2021).

This model shows a natural preference for alcohol, reaching pharmacologically significant blood alcohol concentrations (BAC) with spontaneous binge-drinking when exposed to

alcohol (Ciccocioppo et al., 2006). It has been extensively employed to develop new medications for AUD (Cannella et al., 2019) , and to discover the mechanisms underlying the excessive alcohol drinking (Economidou et al., 2008) and withdrawal symptoms (Economidou et al., 2011). Previous work in our laboratory has used a machine learning approach based on white matter data from these rats to obtain clinically useful biomarkers of early and protracted abstinence (Cosa et al., 2017).

Induced intake: PostDependent model (PD rats)

Other than orally, alcohol can be administrated intravenously (Thomsen and Caine, 2007) and by inhalation. In this thesis, it has been also employed the Postdependent model (Meinhardt and Sommer, 2015), which consists in wild-type rats exposed to chronic intermittent ethanol (CIE) vapor (more details in material and methods) during several weeks. In this manner, rats become alcohol-dependent at the end of the process, showing typical addiction-prone behavioral responses, such as craving and hypersensitivity to stress (Meinhardt and Sommer, 2015).

Thanks to the several intoxication cycles, this model is well suited to study the behavioral and neuroadaptive processes underlying vulnerability to relapse. These rats, unlike the previous ones, are dependent on alcohol, and they are therefore interesting to study the neuroadaptations underlying the addiction and their evolution throughout abstinence (i.e. if they recover or progress as abstinence persists over time).

1.5. Diffusion tensor imaging

Magnetic Resonance Imaging (MRI) is the imaging technique most employed to study the brain. One of its advantages is that it is a non-invasive tool, allowing translational studies with humans and non-human animals longitudinally (i.e. acquiring data at several time points). Briefly, MRI is based on the capacity of protons to resonate under a magnetic field. In this state, protons are able to release energy with the application of a radiofrequency signal. Then, we can measure this energy in different locations of the brain. Since the intensity of the energy signals varies depending on the tissue composition (i.e. white matter, gray matter or cerebrospinal fluid (CSF)), an image of the brain can be formed. As such, the image is composed by a collection of unit elements where the signal is measured, called voxels, which is the analog of the pixel but with three dimensions.

Diffusion-weighted MRI (dw-MRI) is an MRI technique focused on the diffusion of water molecules. As the cerebral tissue is composed by different cellular and tissular compartments (myelinated and unmyelinated axons, cell somas, dendrites, extracellular space, CSF) in which water molecules continuously collide, the net displacement of these molecules will be determined by this local environment. If the barriers encountered by water molecules have a different distribution along the three spatial directions, as it happens in the white matter fibres, the diffusion will be anisotropic; if instead the molecules can equally move in any direction, the diffusion will be isotropic, like in the CSF in the ventricles and, to some extent, in grey matter.

To describe more specifically how molecules diffuse in each voxel, it can be used a technique called Diffusion Tensor Imaging (DTI), which employs a mathematical structure called tensor. Dw-MRI has the capability of measuring these tensor elements by looking at the MRI signal attenuation, given that such signal is weighted by the amount of diffusion that the water molecules experience. However, since it is an indirect measure of water molecules behavior, it shows limitations. For example, the tensor works

properly with axonal bundles that present a main directionality, but is biased by crossing or branching fibers. Similarly, in voxels where reside white matter with grey matter or CSF, the diffusion measure is also distorted. So, it is necessary a careful interpretation of the results and, as far as possible, combining this technique with more direct measurements.

Relevance in AUD

Previous studies have employed DTI to answer how alcohol impacts on the white matter, obtaining widespread microstructural alterations in the whole-skeleton (Pfefferbaum & Sullivan, 2005; Pfefferbaum, et al., 2009; Yeh, at al., 2009). Focusing on specific bundles, some have received more attention such as the corpus callosum, the fimbria/fornix, internal and external capsules and cingulate and longitudinal fasciculi fasciculi (Pfefferbaum et al., 2009, 2014; Yeh et al., 2009; Bühler and Mann, 2011). However, a robust comparison of effect sizes and vulnerability in different tracts has not been still performed.

2. OBJECTIVES

The aim of this PhD thesis is to study alcohol-induced structural and functional alterations in the brain and their evolution in the early phases of abstinence, employing a truly translational design including human AUD patients and animal models. We use two rat models covering genetic and environmental aspects of the disease, brain MRI to facilitate translation, and invasive histological and electrophysiological tools in the animal models to investigate the underlying mechanisms.

The specific objectives are:

1. To characterize white matter integrity in chronic alcohol consumption in both humans and rats.
2. To study how the structural and functional effects of alcohol consumption progress along the abstinence.
3. To investigate the mechanism underlying the structural impairment of the fimbria/fornix pathway.
4. To analyze the physiological consequences that the alcohol consumption produces into the hippocampus.
5. To check the functional coupling between prefrontal cortex and hippocampus, which are connected by the fimbria/fornix.

3. MATERIAL AND METHODS

This thesis consists of two independent but related studies, one of which has been already published in De Santis et al., 2019. The experimental design of both studies is explained in figure 3.1.

3.1. Animal models and preparation for the experiment

All experiments were approved by the Animal Care and Use Committee of the Instituto de Neurociencias de Alicante, Alicante, Spain, and comply with the Spanish (law 32/2007) and European regulations (EU directive 86/609, EU decree 2001-486, and EU recommendation 2007/526/EC).

msP rats

A) Alcohol exposure

Rats were obtained from the breeding facility at the School of Pharmacy University of Camerino, Camerino, Italy. When they arrived at our facilities, rats were individually housed in transparent polycarbonate cages with bedding material, and a wooden stick and nesting material were given as enrichment. They had ad libitum access to food and water and were housed under controlled temperature (22±2°C) and relative humidity (55±10 percent) on a 12-hour light/dark cycle.

A total of 36 male rats (370–480 g, 8 weeks of age) of msP line were used, and 27 rats of them had access to alcohol in a 2-bottle free-choice paradigm for 30 days. Briefly, animals had access *ad libitum* to two bottles, one with water and another one with 10% (v/v) alcohol in water. Fluid consumption and animal weight were registered every 2–3 days concomitantly with replacement of the bottles' content. After one month of two-bottle free-choice drinking regime, the alcohol-containing bottle was removed.

Eighteen rats underwent DTI before alcohol access (TP0r-A), after 4 weeks of alcohol access (TP1r-A), and after 6 weeks of abstinence (TP3r-A). Nine rats underwent DTI twice: after 4 weeks of alcohol drinking (TP1r-B) and after 2 weeks of abstinence (TP2r-B). Nine rats were used as age-matched alcohol-naïve controls.

B) Anesthesia and preparation

Imaging experiments were performed under isoflurane anesthesia. Anesthesia was induced with 4–5 percent isoflurane in oxygen (0.8–1L/min) and animals were secured in a custom-made holding apparatus with a tooth bar and a nose cone. During scanning, the isoflurane concentration was maintained at 1.2 percent, the body temperature was kept constant with a heating pad and physiologic parameters as oxygen saturation, pulse distension, breathing and heart rate were monitored (MouseOx, Starr Life Sciences, Oakmont, PA, USA). At the end of the electrophysiological experiment animals were sacrificed and their brains process for histological analysis.

PD rats

A) Alcohol exposure

Forty-eight male Wistar rats, initial weight 220 to 250 g, were used in the study (Charles River). Animals were housed 2-4 per cage (Type-IV; Ehret) under a 12 hours light/dark cycle with *ad libitum* access to food and water. The PD state was induced in the lab of our collaborators (Prof. Wolfgang Sommer) in the Central Institute for Mental Health in Mannheim, Germany. One week after completion of the protocol (described below), animals were shipped to Alicante, Spain, and were employed with 10-14 days of abstinence.

The CIE ethanol exposure was performed as described previously (Rimondini et al., 2002). Briefly, rats were exposed to either ethanol vapor (post-dependent rats) or normal air flow (control rats), obtaining in this way two independent groups. The alcohol was

delivered by dosing pumps (Knauer) into electrically heated stainless-steel coils (60°C) connected to an airflow of 18 L/min. Exposure was for 7 weeks, combining daily intoxication during 14 hours of ethanol vapor with 10 hours of withdrawal. Blood alcohol levels were constantly monitored and maintained around 250 mg/dL, also the BAC was controlled twice per week.

B) Anesthesia and preparation

To perform the experiments, animals weighing 400 to 500 g were intraperitoneally anesthetized with urethane (1.4 g/kg). When necessary, anesthesia was reinforced with a fifth of the initial dose to assure absence of reflexes. The change to the urethane anesthesia, compared to isoflurane in the study with msP rats, was due to the introduction of electrophysiological recording sessions in the PD rat experiments to investigate the functional consequences of white matter alterations. Urethane has been demonstrated to be an optimal anesthesia for electrophysiological recordings since it produces a stable aesthetique state and preserves brain oscillations (Maggi and Meli, 1986; Mallet et al., 2008; Brazhnik et al., 2014). The experiment started with the MRI session. Animals were then secured in an MRI-compatible cradle, constantly supplied with 0.8 L/m oxygen in air, with a face mask, and their temperature maintained at 37±5 °C with a water heating pad, as before. Breath distention, heart rate, blood oxygen saturation, and breathing rate were monitored (MouseOx, Starr Life Sciences, Oakmont, US). After MRI data acquisition, animals were transferred to a stereotaxic frame for the electrophysiological recordings in the HC and PFC. Physiological monitoring, temperature control and oxygen supply continued as before. At the end of the electrophysiological experiment animals were sacrificed and their brains processed for histological analysis.

3.2. Human subjects

The participants were 127 men enrolled in three different groups:

(1) a cohort of 36 healthy patients;

(2) 48 treatment-seeking patients with AUD (cohort A) undergoing DTI at 1 week after admission into the clinic and completion of detoxification treatment (TP1h-A) and after 2 to 3weeks (TP2h-A).

(3) 53 treatment-seeking patients with AUD (cohort B) undergoing DTI after 2 to 3 weeks of admission into the clinics (TP2h-B), 20 of whom underwent scanning again after 4 to 6 weeks of admission (TP3h-B).

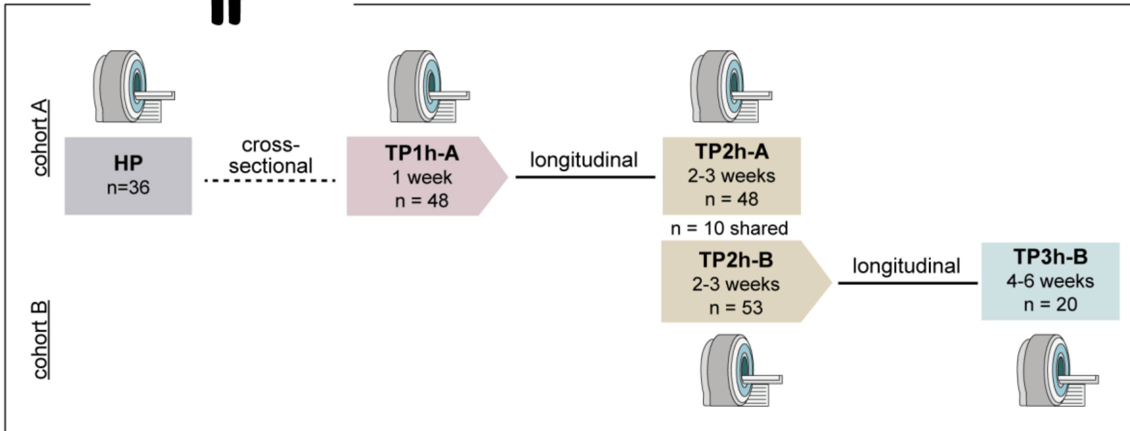
Cohorts A and B shared 10 patients, and all participants were male because the vast majority of AUD patients admitted to inpatient care are males. The study was conducted at the Central Institute for Mental Health in Mannheim, Germany. The local ethics committee approved study procedures and all participants provided informed written consent. Descriptive statistics of demographic data and clinical descriptors appear in Table 3.

	Control (n = 35)	**AUD Cohort 1 (n = 48)**	**AUD Cohort 2 (n = 54)**	**Statistics**	**Significance**
Demographical variables					
Age (years)	40.9 (9.8)°	47.5 (10.1)°	45.0 (8.9)	$F_{(2,136)} = 4.731$	p = 0.010*
Education (no post-secondary educ./apprenticeship only/attended college or higher)	1/20/14	4/27/17	10/32/10	$Chi^2_{(6)} = 12.886$	p = 0.043*
Substance use patterns					
Ethanol (g/day; mean of last 90 days)	6.1 (5.8)°,#	202.5 (196.8)°	196.3 (131.5) #	$F_{(2,134)} = 23.060$	p < 0.001*
ADS (total score)	2.1 (2.4)°,#	15.3 (6.8)°	13.9 (6.5)#	$F_{(2, 131)} = 57.264$	p < 0.001*
OCDS (total score)	1.5 (1.4)°,#	16.9 (7.7)°	16.3 (6.9)#	$F_{(2, 131)} = 67.851$	p < 0.001*
Smoker (yes/no)	3:31	31:17	41:11	$Chi^2_{(2)} = 43.155$	p < 0.001*
Cigarettes per day in smokers (0 to 10/11 to 20/21 to 30/ > 30)	1/0/2/0	2/8/14/8	4/8/15/15	$Chi^2_{(6)} = 5.653$	p = 0.469
FTND in smokers (total score)	5.3 (4.6)	6.1 (2.1)	6.0 (2.5)	$F_{(2, 76)} = 0.870$	p = 0.862

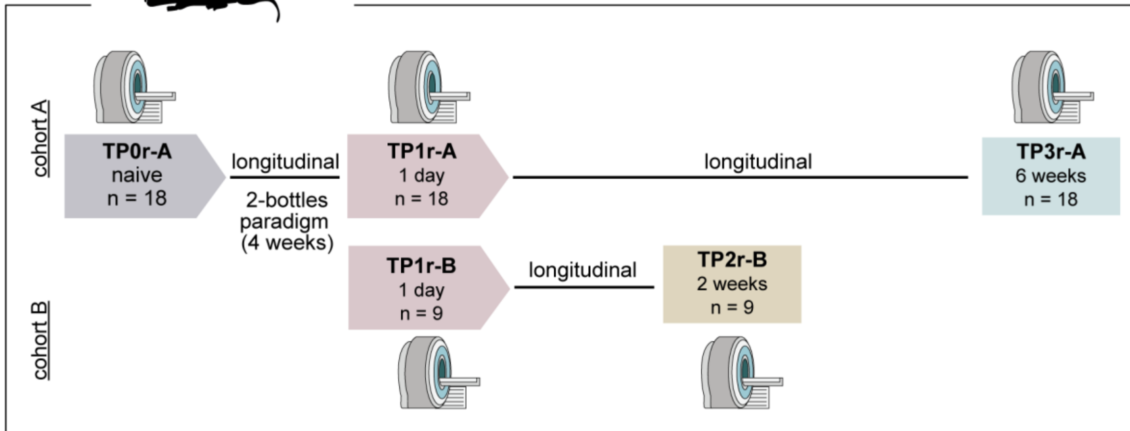
ADS = Alcohol Dependence Scale (missing values for N=1 HC, N=1 AUD Cohort 1, N=3 AUD Cohort 2); Education (missing values for N=0 HC, N=0 AUD Cohort 1, N=2 AUD Cohort 2); FTND = Fagerstroem Test for Nicotine Dependence (missing values for N=32 HC, N=16 AUD Cohort 1, N=12 AUD Cohort 2); OCDS = Obsessive-Compulsive Drinking Scale (missing values for N=3 HC, N=0 AUD Cohort 1, N=2 AUD Cohort 2); Smoking status (missing values for N=1 HC, N=0 AUD Cohort 1, N=2 AUD Cohort 2); SD = standard deviation; * = significant group main effect $p < 0.05$; ° = significant post-hoc difference (post hoc test using Games-Howell procedure) between Control and AUD Cohort 1 $p < 0.05$; # = significant post-hoc difference (post hoc test using Games-Howell procedure) between Control and AUD Cohort 2 $p < 0.05$.

Table 3. Demographic and clinical data for healthy and AUD patients.

1. Humans



2. msP rats



3. PD rats

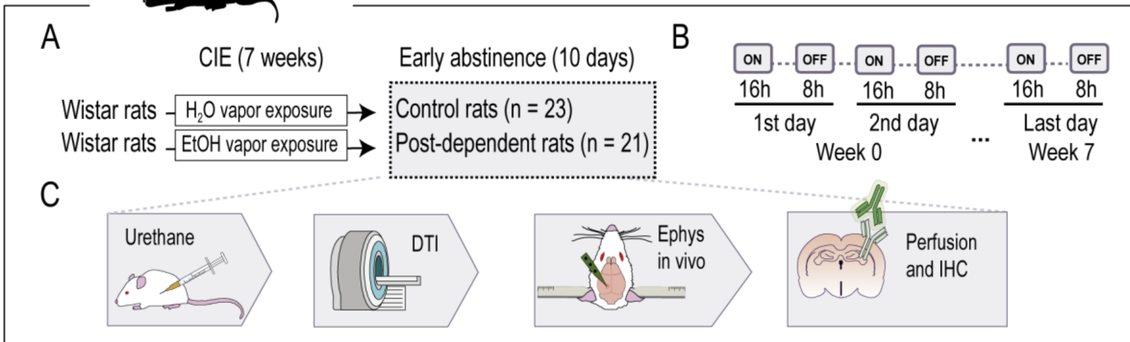


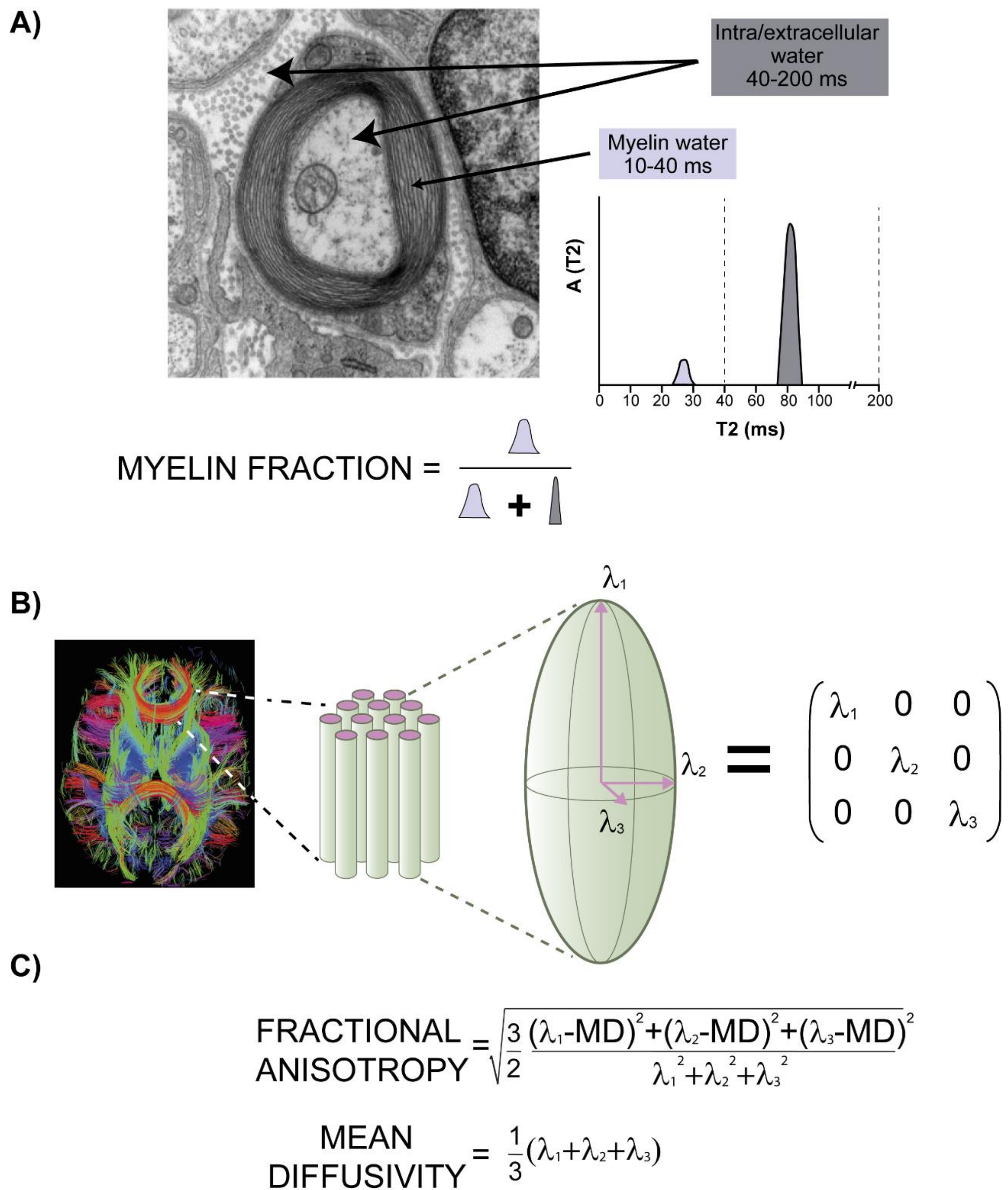
Figure 3.1. Experimental design. 1. The work with humans consisted in an MRI scanning in healthy patients (HP), which was compared cross-sectionally with a group of alcoholic subjects who were 1-week abstinent (TP1-A). This group was again scanned after 1-2 weeks to check longitudinally the microstructural changes (TP2-A). To study deeply these modifications, a group in these same circumstances (2-3 weeks of abstinence; TP2-B) was scanned at this timepoint and again some weeks later (TP3-B). **2.** A similar procedure than humans was developed with msP rats, but in this case was fully longitudinal. A group of 18 naïve rats was scanned (TP0-A) and then exposed to alcohol consumption during 4 weeks. After that, they were scanned again at two timepoints: 1 day (TP1-A) and 6 weeks of abstinence (TP3-A). Parallelly, a cohort B of 9 rats was scanned at 1 day (TP1-B) and 2 weeks of abstinence (TP2-B), to check earlier abstinence as we made in humans. **3A)** A group of naïve rats was exposed during 7 weeks to different protocols: water vapor exposure, becoming control rats; and alcohol vapor exposure, becoming post-dependent (PD) rats. **3B)** The protocol in both cases was a chronic intermittent exposure, which consisted in 16h of vapor ON and 8h of vapor OFF per day. **3C)** Each rat was anesthetized with urethane and, once it was properly induced, was introduced into the magnet to acquire MRI data. After that, it was replaced in a stereotaxic to perform the electrophysiological study and, finally, it was perfused. The brain was extracted and preserved to develop the immunohistochemistry technique.

3.3. Magnetic Resonance Imaging

One of the parameters measured in this thesis is the Myelin Fraction (MF; (fig. 3.2a). Theoretically it is based on how the myelin water is distributed in the brain. This parameter is obtained from a T2 image (one of the most common MRI sequences), which is related with the relaxation time of the protons after having been stimulated by radiofrequency pulses. This relaxation time will be shorter in a voxel with water in contact with the myelin sheaths, while will be longer in molecules belonging to intra/extracellular water. As each voxel has water in different environments, we are able to decompose the T2 decay curve of an image in its exponential components. The amplitude of water signal associated to the myelin subcomparment, calculated as the ratio of the area in the T2 distribution corresponding to the myelin water divided by the area of the full T2 distribution, is interpreted as a proxy for the myelin content.

As we have explained above, we have also applied DTI to further characterize the microstructure of the white matter. The tensor is a three-dimension structure visualized by an ellipsoid and quantified by a matrix (fig. 3.2a). After diagonalizing the matrix, we obtain three different vectors which inform about the diffusion from different perspectives. There are too many parameters that we can obtain from the tensor; in this thesis, we have studied the following ones (fig. 3.2b):

- Fractional Anisotropy (FA). It goes from 0-1, and evaluates the vectors' length, to see if there is any predominant. If FA = 0, the ellipsoid will be a sphere which represents perfectly isotropic diffusion; and if FA = 1, the ellipsoid will be pointier, representing highly anisotropic structures. When measured in white matter, FA is interpreted so that high values represent well-ordered and likely intact structures, while experimental or disease-associated decreases in FA values are commonly interpreted as signs of microstructural damage. However, some limitations exist to this simplistic interpretation, since different changes in the tissue can produce comparable FA alterations (De Santis et al., 2014).
- Mean Diffusivity (MD). It does not consider the directionality, but the amount of diffusion. It is an average of the diffusivity in the three directions. An increase in this parameter is commonly found in pathologic conditions, and suggests a reduction in barriers for the water diffusion in the tissue, which in turn is interpreted as an alteration in the underpinning microstructure.



$$\text{FRACTIONAL ANISOTROPY} = \sqrt{\frac{3}{2}\frac{(\lambda_1-MD)^2+(\lambda_2-MD)^2+(\lambda_3-MD)^2}{\lambda_1^2+\lambda_2^2+\lambda_3^2}}$$

$$\text{MEAN DIFFUSIVITY} = \frac{1}{3}(\lambda_1+\lambda_2+\lambda_3)$$

Figure 3.2. Diffusion-weighted MRI methodology. A) On the left, there is a transversal section of a myelinated axon, where water molecules are both between myelin sheaths and occupying intra- and extracellular space. On the right, we can see a plot of signal amplitude vs T2 time (adapted from MacKay et al., 2006). **B**) The tensor models the microstructure of the brain, which is explained by three vectors. These ones can be extracted from a matrix, from which we can also extract different parameters, for instance fractional anisotropy and mean diffusivity (**C**). Design by Dr Silvia De Santis.

Acquisition

Humans

Scanning was performed in the Central Institute for Mental Health in Mannheim, Germany, within a 3 T whole-body tomograph (MAGNETOM Trio with TIM technology; Siemens, Erlangen, Germany). In order to assess the individual brain morphology of each participant, high-resolution three-dimensional T1-weighted anatomical images (MPRAGE) were collected with 192 contiguous sagittal slices [slice thickness, 1.0 mm; field of view = 256 × 256 mm^2, time of repetition (TR) = 2.3 seconds, time of echo (TE) = 3.03 milliseconds 2 secods, time of inversion = 900 milliseconds, flip angle = 9°]. DTI data was acquired using an Echo Planar Imaging spin-echo diffusion sequence with the following parameters: repetition time (TR) 14 ms, echo time (TE) 84 ms, 41 gradient orientations uniformly distributed plus one non-diffusion weighted images, b-value 1000 s/mm^2, matrix size = 128X128X64, isotropic resolution of 2 mm^3.

Rats

The experiments were carried out in Alicante, Spain, in a horizontal 7 T scanner with a 30 cm diameter bore (Biospec 70/30, Bruker Medical, Ettlingen, Germany). DTI data were acquired using an Echo Planar Imaging spin-echo diffusion sequence. For the group of rats from the first work (msP rats; De Santis et al., 2019), a diffusion protocol was employed with the following parameters: TR = 8000 ms, TE = 29 ms, 30 gradient orientations with b-value 1000 s/mm^2 plus three non-diffusion weighted images, matrix size = 128 x 128 x 16, in-plane resolution = 0.225 x 0.225 mm^2, slice thickness = 1 mm. For the group of rats from the second work (PD rats), we employed a protocol with 30 uniform distributed gradient directions, b-value 670 s/mm^2 plus four non-diffusion weighted images, TR = 4000 ms, and TE = 23 ms. Fourteen horizontal slices were planned for every subject field of view = 32 x 32 mm^2, matrix size = 128 x 128 x 14, in plane resolution = 0.25 x 0.25 mm^2, slice thickness = 1 mm). In addition, in PD rats,

multi-compartment relaxometry data were acquired using a Multi-Slice Multi-Echo protocol with the same geometry of the diffusion scan and the following sequence-specific parameters: TR 6000 ms, TE varied in the range 5-155 ms in steps of 5 ms, 2 repetitions.

Analysis

Both species data were preprocessed to correct for Eddy current and motion distortions using affine registration. DTI analysis was done with the software ExploreDTI v.4.8.4 (Leemans et al., 2009).

FA maps computed through the DTI models were fed into an in-house modified version of the Tract-Based Spatial Statistics (TBSS) routine of FSL19 (Smith et al., 2006), in which the normalization to MNI standard space is performed using more accurate normalization tools (ANTs package) (Klein et al., 2009). After skeleton extraction, skeletonized maps were obtained for FA by applying the pre-computed registration and skeletonization steps. The normalization and skeletonization routine was combined with an automatic region of interest (ROI)-based aggregation based on WM labelling in standard space (JHU ICBM DTI 81 Atlas by Mori et al., 2008, also available in FSL, total 50 ROIs) as previously described (Toschi et al., 2020). This allows to simultaneously minimize registration- and interpolation-related biases (through skeletonization) and increase sensitivity through ROI-wise averaging (Coutu et al., 2014). After skeleton extraction, the skeleton was combined with the ICBM DTI 81 Atlas, and for all participants, mean FA values were calculated in each ROI belonging to the WM parcellation.

For whole brain analyses, and in order to compare the effect size across the time, we calculated voxel-wise, in voxels with significant differences across conditions, the percent change in biomarkers according to the following formula:

$$P=(P_2-P_1)/P_1$$

Where P is FA, 1 stands for initial/healthy condition and 2 for final/pathology condition. We calculated: 1) P for healthy patients versus TP1; 2) for the scan at TP1 versus the scan at TP2a; and 3) for the scan at TP2b versus the scan at TP3.

Whole brain tractography was calculated in native space using a deterministic, tensor-based approach; tract termination criteria were FA<0.15 and angle>30 degrees. The fimbria was then manually reconstructed both in humans and rats using endpoints placed in anatomically plausible positions. Average values of FA, MD and MF were calculated in the fimbria for each species.

In order to specifically test the myelin status, the diffusion data of PD rats were additionally non-linearly registered to Multi-Slice Multi-Echo data to correct for EPI distortion using ANTs (Klein et al., 2009). These multi-compartment relaxometry data were fitted to a bi-exponential decay (representing water trapped into myelin sheets and intra-extra cellular water) using in-house Matlab (R2015b, The Mathworks Inc., Natick, MA) code, and the fraction of the signal associated to the fast-relaxing component was interpreted as a proxy for myelin content (MacKay and Laule, 2016).

3.4. *In vivo* extracellular electrophysiology

Electrophysiology is the branch of physiology which studies the electrical signals employed by the neurons to communicate. When we introduce a recording electrode into the brain we obtain a voltage readout, which reflects the movement of currents across the neuronal membranes occurring around the tip of our electrode. This activity is produced by both local and remote neuronal populations contacting our targeted cells, and is called Local Field Potential (LFP). This is in part a blind technique, reason by which is necessary to ensure the position of the electrode by coordinates and to be familiar with the characteristic electrical signals of the regions.

One way to facilitate the location of the recording electrode is to add a stimulation electrode which evokes an expected evoked field potential in the targeted population. For instance, the hippocampus has a clear cytoarchitecture, with well-differentiated dendritic

and somatic layers. If we electrically stimulate the perforant pathway, which is the principal excitatory affluence to hippocampus, we will obtain different responses depending on which specific layer are we recording. In the dendrites, we will depolarize the membranes obtaining a negative shift in the LFP, called field Excitatory Post-Synaptic Potential (fEPSP). By contrast, in the soma layer, if the excitatory input is strong enough, the neurons will be depolarized and will respond with an action potential. In the LFP, we will record this population synchronized firing as a negative shift in the voltage, called Population Spike (PS).

Preparation

Surgical and stereotaxic procedures were performed as described previously (Canals, et al., 2005; Figure 3.3). Two concentric bipolar stimulating electrodes (WPI, London UK) were used to stimulate the medial perforant pathway (MPP; from lambda: AP: 0; ML: 4.1; DV: 2.3-2.7 mm) and the fimbria (from bregma AP: -1.5; ML: 0.4; DV: 3,2 mm), according to Paxinos and Watson (2007). Two multisite silicon probes (single shank, 32 channels, 100 µm spacing; Neuronexus Technologies) connected to multiple high impedance head-stages were lowered into intermediate hippocampus and medial prefrontal cortex using coordinates with respect to bregma: AP -4.4; ML 2.6; DV 3.5 mm and AP 3.4; ML 0.5; DV 4.5 mm, respectively. Before brain insertion, probes were immersed in a saturated solution of DiI in ethanol, for posterior postmortem confirmation of probes placement. A silver chloride wire in contact with the neck skin worked as a ground for the recordings. Electrophysiological signals were filtered (high-pass 0.1 Hz), amplified and digitalized using Multi Channel Systems recording hardware and software (10 kHz sampling rate).

Recordings

After surgery, we let the tissue rest for 30 minutes before starting with the recordings. First, ten minutes of spontaneous activity were acquired both in the prefrontal cortex and hippocampus. Then, different protocols of electric stimulation of the perforant pathway and the fimbria were applied using a pulse generator and current source

(STG2004, Multichannel Systems, Reutlingen, Germany). First, a stimulus-response curve protocol consisted in single biphasic 100 µs duration pulses at different intensities (ranging from 60 to 900 µA) delivered every 15 s, with each intensity presented four times. Second, a paired-pulse protocol with two pulses of suprathreshold and identical intensities applied at varying inter-pulse time intervals (from 10 to 80 ms) was used to investigate feed-back inhibition (around the time of maximal $GABA_A$ conductance) and facilitation (around the time of maximal $GABA_B$ conductance) (Davies et al., 1991). Finally, a long-term potentiation (LTP) protocol consisting in six trains of pulses 400 Hz lasting 20 ms, delivered at a 10 s interval, and repeated six times at an interval of 2 minutes, was used to investigate long-term synaptic plasticity.

Neuronal firing in response to the stimulation protocols was quantified as the amplitude of the population spikes (PS) recorded in the DG and CA1 soma layers, respectively. Similarly, the synaptic activity evoked by stimuli was measured as the slope of the fEPSP recorded in the DG molecular layer and the CA1 *stratum radiatum*. The effect of pair-pulse stimulation was measured as the ratio of the second PS divided by the first PS and that of LTP as the percentage increase in the PS after LTP induction vs. the baseline. Activity propagation from the HC to the PFC was computed as the amplitude of the evoked potential in the PFC divided by the simultaneously recorded PS in the CA1.

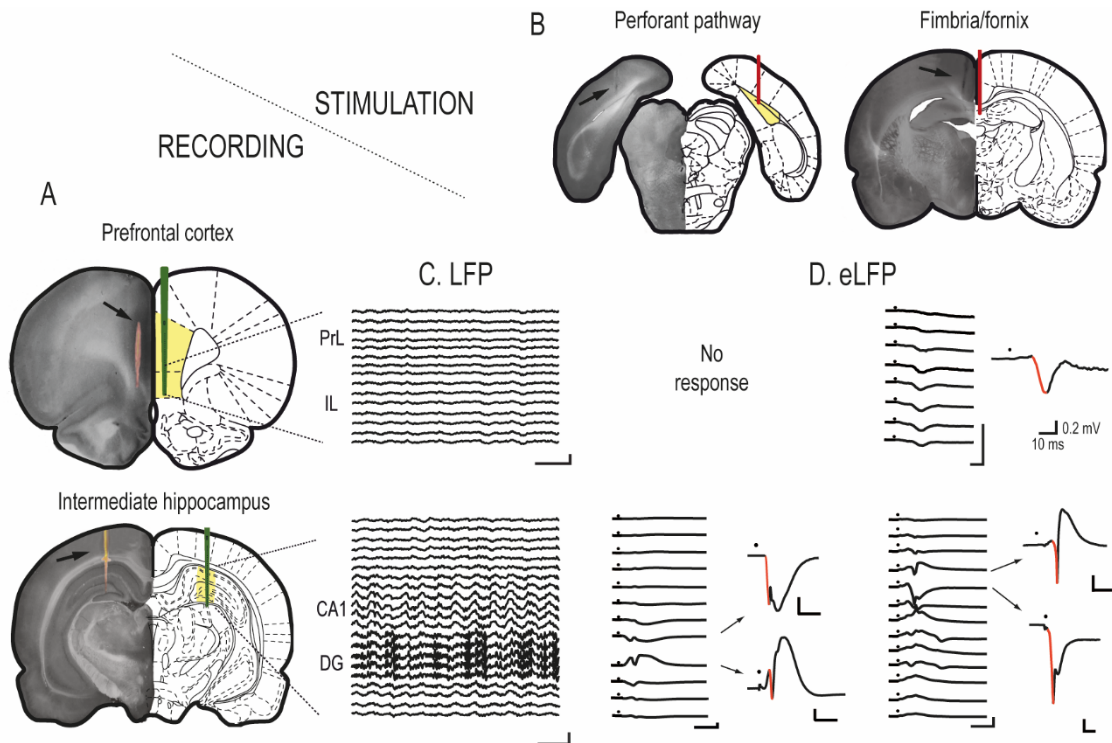


Figure 3.3. Electrophysiological methods. Histological images represent the location of the recording electrode **(A)**, indicating the DiI trace with an arrow, and the corresponding atlas section on the right, with the recorded area of interest in yellow. Also, is showed an example of Local Field Potential recorded by each electrode in every channel **(C)**. The same is showed for the stimulating electrodes **(B)**, but in this case the arrow is pointing to the tissue damaged due to the introduction of each probe. **(D)** Evoked Local Field Potentials (eLFP) produced by stimulating PP and fimbria/fornix with a single pulse protocol are shown for each recorded region. Specifically, is represented the evoked electrophysiological profile and the specific response that we measured, highlighting in red the voltage deflections employed for quantification.

3.5. Tissue processing for histology and immunohistochemistry for myelin basic protein

DTI, while sensitive to changes in myelin, provides indirect measurements which might be masked by uncontrolled factors. The concomitant application of microscopic imaging tools, such as immunohistochemistry, provides direct information from the tissue. In our study, we immunostained the myelin basic protein (MBP, figure 3.3) to obtain direct information on myelin status in the same animals.

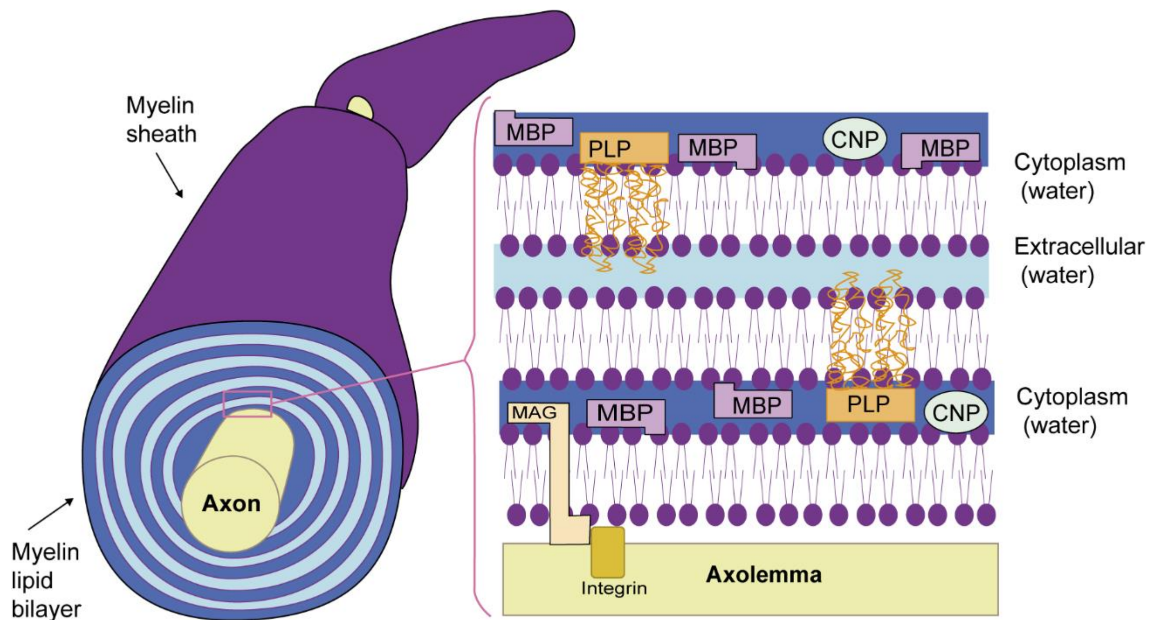


Figure 3.4. Substrates checked by immunohistochemistry. On the left, myelin sheath is represented surrounding an axon. On the right, is showed the molecular composition of the myelin lipid bilayer, including myelin basic protein (MBP), proteolipid protein (PLP), cyclic nucleotide phosphodiesterase (CNP) and myelin-associated glycoprotein (MAG). Adapted from Laule et al., 2007.

After completion of each experiment, anesthetized-rats were intracardially perfused with 100 mL of 1% phosphate-buffered saline (PBS) solution followed by 50 mL of cold 4% paraformaldehyde (PFA) in PBS. Brains were kept for 24 h on 4% PFA post-fixation at 4° and prepared for coronal sections (50 µm) using a vibratome (VT1000S, Leica Microsystems). Slices were collected into 24-well plates containing 1% PBS. For each rat, tissue sections were selected for the fimbria, between 2- and 3-mm posterior to Bregma. Then slices were kept in a solution of sodium citrate (pH 6.0) and introduced in a thermomixer (Eppendorf Ibérica) once the temperature has reached 85 °C. After rinsing with 1% PBS, sections were incubated with a blocking solution [10% goat serum donor herd + 4% bovine serum albumin in PBS supplemented with 0.5% Triton X-100] for 2 h. Sections were then incubated with mouse anti-MBP antibody (1:250, Merk Millipore, Darmstadt, Germany) in the blocking solution overnight at 4°C. After rinsing again in PBS, sections were incubated for 90 minutes with Alexa-488-conjugated goat antibody (anti-mouse, Termofisher, 1:500), rinsed in PBS and mounted for photography under a

fluorescence stereo microscope (Leica Microsystems, Germany) or a fluorescence light microscope (Neurolucida, MBF Bioscience, Netherlands). Images were analyzed with the ImageJ program (National Institute of Health, USA) (Schindelin et al., 2012). Intensity of fluorescence was determined considering the area of the region selected (Integrated density = mean fluorescence * area) and the intensity of the background. In this way, we obtained the corrected total fluorescence (CTF) with this formula: CTF = Integrated density – (area * mean background fluorescence).

3.6. Statistical analyses

Experimenters were blind to the experimental group until the completion of individual data collection and analysis of data. Regarding the sample, the study in humans was performed with the absence of some subjects at point TP3-B, due to patient relapses or other personal reasons. In rats, a small part of the sample was lost due to anesthesia, specifically two rats were lost from the msP group and four from the PD group for this reason.

All statistical analyses were conducted in Matlab (The Mathworks Inc., Natick, MA), SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM) or GraphPad Prism version 7.00 for Windows, (GraphPad Software, La Jolla, CA, USA). Data are expressed as mean ± SEM.

<u>DTI</u>

A general linear model (GLM) was used within a voxel-wise, permutation-based, non-parametric statistical framework (Smith et al., 2006) to test for significant differences between alcohol and control, controlling for age in humans and multiple comparisons across clusters using Threshold Free Cluster Enhancement. We employed 10000 permutations, and a corrected voxel-wise p-value <0.05 was considered statistically significant.

Two-sample t-tests were performed in each ROI to test for significant differences in alcohol versus control, and the p-value was corrected for multiple comparisons using a false discovery rate approach. Cluster location has been assessed using a WM DTI-driven parcellization for humans (Mori et al., 2008) and the Paxino-Watson atlas for rats (Paxinos and Watson, 2009).

Significant differences between healthy and alcoholic humans were calculated using a multivariate analysis of variance (ANOVA) on all microstructural parameters, with age and gender as covariates of no interest. In msP rats, significant differences between different time points were tested using a repeated measures multivariate ANOVA on all microstructural parameters. Both ANOVAs were followed by post-hoc t-tests, corrected for multiple comparisons using false discovery rate (Groppe et al., 2011), to assess which parameter was statistically different between the two conditions. In PD rats, unpaired one-tailed t-tests were applied to test for significant differences across conditions (alcohol exposed and controls), based on the premise that alcohol consumption affects axonal and myelin integrity.

Electrophysiology

For electrophysiological analyses, criteria for inclusion in final analysis were correct location of recording and stimulating electrodes (all remaining animals were included). On one hand, analysis of coherence and broad band correlation were applied on the spontaneous signals. The significance of the analysis was tested with a surrogates analysis and with an unpaired two-tailed t-test. On the other hand, for the evoked-activity, the dependent variables were the amplitude of the evoked potential in the PFC, the PS and EPSPs recorded in CA1 and DG, the PPRs, the percentage of potentiation after LTP and the propagation index. These variables were analyzed with unpaired two-tailed t-tests or ANOVAs, applying Bonferroni's *post hoc* tests when significant effects were obtained.

Histology

For histological analysis, we decreased the variability of the measure averaging the mean intensity of two consecutive slices per animal. Three outliers were found applying the ROUT module set at 1. Then, an unpaired two-tailed t-test was applied. All distributions were checked to be normal using D'Agostino-Pearson omnibus normality test, and a *P*-value < 0.05 was considered statistically significant.

4. RESULTS

4.1. Continuous alcohol exposure alters the whole white matter skeleton

Our first aim was to compare the microstructural parameters between control and alcohol conditions. In humans, as expected, alcohol dependent subjects presented microstructural abnormalities, such as reduced FA and increased MD compared to controls (fig 4.1A, B left) at $p<0.05$ level, corrected for multiple comparisons. Specifically, FA is reduced in the genu and body of the corpus callosum, in the fornix, in the cerebral peduncle bilaterally, in the right cortico-spinal tract, in the corona radiate bilaterally, in the thalamic radiation bilaterally, in the right sagittal stratum, in the right cingulum, in the internal and external capsule bilaterally, in the superior longitudinal fasciculus bilaterally, in the inferior fronto-occipital fasciculus bilaterally, in the uncinate fasciculus bilaterally and in the right tapetum. On the other hand, increased MD is observed in the body and genu of the corpus callosum, in the internal and external capsule bilaterally, in the corona radiate bilaterally and in the right tapetum. The mean percent difference over the significant voxels was -7% for FA, and 6% for MD. The differences in white matter microstructure between healthy subjects and alcoholics a few days after withdrawal follow a complex pattern, which depends on condition and region, with a preferential involvement of frontal and superior fiber bundles, and are most evident in FA.

In rats, one month of excessive alcohol drinking also caused widespread microstructural abnormalities, like reduced FA and increased MD (fig. 4.1A, B right). FA is decreased in the anterior commissure, in the cingulum, in the corpus callosum, in the internal capsule, in the fimbia/fornix, in the dorsal hippocampal commissure, in the cerebral peduncle and in the thalamic radiation. MD is increased in the cingulum and in the corpus callosum. The mean percent difference P over the significant voxels is -6% for FA

and 4% for MD. The nature of these changes is comparable to the human findings and again, most evident in FA.

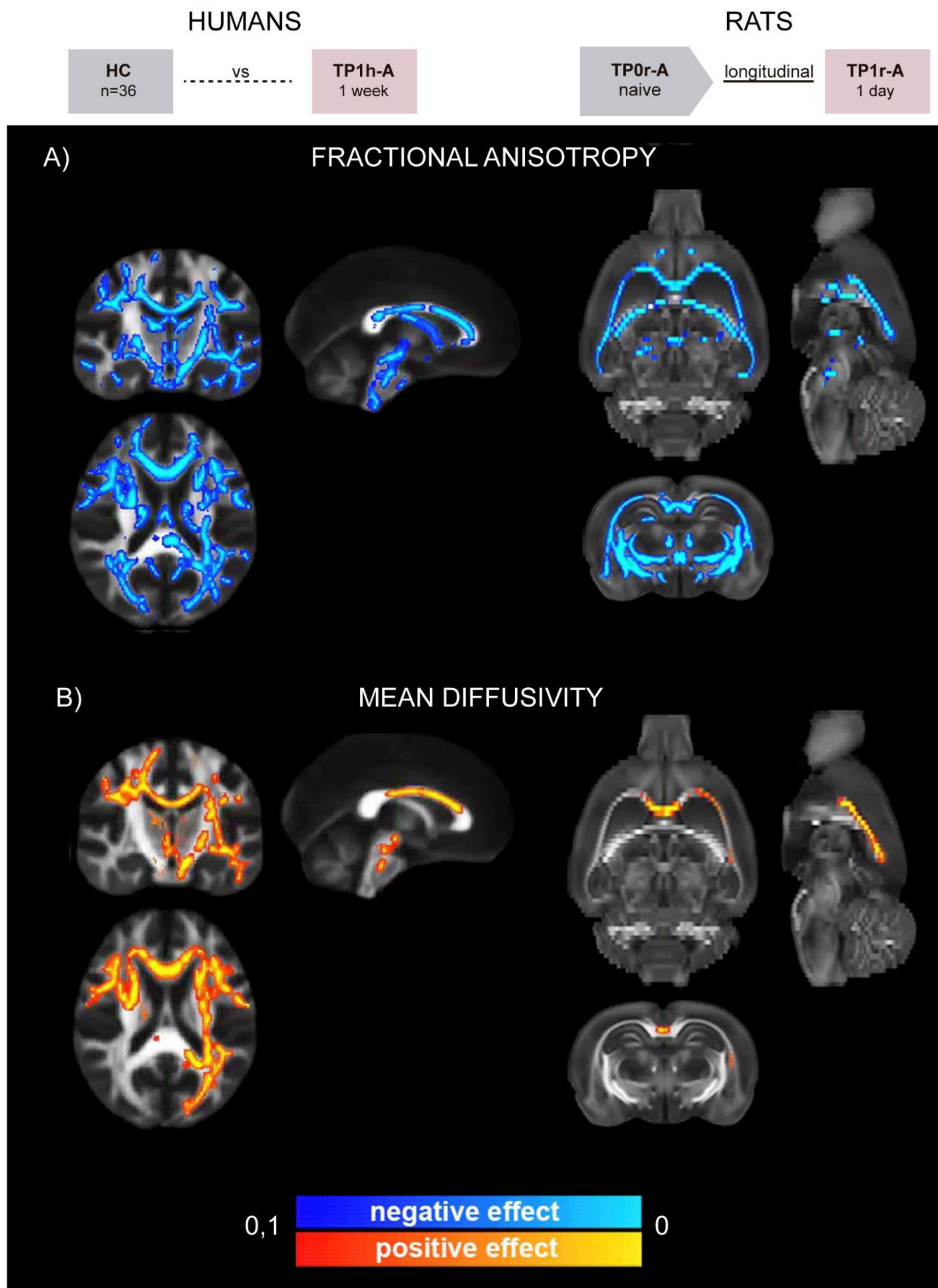


Figure 4.1. Comparable alterations in the white matter whole-skeleton between humans and rats. TBSS analysis shows cross-sectional differences between control and alcohol-dependent

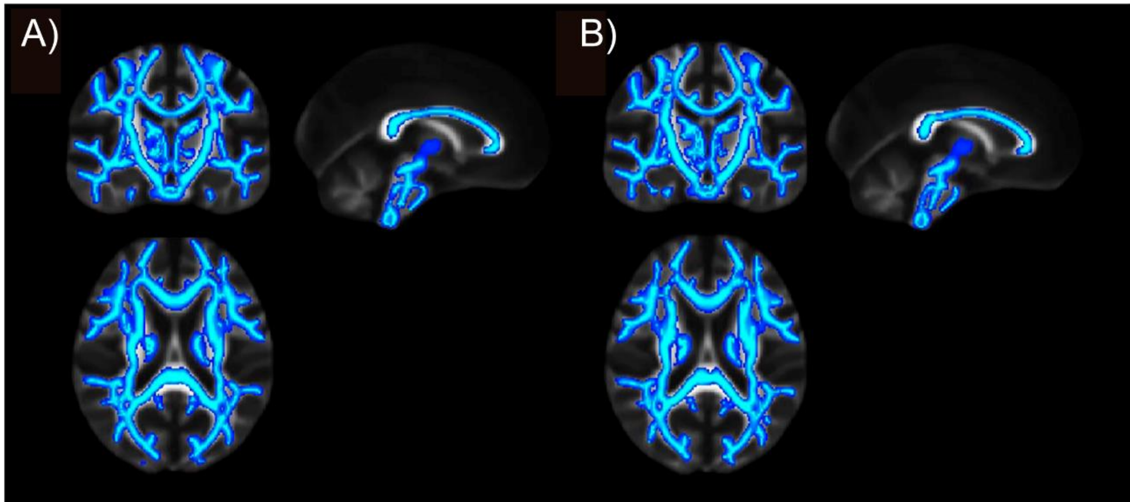
subjects at TP1-A for FA **(A, left)** and MD **(B, left)**, and longitudinal differences between baseline and four weeks of two-bottle free-choice paradigm in rats for FA **(A, right)** and MD **(B, right)**. Thick tracts are significant tracts (P<0.05 corrected), thin tract are points just below p-value threshold (p=0.05-0.1)

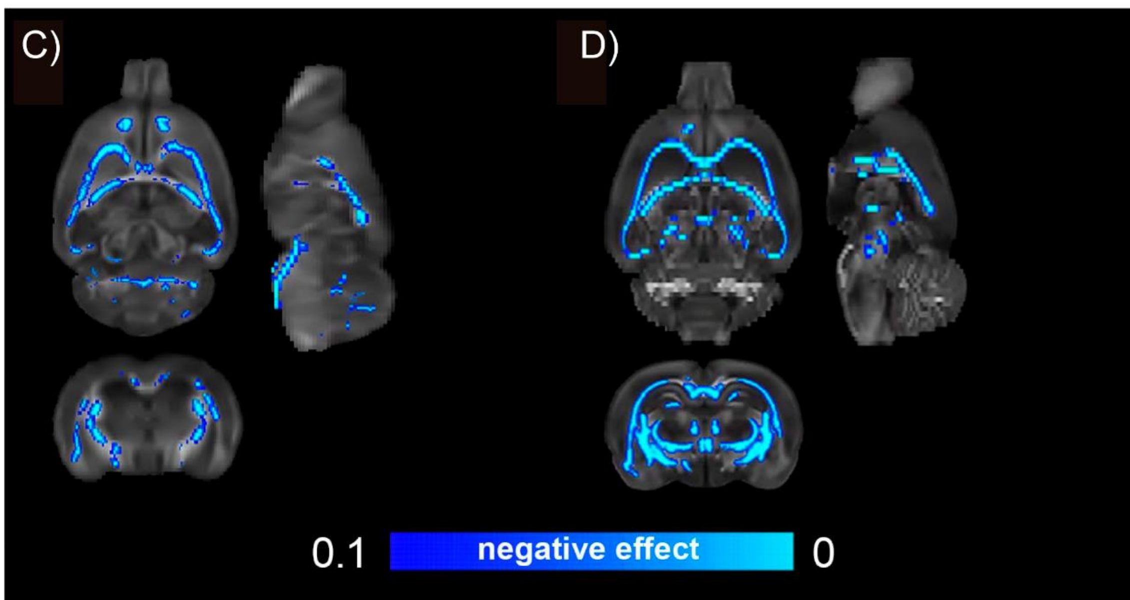
4.2. Microstructural alterations in the white matter whole-skeleton progress along the alcohol abstinence

We next checked how DTI parameters progressed at different time points of abstinence in both species. On one hand, humans were compared after 2-3 weeks of abstinence and after 4-6 weeks. At both time points TP2-A vs TP1-A, and TP3-B vs TP2-B, respectively, we found a pattern of decreased FA (fig. 4.2A, B) and increased MD (fig. 4.2E, F) in most WM tracts. The mean percent change P over the significant voxels for TP2-A vs TP1-A was -6% for FA and 4% for MD; the mean difference for TP3-B vs TP2-B was -7% for FA and 4% for MD. These results indicated that neither FA nor MD did normalize towards levels measured in healthy patients, but rather progressed during early abstinence.

In rats, we compared microstructural alterations measured after 2 weeks and 6 weeks of abstinence against FA and MD measured immediately after alcohol exposure (fig 4.1). We found a significant decrease in FA at TP2-B compared to TP1-B (fig. 4.2C) and an even more widespread decreased FA at TP3-A compared to TP1-A (fig. 4.2D). Differences in MD were less apparent, reaching significant differences at 2 weeks only in the cingulum (fig. 4.2G, H). Compared to the condition measured immediately after alcohol drinking, the mean percentage change P over the significant voxels for TP2-B versus TP1-B was -4%; and for TP3-A versus TP1-A was -0.2%, both for FA. This further progression of microstructural changes away from baseline during abstinence is compatible with the results obtained in humans.

FRACTIONAL ANISOTROPY





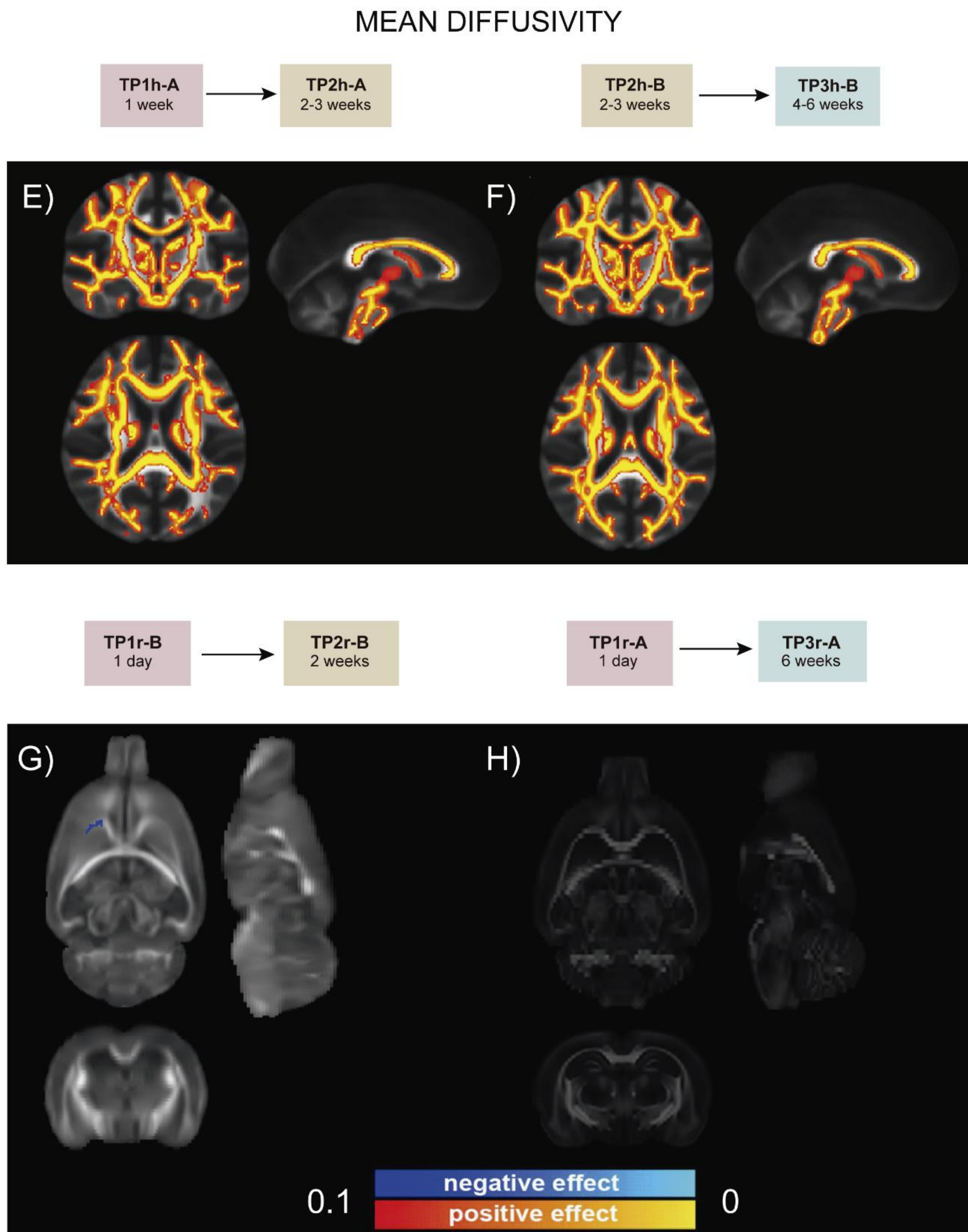


Figure 4.2. Evolution of white matter alterations into early abstinence both in humans and rats. Tract-based statistical analysis shows longitudinal differences in the white matter skeleton between patients in alcohol use disorder (AUD) undergoing diffusion tensor imaging (DTI) at 2 weeks after detoxification (TP2h-A) vs 1 week after detoxification (TP1h-A) for FA **(A)** and MD **(E)**. Also, longitudinal DTI differences between patients in AUD undergoing DTI 4 to 6weeks after admission (TP3h-B) vs 2 to 3 weeks after admission (TP2h-B) for FA **(B)** and MD **(F)**. The same analysis is shown in rats that underwent DTI after 4 weeks of alcohol access (TP1r-B) vs after 2 weeks of abstinence TP2r-B for FA **(C)** and MD **(G)**, and after alcohol access (TP1r-A)

vs after 6 weeks of abstinence (TP3r-A) for FA **(D)** and MD **(H)**. Thick tracts are significant tracts (P<0.05 corrected), thin tract are points just below p-value threshold (p=0.05-0.1)

4.3. Effect size of FA decreases across WM skeleton regions highlights fornix vulnerability.

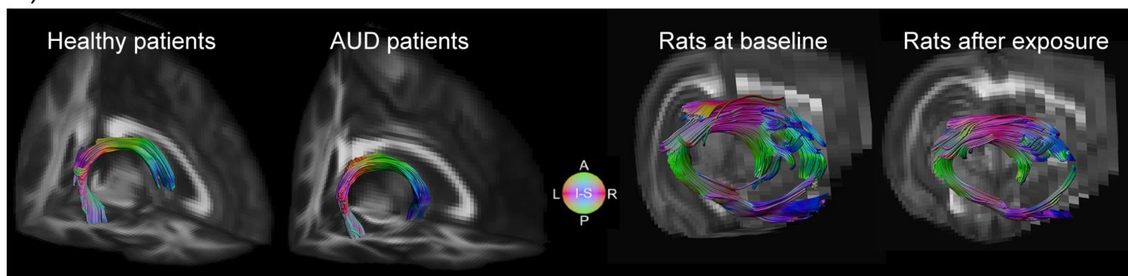
An analysis of effect size was carried out to quantify how alcohol consumption affected FA in individual fiber tracts at 1 and 2-3 weeks of abstinence in patients compared to healthy patients, controlling for age differences at p<0.05 level and corrected for multiple comparisons. Interestingly, the fornix demonstrated the largest reduction in FA in AUD patients compared to control subjects in both cohorts (51% more than the second most affected tract in cohort A, and 54% more than the second most affected tract in cohort B; fig. 4.3A, B). This result uncovered a higher vulnerability of the fornix as compared with other white matter tracts.

Figure 4.3. Effect size of FA alterations in AUD patients. Histograms (mean and standard error) of effect size across all the 50 ROIs of the ICBM DTI 81 Atlas for Cohort A **(A)** and B **(B)**. The asterisk represents significant difference in effect size between groups (*p<0.05).

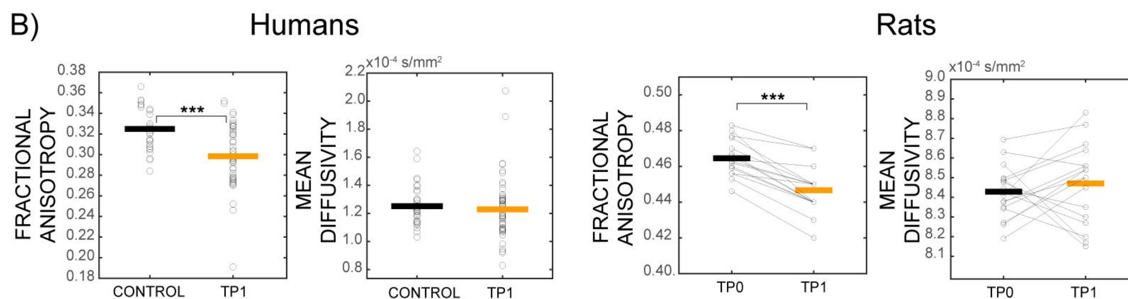
4.4. Continuous alcohol exposure alters the microstructural integrity of the fimbria/fornix tract

Being the fimbria/fornix a vulnerable tract in AUD patients, we applied a tractometry analysis to deeply study its specific alterations after a continuous alcohol exposure (fig. 4.4A). We compared, then, tract-specific DTI parameters (FA and MD) between TP1h-A and controls in humans and TP1r-A and TP0r-A in rats. We found that alcohol exposure was associated with microstructural alterations in the fornix, obtaining a statistically significant reduction in FA (fig. 4.4B) both in humans (d = -0.92), and rats (d = -1.24). Regarding MD, no significant differences were found (fig. 4.4B).

A)



B)



4.4. Fimbria/fornix-specific differences between healthy patients and AUD patients. (A). Example of fimbria/fornix reconstruction in native space for one healthy volunteer, one age- and sex-matched AUD patient, and for one rat both at baseline and after alcohol exposure. The tract is displayed using DTI color conventions, superimposed on the FA maps. **(B).** Values of FA and MD in the fimbria/fornix are shown for humans at the left and for rats at the right. Mean values in each population are reported in blue for control conditions and in orange for alcohol conditions. Asterisks represent significant difference in the ANOVA test statistic, corrected for the false discovery rate (***=P<0.001).

4.5. Alcohol intake is negatively correlated with microstructural alterations

Next, we checked whether the microstructural alterations found in humans were associated with the consumption level. Correlation analysis between the microstructural parameters and alcohol use history in the combined AUD patients from both cohorts (TP2-A TP2-B) unveiled a significant negative association between FA and the mean ethanol daily intake before treatment (fig. 4.5A, B; r^2 = 0,31, p-value <0,0001). Importantly, we found no significant correlations with smoking.

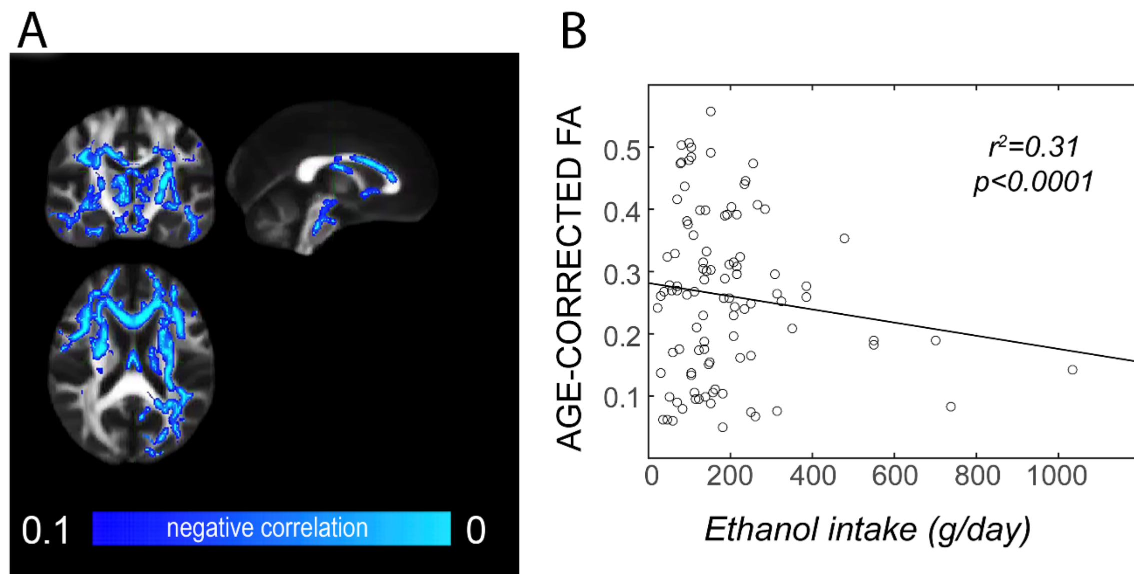


Figure 4.5. Correlation between FA in fimbria/fornix and ethanol daily intake. A) Tract-based statistical analysis of the correlation between FA measure in AUD patients and ethanol intake at baseline, corrected for age. Thick tracts are significant tracts (P < .05, obtained using tbss_fill); thin tracts are points just below P value threshold (P = .05 to P = .10). **B)** Scatterplot between age-corrected FA (using the multiparametric regression $\alpha + \beta \times$ age $+ \gamma \times$ ethanol) and ethanol intake for the fimbria/fornix specifically.

4.6. Chronic intermittent exposure to alcohol alters the microstructure of the fimbria tract.

Having obtained the major damage in the fimbria/fornix both in humans and a genetic model of alcohol-preferring rats, we decided to investigate the issue in a model of alcohol-dependent but wild type rats, the PD rat model.

We investigated the microstructural integrity of the fimbria/fornix tract in PD rats vs. age-matched controls (fig. 4.6A, B). We found significantly lower fractional anisotropy in PD rats (FA; fig. 4.6C; unpaired t-test, $t(29) = 1.88$, $p = 0.035$) with preserved mean diffusivity (MD, fig. 4.6D); unpaired t-test, $p = 0.625$), corroborating previous findings. We also found that the myelin fraction (MF) was significantly smaller in alcohol abstinent animals (Fig. 4.6E; unpaired t-test, $t(28) = 1.90$, $p = 0{,}033$), suggesting demyelination as the underlying mechanism.

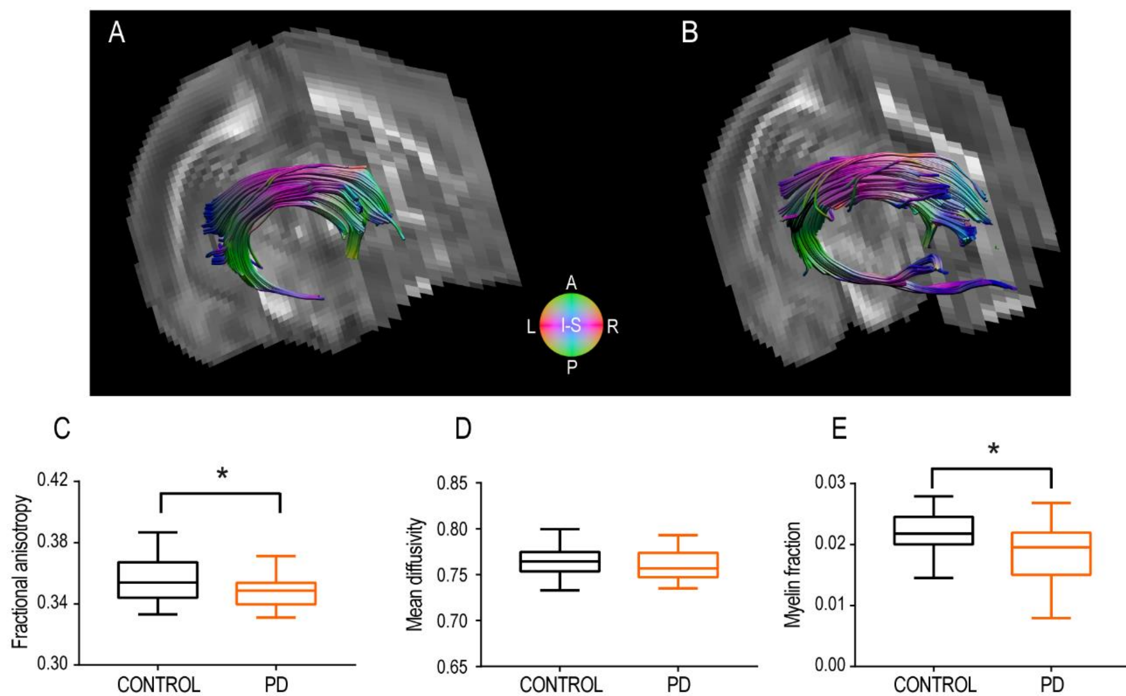


Figure 4.6. Effects of chronic alcohol exposure in DTI parameters measured in the fimbria/fornix. (A, B) Representative DTI reconstruction of the fimbria/fornix in native space for one control rat (A) and one post-dependent rat (B). Both representations are displayed using DTI color conventions, superimposed on the FA maps. **(C-E)**. Group-level values of fractional anisotropy **(C)**, mean diffusivity **(D)** and myelin fraction **(E)** in the fimbria/fornix are shown for control and PD rats. Box-and-whisker plot showing the first and third quartile (box) and min/max values (whiskers). The horizontal line in the box represents the median. The asterisk represents significant difference in the unpaired t-test statistic ($*p<0.05$).

4.7. Myelin basic protein content in the fimbria/fornix is lower in abstinence.

Using the PD rat model, which recapitulates important human features of alcohol addiction and withdrawal, as explained in the introduction, we investigated the mechanisms underlying the found white matter alterations, as well as the potential physiological consequences. We first investigated white matter structure using quantitative immunohistochemistry. Myelin basic protein (MBP) is a component of the myelin sheath commonly used to quantify, based on its staining intensity, the integrity of the white matter (Rice et al., 2019). We immunostained MBP in the fimbria/fornix of the same animals and quantified its content as fluorescence staining intensity (fig. 4.7A). We found significantly lower levels of MBP in the PD rats at two weeks of abstinence compared to the control animals (fig. 4.7B; unpaired t-test, $t(34) = 2.469$, $p = 0.018$). This result confirms the DTI finding and the hypothesis of an alcohol driven demyelination in early abstinence.

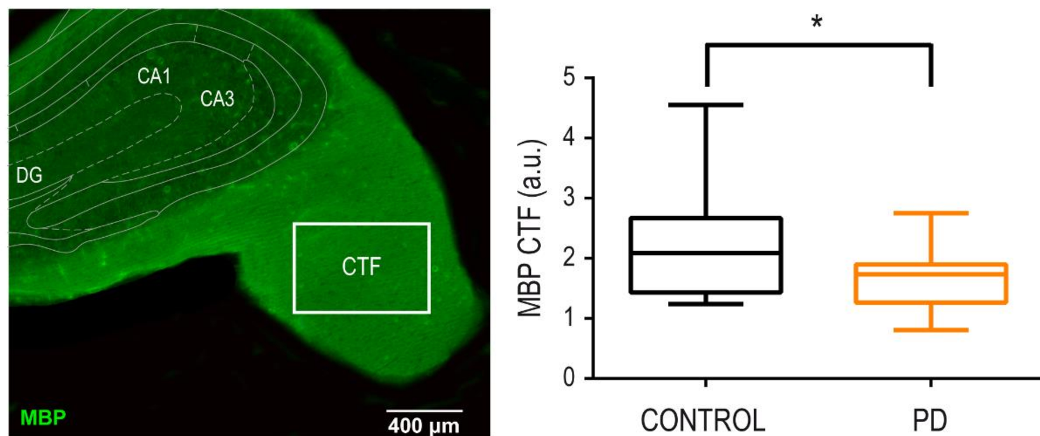


Figure 4.7. CIE-induced alteration of the MBP amount in the fimbria tract. (A). Representative section of the fimbria of a rat labeled for MBP (green) to assess white matter integrity, taken from a control subject. **(B).** Quantification of the MBP corrected total fluorescence (CTF) in control and PD rats and expressed in arbitrary units (a.u.). Box-and-whisker plot showing the first and third quartile (box) and min/max values (whiskers). The horizontal line in the box represents the median. The asterisk represents significant difference in the unpaired t-test statistic (*$p<0.05$).

4.9. Chronic intermittent alcohol exposure increased hippocampal excitability.

We next investigated the synaptic transmission and plasticity in the hippocampus, carrying out multiple *in vivo* electrophysiological recordings using 32-channel electrodes implanted in the hippocampus and spanning CA1 and the DG.

Electrical field potentials recorded in the DG and CA1 evoked by stimulation of the perforant pathway or the fimbria, respectively, demonstrated facilitated principal cell firing in both regions, as measured by the larger amplitude of the PS in abstinent PD rats *vs.* controls (Fig. 4.9B, two-way repeated-measures ANOVA condition effect $F(1, 34) = 4.151$, $p = 0.049$, intensity effect $F(5,170) = 167$, $p<0.0001$, interaction $F(5,170) = 2.49$, $p = 0.033$; and Fig. 4.9E, two-way repeated-measures ANOVA condition effect $F(1, 19) = 6{,}779$, $p = 0.0174$, intensity effect $F(5,95) = 114{,}6$, $p<0.0001$, interaction $F(5,95) = 5.48$, $p = 0.0002$) with comparable EPSPs in both experimental groups (Fig. 4.9C, two-way repeated-measures ANOVA condition effect $F(1, 33) = 1.295$, $p = 0.263$, intensity effect $F(5,165) = 178.2$, $p<0.0001$, interaction $F(5,165) = 0.626$, $p = 0.679$; and Fig. 4.9F, two-way repeated-measures ANOVA condition effect $F(1, 18) = 0{,}1082$, $p = 0.746$, intensity effect $F(5,90) = 54.11$, $p<0.0001$, interaction $F(5,90) = 1.148$, $p = 0.341$). Facilitated PS and constant EPSP reflects enhanced neuronal excitability, rather than enhanced synaptic activity, in the abstinent condition.

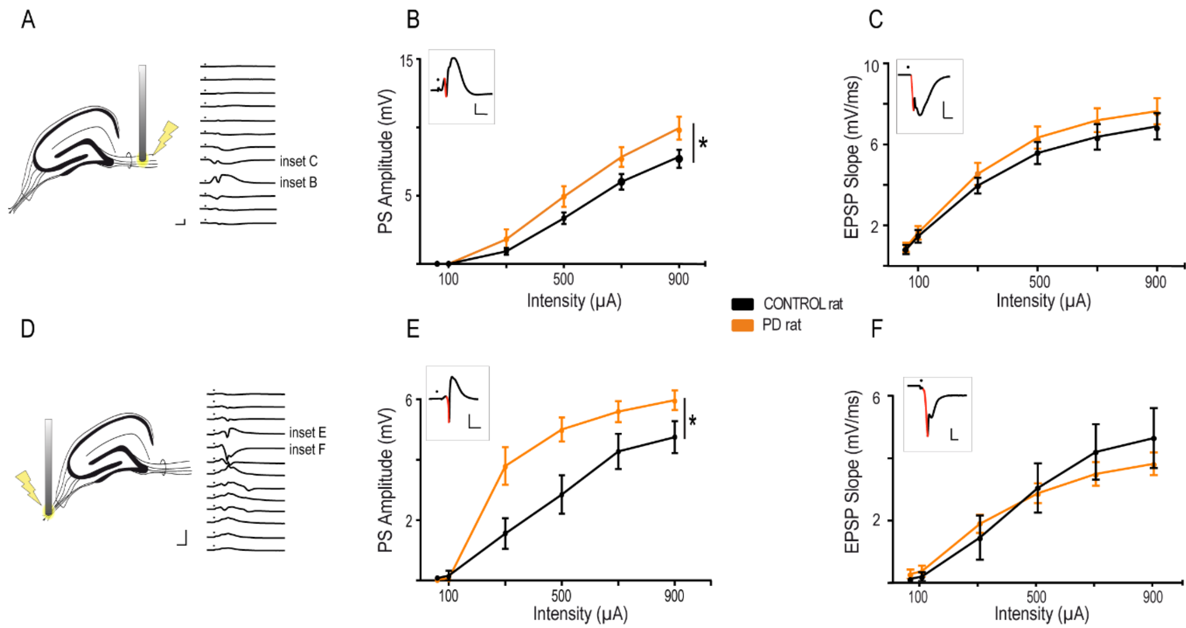


Figure 4.9. Chronic intermittent alcohol exposure increased hippocampal excitability. (A) Representative evoked profile recorded upon stimulation of the perforant pathway. The recording channels used for the group-level quantification are labeled and amplified in the respective insets in panels B,C,E,F. **(D)** same as A but for the fimbria stimulation. **(B,C)** PS amplitudes and EPSP slopes, respectively, recorded in the DG in response to perforant path stimulation of increasing intensities in PD (orange) and control (black) rats. Insets show enlarged representative field potentials highlighting in red the voltage deflections used for quantification. **(E,F)** Same as B,C but for fimbria stimulation and CA1 recordings. Data in B,C,E,F represents mean ± SEM. The asterisk represents significant difference in the two-way repeated-measures ANOVA for PD vs. control groups (*$p<0.05$). Scale bar: A, D: 10mV, 5ms; insets B,C,E,F: 3mV, 5ms.

Increased neuronal excitability can be the result of a change in the intrinsic electrophysiological properties of neurons (Jung et al., 2007), or a decrease in the inhibitory tone (Coulter and Carlson, 2007). We used paired-pulse stimulation protocols to investigate the inhibitory tone in PD animals. In a stimulation pair at 20 ms inter-stimulus interval (ISI), the second synaptic input occurs at the maximal $GABA_A$ conductance driven by the firing of interneurons recruited by the first pulse. The depression of the response to the second pulse relative to the first (fig. 4.10A), is thus proportional to the $GABA_A$-mediated inhibitory tone (Davies et al., 1991). For longer delays, in the range of 50 to 100 ms, a facilitation of the response to the second pulse dominates (fig. 4.10A), which corresponds to a disinhibitory effect mediated by

presynaptic $GABA_B$ receptors (Davies et al., 1991). As shown in fig. 4.10A, we found no statistically significant differences between abstinent PD rats and controls neither for synaptic depression (10-20 ms inter-pulse interval) nor facilitation (50-80 ms), suggesting that the inhibitory tone in the hippocampus was intact during abstinence in this animal model (two-way repeated-measures ANOVA $F(1,24) = 0.003$, $p = 0.950$, latency $F(7,168) = 42.04$, $p < 0.0001$, interaction $F(7, 168) = 0.476$, $p = 0.850$).

Finally, we tested whether long term synaptic plasticity in the hippocampus was affected by a history of chronic intermittent alcohol drinking in the PD rats. We applied a high frequency stimulation protocol known to induce long-term potentiation (LTP) in the perforant pathway (Davis et al., 2000) and measured the amplitude of the PS in the DG before and one hour after LTP induction. We found that LTP in PD animals is indistinguishable from that found in control animals (Fig. 4.10B, two-way repeated-measures ANOVA $F(1,8) = 0.001$, $p = 0.969$, intensity $F(5,40) = 35.76$, $p < 0.0001$, interaction $F(5, 40) = 0.252$, $p = 0.936$). Overall, we can conclude that the inhibitory tone and long-term synaptic plasticity in the hippocampus are not affected during early abstinence in PD rats.

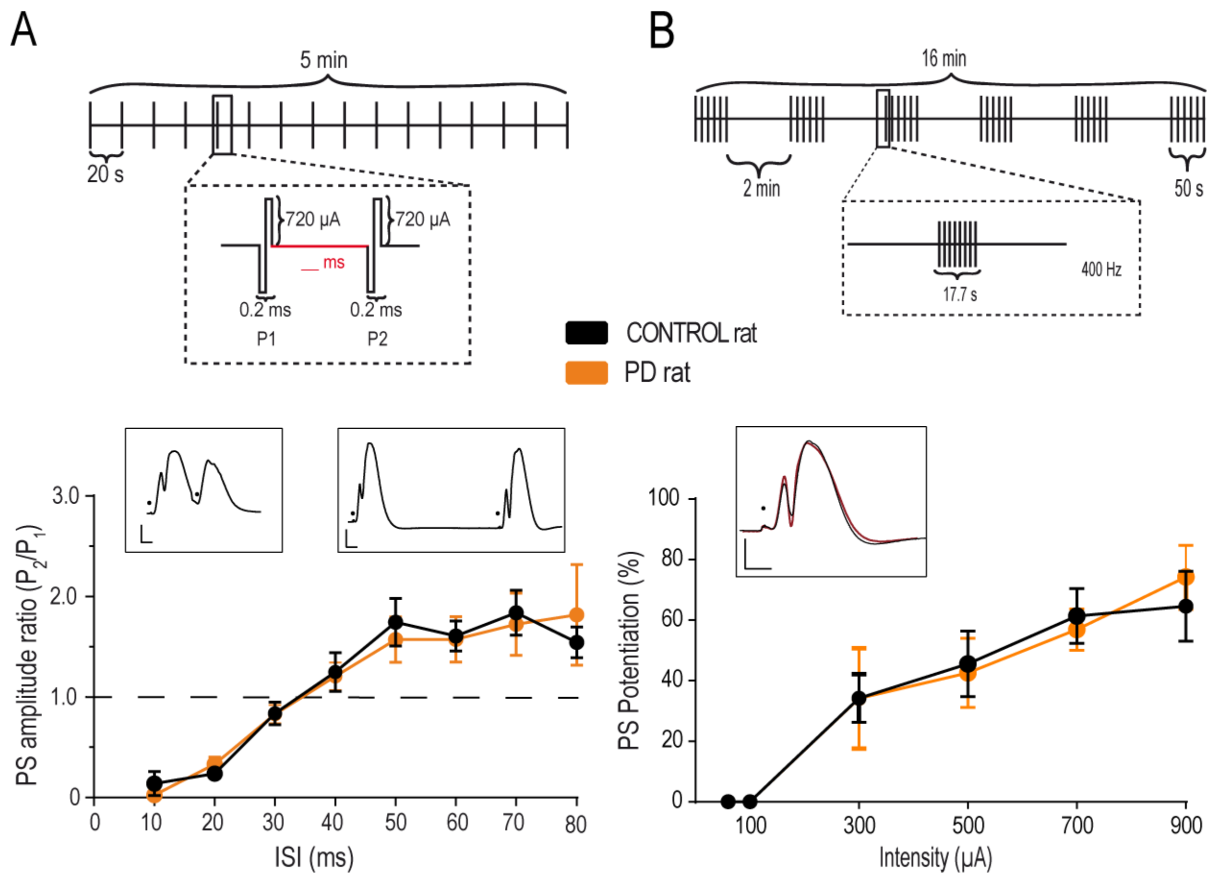


Figure 4.10. Short and long-term synaptic plasticity are preserved in the hippocampus of PD rats. (A) Pair pulse stimulation of the perforant pathway. Suprathreshold and equal intensity pairs of pulses (upper panel) are delivered at varying inter-stimulus time intervals (ISI). The results (lower panel) are expressed as the PS amplitude ratio (PS2/PS1). PS ratios below 1 indicate response depression and *vice versa*. Insets show pair pulse inhibition (left) and facilitation (right). **(B)** LTP of the perforant pathway. The upper panel shows the stimulation protocol used to induce LTP. Lower panel: synaptic potentiation in PD (orange) and control (black) rats quantified as the percentage increase of the PS amplitude after *vs.* before LTP induction. The inset shows representative evoked potentials before (black) and after (red) LTP induction in one control animal. Dots in all insets denote the position of the eliminated stimulation artefacts. Scale bar in both insets: 4mV, 3.5ms.

4.10. Effective connectivity from the hippocampus to the prefrontal cortex is lower during alcohol abstinence.

Finally, we investigated the functional consequences of the structural alterations in the fimbria myelin sheath and the altered hippocampal activity. We investigated the effective connectivity from the HC (CA1 region) to the PFC (prelimbic and infralimbic) using

simultaneous electrophysiological recordings. To do that, we electrically stimulated the most dorsal part of the hippocampal commissure, where efferences from the CA3 region of the hippocampus travel (Wyss et al., 1980) and recorded the evoked potentials in the CA1 region and the PFC. Firing of CA3 neurons reaches the PFC through a polysynaptic activation chain involving CA1 (Wyss et al., 1980, Andersen P., et al, 2007) (fig. 4.11A). A first analysis comparing the amplitude of the PFC evoked potentials showed a significant difference, with larger potentials evoked in control animals (fig. 4.11B, unpaired t-test, $t(21) = 2.344$, $p = 0{,}003$). Then, we quantified the functional coupling between the HC activation and the PFC response, as the propagation ratio obtained by dividing the amplitude of the PFC response by the corresponding amplitude of the CA1 PS. The results demonstrated a reduced propagation ratio in the abstinent rats (fig. 4.11C, unpaired t-test, $t(13) = 2.721$, $p = 0{,}01$). Furthermore, no differences were found in the coherence (fig. 4.11D) or correlation (fig. 4.11E) between spontaneous local field potentials (LFPs) recorded in CA1 and the PFC (fig. 4.11D inset).

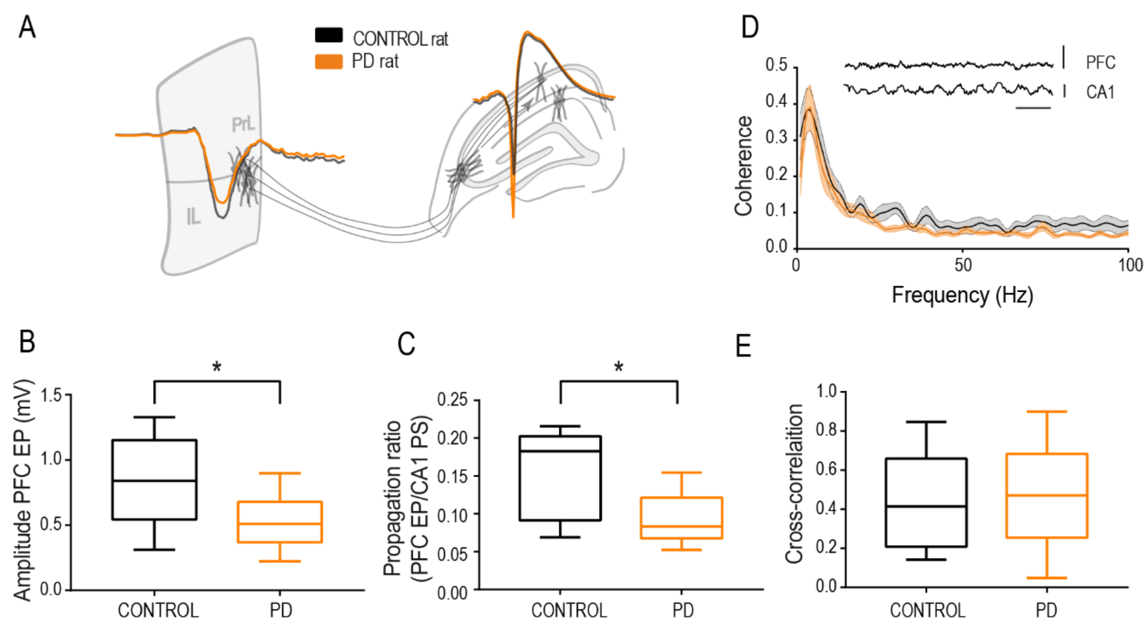


Figure 4.11. Functional connectivity from the HC to the PFC is impaired in PD abstinent rats as compared to controls. **(A)** Representative evoked potentials (EP) in the PFC and PS in CA1 in response to stimulation in control (black) and PD (orange) animals, overlaid on a schematic representation of the respective structures. **(B)** Group level quantification of the amplitude of the evoked potentials in the PFC. **(C)** Effective connectivity between the HC and PFC measured as the evoked response in the PFC divided by the corresponding CA1 PS amplitude

(input/output ratio). Box-and-whisker plot showing the first and third quartile (box) and min/max values (whiskers). The horizontal line in the box represents the median. Asterisks represents significant difference in the unpaired t-test statistic (*p<0.05, **p<0.01). (D-E) Spectral coherence **(D)** and broad band cross-correlation analysis **(E)** between CA1 and PFC spontaneous recordings in control (black) and PD (orange) rats. The inset shows representative spontaneous LFP traces recorded in CA1 (pyramidal layer) and PFC (prelimbic region). Scale bar in spontaneous LFPs: 1mV, 0.5ms.

5. DISCUSSION

5.1. Alcohol consumption impairs microstructural integrity in humans, and it is generalized across rat models

In this thesis, we have uncovered alterations in brain microstructure due to alcohol consumption both in humans and in two rat models, using a non-invasive neuroimaging technique: DTI. The convergence of results between species (i.e., between humans and alcohol-preferring rats) allows us to establish a causal link between alcohol and brain damage, since the experimental conditions in the model are controlled and there are no comorbid factors, as occurs in human patients. Interestingly, both models present a quite similar result being very different from each other. msP rats (alcohol-preferring rats) have been selected generation after generation for a very specific behavioral response to alcohol, ultimately resulting in a very uniform outcome. However, in the case of PD rats, they are wild-type rats that will respond more heterogeneously to alcohol, which translates into a more variable and, therefore, less robust phenotype. Therefore, we can confidently state that there is an effect of alcohol *per se* on the white matter microstructure.

Although the results with both rat models converge, the magnitude of the effects were quite different. A whole-skeleton TBSS analysis in PD rats revealed milder microstructural alterations compared to msP rats (not shown). This may be due to several reasons, the most obvious being genetics. Alcoholism is known to have a genetic component (Edenberg and Foroud, 2013), and indeed it is relevant to the effectiveness of treatment. For example, patients with variations in the mu-opioid receptor gene (OPRM1) respond positively to treatment while those without do not (Kranzler et al., 2013). Epigenetics also appears to produce changes in different cell types as a consequence of chronic alcohol consumption, such as DNA and histone methylation (Ponomarev, 2019). Taken together, this suggests that the genetic component will play a determining role in the individual's drug behavior, which may in turn produce differential effects in the brain.

Further reasons for these differences between models may be age differences, as DTI is sensitive to this factor (Engvig et al., 2012; Sexton et al., 2014), or the anesthesia,

because transient changes in DTI parameters have been shown employing sevoflurane (Tang et al., 2021). Another difference is the intoxication route, being by inhalation in PD rats and oral in msP rats. Considering that in PD rats, intoxication protocol was longer and blood alcohol concentrations achieved higher, but microstructural alterations milder, it suggests that the inhaled route is less harmful than the oral one.

A relevant aspect to be considered in this difference of effects in both animal models is the influence of gut-microbiota over the brain, because by the inhaled via only some portion of the drug is metabolized by the gastrointestinal tract (Rohatagi et al., 1999). Alcohol and its principal metabolite, acetaldehyde, are capable to disrupt intercellular junctions of the epithelium and increase the intestinal permeability (Worthington et al., 1978; Bode and Bode, 2003), giving rise to the release of inflammatory microbial products which, through the bloodstream, reach and alter the brain (Leclercq et al., 2012, 2014). Alcohol abuse drives to a chronic inflammatory state both in humans (Bala et al., 2014; Leclercq et al., 2014), and animal models (Maraslioglu et al., 2014; Pascual et al., 2015; De Santis et al., 2020), which has been seen to correlate with symptoms such as anxiety or cognitive dysfunction in Parkinson disease (Smith and Parr-Brownlie, 2019) or major depressive disorder (Carlessi et al., 2021). This neuroinflammation from the gut could contribute to the inflammatory state generated by alcohol *per se* (as we will mention below), producing stronger brain alterations in rats ingesting alcohol (msP rats).

5.2. Alcohol consumption produces microstructural alterations which progress during the abstinence

Is the damage caused by alcohol consumption recovered when abstinence is started? Nowadays, neither the reasons for relapse nor the mechanism underlying the progression of white matter alterations during withdrawal have been discovered. In late abstinence, specifically during the first year, there is already a neuropsychological improvement in patients (Ende et al., 2005; Fein et al., 2006; Bartsch et al., 2007), and at 6-7 years patients show no differences with healthy subjects when performing in cognitive tasks (Fein et al., 2006). In contrast, early abstinence, which lasts up to two weeks from the

last drink, is the period in which there are more deficits in all psychological domains and in which the probability of relapse is higher. In this period, we have reported translational microstructural alterations in the whole white matter that progress in time (De Santis et al., 2019). This finding has a high clinical relevance given the susceptibility to relapse that patients present during this period.

The interpretation of this progression is challenging because water diffusion is highly sensitive to microscopic changes in the architecture of the brain. Processes such as myelination, axonal density, gliosis or inflammation alter DTI measurements, making difficult to dissect the specific cause underlying the changes. The first work, being translational, required acquisition protocols both short and comparable between species, reason by which we were unable to obtain more specific measures such as myelin fraction. The second work performed in PD rats suggests that a loss of MBP could be partially contributing to such damage progression. A possible mechanism explaining these alterations could be an inflammatory process (which we will discuss later), which is able to increase the mean diffusivity in the gray matter of alcoholic rats (De Santis et al., 2020), and has been shown in AUD models (Montesinos et al., 2016; Guerri and Pascual, 2019).

As our effect size analysis reveals and in agreement with other literature (Fortier et al., 2014), alcohol does not impact white matter uniformly but some tracts are more vulnerable than others. We have reported microstructural alterations in the fimbria/fornix at 10 days of abstinence in PD rats, with no changes in the perforant pathway (not shown). This differential impairment in white matter is interesting, but its explanation unknown, although intrinsic properties of the tracts may probably play a role. This will be an aspect to explore in the future, making a whole-skeleton comparison of the myelination properties, the tract length and diameter, the genetic differences between them, or even their plasticity capability.

5.3. Myelin content in the fimbria/fornix is decreased in alcohol abstinence

Once we uncovered the alteration in the microstructural integrity as a consequence of the alcohol consumption, we tried to discern the neurobiological substrate. Our hypothesis was that alcohol consumption produces demyelination in white matter (Pascual et al., 2014; Vargas et al., 2014; Rice and Gu, 2019; Guo et al., 2021), and we tested it by combining MRI with immunohistochemistry.

Myelin fraction (MF) obtained with dw-MRI represents water restricted to the myelin sheaths. Our results showed a decrease in the MF of PD rats, supporting our hypothesis. Some work has suggested that MF from rodent spinal cord is not able to give a sufficiently accurate measure of myelin, due to the rapid exchange of water molecules (Dula et al., 2010; Harkins et al., 2012). However, these authors employed acquisition protocols that were slow enough to not capture this kind of biological processes. Moreover, in humans, MF was shown to be more sensitive to myelin variations than to water exchange rates (Kalantari et al., 2011), supporting the validity of our finding.

Although MF can be a good biomarker for myelin, it still is an indirect measure which could be influenced by other factors. Therefore, we performed a direct measure of the myelin compartment: immunohistochemistry against Myelin Basic Protein (MBP). This is the second most abundant protein in myelin, and is indispensable for its assembly (see Boggs, 2006 for a review). MBP is altered in neurodegenerative diseases, as Alzheimer (Zhan et al., 2015) and multiple sclerosis (Kuhlmann et al., 2017), and during aging (Ansari & Loch, 1975) but, to our knowledge, MBP has not yet been conducted with immunohistochemistry on brain tissue from alcoholic patients. It has been studied with Western blot, obtaining a decrease of this protein in the cortex of patients (Lewohl et al., 2005) but in general the study of myelin in human tissue is challenging due to the rapid deterioration of myelin membranes after death. Additionally, comorbidities have shown their own impact on the myelin, such as the co-abuse of ethanol and tobacco (Papp-Peka et al., 2017), masking the alcohol-specific alterations.

Therefore, immunohistochemistry is more employed in animals to test myelin. Typically, a decrease in MBP antibody fluorescence is interpreted as myelin alteration (Weil et al., 2016; Rice et al., 2019). Recently, it has been shown a reduction of this protein in binge-drinking mice in the gray matter both of hippocampus and prefrontal cortex (Rice et al., 2019). Interestingly, our results showed a decrease in the fluorescence of MBP during the early abstinence in the fimbria fornix, the pathway that is connecting both areas, so it appears that an early demyelination underlies the finding of DTI in PD rats.

A potential mechanism responsible for early demyelination is neuroinflammation. It has been demonstrated that alcohol consumption predisposes to infection and affects the inflammatory and immune system (Macgregor, 1986) by numerous ways (Blanco and Guerri, 2006). An important point is that astrocytes respond directly to alcohol, contributing to an inflammatory scenario (Blanco et al., 2004; Vallés et al., 2004) where macrophages both phagocyte the myelin sheaths (Bruck et al., 1995) and produce reactive oxygen species (ROS), which also produce demyelination (Konat and Wiggins, 1985). Additionally, ethanol affects oligodendrocytes and their development (see Rice & Gu, 2019 for a review), and it is able to alter the lipid membranes (Peoples et al., 1996), which ultimately may lead to myelin destruction (Ohler et al., 2004).

The proliferation of inflammatory cells (e.g. microglia, astrocytes or endothelial cells) and their increase in size as a consequence of a noxious event could be interpreted as an increase in physical barriers and thus a difficulty in water diffusion (and vice versa). In alcohol-preferring rats it has been shown that a continuous period of alcohol consumption increases mean diffusivity in the parenchyma and decreases the number and complexity of microglial cells (De Santis et al., 2020). They also tested how the state of these cells evolves after 10 days of abstinence, and showed that the number of cells recovered but not their morphology, retaining an ameboid phenotype. Therefore, our hypothesis is that the progression of microstructural alterations throughout abstinence found in this thesis could be produced by changes in the inflammatory system. Further research is needed to characterize the inflammatory process, under what circumstances it occurs and how they

affect the evolution of this disease (a pro-inflammatory vs. an anti-inflammatory profile). If there are different inflammatory phases along abstinence according to the patient's history of alcoholism, for instance, these could serve as biomarkers to prevent and/or predict the evolution of the disease.

5.4. Excitation-inhibition balance impaired in hippocampus without plasticity alterations during alcohol abstinence

Although more homogeneous than human populations, animal models can also vary, especially regarding alcohol intake protocols (acute *vs.* chronic of different durations, inhalation vs drinking), disease stage (during alcohol intoxication *vs.* abstinence) and experimental condition (*in vitro vs. in vivo*). It has been demonstrated, for instance, that continuous consumption affects differently the brain compared to intermittent one (Nelson et al., 1999; O'Dell et al., 2004), because several intoxication cycles with different withdrawal phases promote voluntary alcohol intake and more signs of physical dependence (Rimondini et al., 2002, 2003). It is important to keep this in mind when comparing studies, because different preparations can lead to different results.

Alcohol has a wide variety of direct targets on which it can cause chronic changes with continued use of the drug (see Lovinger & Roberto, 2012 for a review). For example, one of the receptors affected are NMDA receptors, since alcohol blocks their release of glutamate (Lovinger et al., 1989) and another neurotransmitters such as dopamine, norepinephrine, and acetylcholine (Göthert and Fink, 1989; Woodward and Gonzales, 1990), as well as decrease NMDA-induced Ca^{2+} flow (Leslie and Weaver, 1993; Hoffman et al., 1989). Within 10 hours of alcohol intake, glutamate increases, which is related to the hyperexcitability and seizures typical of acute withdrawal (Fadda & Rossetti, 1998). Alcohol also inhibits AMPA/kainate receptors (Dildy-Mayfield and Harris, 1992), binds to GABA receptors and enhances their activity (Tatebayashi et al., 1998), and affects indirectly G-protein signaling in various ways (Lovinger & Roberto, 2012). All this may have an indirect impact on other phenomena at different scales, such as synaptic plasticity or the transmission of information between different brain nuclei.

In this thesis, we have investigated the damage of the hippocampus by quantifying some electrophysiological properties. Thanks to multichannel recording electrodes, we were able to study concomitantly the activity of the DG and CA1, obtaining in PD rats an increased excitation/inhibition balance in both regions without changes in the synaptic plasticity. Focusing on DG, Abraham et al 1984 made a similar preparation in anesthetized-rats after 8 weeks of abstinence and 20 weeks receiving alcohol in the diet continuously (Abraham et al., 1984). They found a significant decrease in PS amplitude with no difference in EPSP and, similar to our results, no significant effect on short- and long-term plasticity. However, in addition to having different times of both alcohol exposure and abstinence, the comparison of alcohol consumption was made with a group of rats receiving sucrose. It is known that sugar produces modifications in the brain that increase the preference for alcohol (Dorofeikova et al., 2018) and cocaine (Carroll et al., 2007) in rats, so we can hardly compare this study with ours, whose control group received water. Durand and Carlen (1984) studied in vitro the response of DG after a shorter abstinence stage (3w, more similar to our period), though the control group received a maltose dextrin diet (Durand and Carlen, 1984). Alcohol exposure reduced the amplitude of intracellularly recorded inhibitory postsynaptic potential (IPSP) without differences in other membrane parameters such as EPSP amplitude. This is suggesting that chronic alcohol is affecting presynaptic release by affecting the GABAergic neurotransmission. However, applying a paired-pulse stimulating protocol in the perforant pathway we inferred an intact feed-forward inhibition. An optimal function of inhibitory neurons is considered extremely important for the proper performance of the dentate gyrus, for instance to avoid epileptic events (Trevelyan et al., 2006, 2007), reason by which a quick recovery of the inhibitory tone during alcohol withdrawal sounds reasonable.

Compared to dentate gyrus, substantially more electrophysiological research has been focused on CA1, stimulating the schaffer/commissural pathway. In 1981, Abraham et al employed a protocol of alcohol in the diet and 8-weeks of abstinence in rats. They performed *in vivo* experiments using urethane anesthesia and again compared alcohol-

fed rats vs. sucrose-fed rats (Abraham et al., 1981). They discovered a significant increase in the short-term plasticity due to the chronic alcohol exposure only in the PS, without significant changes in the EPSP, supporting the previously mentioned decreased recurrent inhibition in this area. Later, a research group studied in depth the consequences of intermittent alcohol consumption (protocol very similar to ours) in the CA1 area by *in vitro* electrophysiology. Stimulating the schaffer/collateral pathway, this group found a reduction both of the PS and EPSP one hour after the end of intoxication, with no change in the short-term plasticity (Nelson et al., 1999) but an impairment in the long-term plasticity (Roberto et al., 2002). One week later, there was a recovery of the LTP but only in the dendritic region (EPSP; Roberto et al., 2002), matching with our differences between neuronal compartments in CA1 and DG.

A possible explanation at the circuit level may be a cellular reorganization. For example, after 18 months alcohol consumption in the diet, rats show death of CA3 neurons and a reduced number of synapses between these cells and mossy fibers (which are the axons of DG cells). However, MF show an increased number of synaptic contacts, that is considered a compensatory mechanism for cell death (Cadete-Leite et al., 1989). In our case, a circuit between MF and hilar interneurons could disinhibit the DG. There could be also enhanced extrahippocampal projections due to the alcohol consumption contacting CA1 and DG principal cells and increasing their activity. On the other hand, we cannot discard an alteration in the inhibitory function with our results, which could be studied in more detail with *in vitro* electrophysiology. More investigation is needed to understand how these plastic changes occur and their impact on cognitive capabilities.

5.5. Effective connectivity in the fimbria/fornix

The fimbria/fornix is the pathway connecting prefrontal cortex and hippocampus, as well as other brain regions (Jay and Witter, 1991; Croxson et al., 2005; Cenquizca and Swanson, 2007). As mentioned above, the hippocampus is divided into three portions in the septotemporal axis: dorsal, intermediate, and ventral. Ventral/intermediate CA1

neurons contact directly (monosynaptically) with prefrontal cortex neurons, whereas dorsal CA1 neurons must make polysynaptic contact with other regions before reaching prefrontal cortex (Fanselow and Dong, 2010; Tannenholz et al., 2014). Functional connectivity commonly refers to statistical interdependence between two time series (Jenkinson, M and Chappell M, 2017), measured in terms of correlation or coherence between signals, and it is usually assessed using techniques like fMRI or EEG (Bullmore and Sporns, 2009). However, these analytical tools cannot assess causal relations between the signals nor directed interactions. Some studies have applied techniques like Granger causality or transfer entropy to investigate causal interactions (López-Madrona et al., 2019). However, the main limitation of these techniques is that, in the absence of a full representation of the interacting regions, something complicated in the brain, a clear causality cannot be inferred as activity can be routed through hidden (non-recorded) nodes in the network. In this thesis, we took advantage of electrically evoked potentials to study directed interactions from the hippocampus to the prefrontal cortex, referred here as effective connectivity. With this technique, electrically evoked activity propagates in the network leaving a signature in the recorded field potential that can be causally linked to the neurons activated by the stimulus. Stimulating in the perforant pathway or the fimbria and recording in the HC and the PFC, we demonstrated an alcohol-driven decrease in effective connectivity between both structures. We hypothesize that the alteration in the fimbria white matter translates into a decrease in effective connectivity from the HC to other brain regions, including the PFC. This alteration may underlie a number of cognitive deficits found in AUD patients, including those we have reported.

As explained in the introduction, reward-associated learning is a fundamental component of the addiction cycle (Koob et al., 2014). By virtue of this process, the subject acquires information about stimuli, actions and contexts (Hyman et al., 2006). It is also well known that the HC, and more specifically the hippocampal projections to PFC, has a key relevance for cue-reward learning (Burton et al., 2009; Benchenane et al., 2010). For instance, it has been shown that the characteristic anticipatory firing produced in the PFC in response to a reward appearance (Goldman-Rakic, 1995; Leon and Shadlen, 1999;

Constantinidis and Qi, 2018) is actually governed by hippocampal activity (Burton et al., 2009). Also, HC and PFC functional coupling was shown to increase in the acquisition of a new learning rule, especially if it was reward-related (Benchenane et al., 2010). Furthermore, this connection is critical for the extinction of learnt associations, once they have lost their adaptative value (Holmes et al., 2012). For instance, the extinction of a fear-context association when the fear stimulus has disappeared relies on an intact hippocampal-PFC function (Ji and Maren, 2007; Corcoran and Quirk, 2007; Garcia et al., 2008; Sotres-Bayon et al., 2012). Finally, in the laboratory it has been shown previously that synaptic plasticity in the HC enhances the connectivity of this region with the PFC and NAcc (Canals et al., 2009; Alvarez-Salvado et al., 2014; Moreno et al., 2016; Del Ferraro et al., 2018) important regions for the development of addiction (Koob and Volkow, 2010; Volkow and Morales, 2015). Overall, these evidences suggest that a functional and/or structural alteration in the fimbria/fornix might be crucial for the cognitive pathology found in alcoholic subjects.

Several psychiatric disorders share an impairment in the HC-PFC communication, such as schizophrenia, major depression or post-traumatic stress disorder (Godsil et al., 2013). Additionally, this pathway appears especially vulnerable to stress, since it impairs working memory (Murphy et al., 1996; Mizoguchi et al., 2000) and causes short, medium- and long-term changes within HC and PFC (Rocher et al., 2004; Cerqueira et al., 2007; Pittenger and Duman, 2008; Yuen et al., 2009). In turn, hippocampal communication with prefrontal cortices is critically important for stress regulation (Sheline et al., 1996; Rocher et al., 2004; Vouimba and Richter-Levin, 2005; Kogan and Richter-Levin, 2008; Pittenger and Duman, 2008), which could support a relationship between fimbria/fornix alteration and relapse proneness (Sinha, 2001). On the other hand, the mental rigidity typical in AUD patients, which has a relevant role in adjusting the self-behavior in a changing environment (Grant and Berg, 1948; Teichner et al., 2001), is tightly associated to alterations in the PFC (Dao-Castellana et al., 1998; Li et al., 2009).

In summary, we suggest a more important role of the hippocampus in the addiction cycle (fig. 5.1) than commonly attributed (Koob et al., 2014). At the beginning of a protracted

alcohol-consumption period and with an intact fimbria/fornix, hippocampal-PFC connections participate in the encoding and consolidation of cue-reward associations. These associations contribute to positive reinforcement and the establishment of appetitive alcohol-related behaviors. With the repetition of the consumption, microstructural alterations in the white matter start to appear, with vulnerable tracts being more affected. Alterations in the fimbria/fornix impact learning abilities, preserving consolidated memories but impairing their correct update or extinction in response to environmental contingency changes. Even in the presence of a reduced rewarding effect, habit formation and maladaptive memories drive the behavior towards consumption. Behavioral options are narrowed down to alcohol-related actions whose association with external stimuli or cues are strongly consolidated in memory. Finally, the subject becomes cognitively rigid and stress-prone, being unable to change the valence that alcohol-cues have. Altogether, makes the subject vulnerable to relapse (Sinha, 2001; Van Gucht et al., 2010; Elkins et al., 2017).

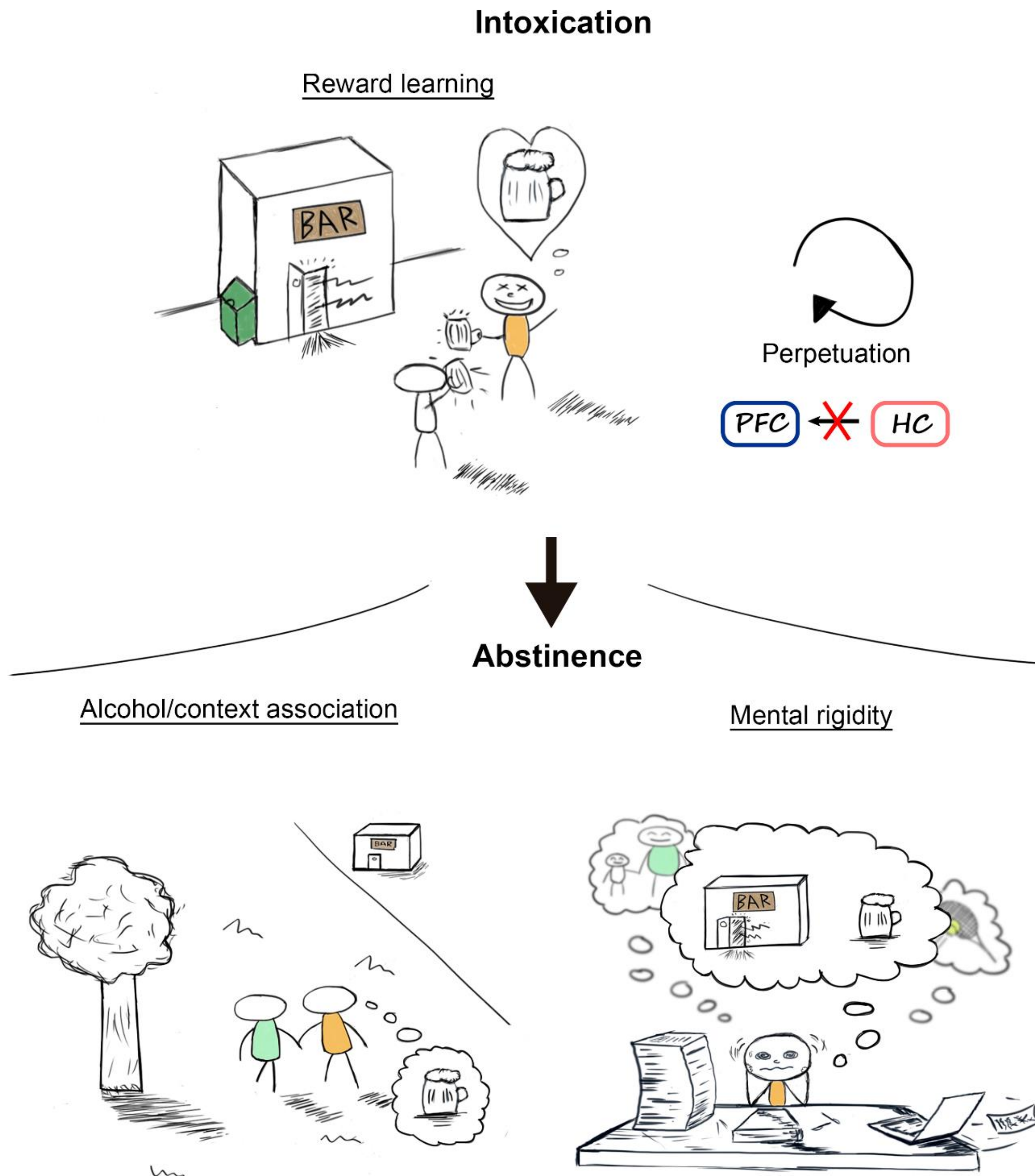


Figure 5.1. Relevance of the hippocampus-prefrontal cortex connectivity for the development of some typical features of AUD patients. Repeated alcohol consumption leads to an alteration in the connectivity between the hippocampus and the prefrontal cortex, promoting behaviors that perpetuate the addiction cycle. For example, the association of alcohol with certain contexts where the consumption took place, or mental rigidity, which emphasizes the pleasurable memories associated with the drug over other activities. All these actions foster the craving and, finally, triggers the relapse.

5.6. Limitations

One limitation of the dw-MRI technique is the contamination of white matter measurements by the proximity of the ventricles and the imaging resolution limit creating partial volume effects. The fornix is in close contact with the third ventricle and therefore the free-water diffusion properties of the cerebrospinal fluid may have contaminated FA measurements.

It is also important to note that an alteration in the fimbria/fornix could also affect communication between other structures not studied here. With respect to the HC, for example, its connections with the septum and nucleus accumbens could also be compromised, which would add to the decreased effective connectivity with PFC further compromising HC function in abstinence.

Finally, the present study was performed in males. However, important sex differences to alcohol exposure have been found (Agabio et al., 2017; Fama et al., 2020) that warrant further work comparing fimbria/fornix vulnerability in males and females.

5.7. Concluding remarks

In the present work we have shown that alcohol consumption produces microstructural alterations comparable between humans and rats, which additionally progress in the early abstinence phase. Furthermore, we have demonstrated that the most vulnerable tract to this alcohol effect is the fimbria/fornix, an important pathway for learning, memory and behavioural flexibility. A more detailed and mechanistic investigation of this finding using the animal model unveiled a reduction in the staining intensity of MBP in this fiber tract, suggesting an alcohol-driven demyelination. Concomitant electrophysiological recordings demonstrated altered hippocampal physiology, with an increased excitation/inhibition ratio, and the functional decoupling between the hippocampus and the prefrontal cortex. We propose that through the described mechanism, the

fimbria/fornix vulnerability could critically contribute to several cognitive alterations found in AUD patients.

5.8. Future steps

1. To unveil why the fimbria/fornix is the most vulnerable tract in this disease.
2. To discover what is the biological mechanism inducing the progression of the microstructural alterations found in this thesis.
3. To deepen in the relationship between structural and functional alteration, with the aim of obtaining an early biomarker to prevent the neurophysiological maladaptations produced by this disease.

6. CONCLUSIONS

1. Convergent evidence from humans and two different rat models of AUD indicate that alcohol drinking is sufficient to cause microstructural changes in the white matter.

2. White matter microstructural alterations progress during early abstinence (at least 6 weeks in rats and 4-6 weeks in humans).

3. Demyelination contributes to the microstructural changes found with MRI in the fimbria/fornix of the PD rat model.

4. Electrophysiological analysis indicates increased excitability in the hippocampus, in the absence of alterations in synaptic plasticity nor inhibitory tone.

5. The effective connectivity from the hippocampus to the PFC is decreased by 1 month of alcohol drinking and concomitant with the microstructural alterations found in the fimbria/fornix.

CONCLUSIONES

1. Resultados de humanos y de dos modelos diferentes de ratas con trastorno por consumo de alcohol indican que el consumo de alcohol es suficiente para causar cambios microestructurales en la materia blanca.
2. Las alteraciones microestructurales de la materia blanca progresan durante la abstinencia temprana (al menos a las 6 semanas en ratas y a las 4-6 semanas en humanos).
3. La desmielinización contribuye a los cambios microestructurales de la fimbria/fornix encontrados con la resonancia magnética en el modelo de rata postdependiente.
4. Los análisis electrofisiológicos indican un aumento de la excitabilidad en el hipocampo, sin cambios en la plasticidad sináptica ni en el tono inhibitorio.
5. La conectividad efectiva del hipocampo con la corteza prefrontal está disminuida tras un mes de consumo de alcohol, y coincide con las alteraciones microestructurales encontradas en la fimbria/fornix.

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