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MASTER IN NEUROBIOLOGY

Alcohol intake triggers aberrant synaptic pruning and synapse loss

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Agradecimentos

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Abstract

Abstract

Alcohol use continues socially well accepted in modern society, although its abuse adversely impacts the life of millions of people worldwide. Following alcohol intake, microglia become activated, dramatically changing their transcriptomic signature and releasing pro or anti-inflammatory mediators to restore brain homeostasis. However, exacerbated or prolonged microglial activation may result in neuronal impairment caused by excessive production of proinflammatory mediators, culminating in neuronal damage.

Deficits in synaptic transmission is a common finding in human alcohol users and in animal models of alcohol intoxication. Here we used genetic ablation of Tnf and conditional ablation of microglia to assess their influence on synapses following alcohol intake. Our paradigm of moderate alcohol intake over ten consecutive days resulted in substantial loss of excitatory synapses in the prefrontal cortex, a consequence of aberrant synaptic pruning. Additionally, *in vitro* assays using a microglial cell line corroborate that alcohol exposure increase their capacity to engulf synaptic structures.

Overall, our data suggest that both TNF and microglia ablation attenuate aberrant synaptic pruning preventing excitatory synapse loss, restraining the disruption of synaptic transmission during alcohol use.

Keywords: CNS, microglia, alcohol, synaptic pruning, synapses loss

Resumo

Resumo

O consumo de álcool continua a ser socialmente bem aceite na sociedade moderna, apesar do seu consumo abusivo influenciar de forma negativa a vida de milhares de pessoas em todos o mundo. Durante o consumo de álcool, a microglia transita para um estado ativo, alterando drasticamente o seu transcriptoma e liberta mediadores pro ou anti-inflamatórios para reestabelecer a homeostasia do cérebro. No entanto, a ativação exacerbada ou prolongada da microglia pode levar ao comprometimento sinático causado pela excessiva produção de mediadores proinflamatórios, culminando em danos neuronais.

Défice na transmissão sinática é comummente encontrada em humanos que consomem álcool assim como em modelos animais de intoxicação com álcool. Neste estudo recorremos a modelos de ablação do Tnf e da microglia de modo a estudar a sua influência nas sinapses durante o consumo de álcool. O nosso paradigma de consumo moderado de álcool durante dez dias consecutivos resultou na perda substancial de sinapses excitatórias no córtex pré-frontal, como consequência da supressão aberrante de sinapses. Adicionalmente, foi feita uma análise *in vitro* usando uma linha celular de microglia pra corroborar a sua capacidade de englobar estruturas sináticas.

No geral, os nossos dados sugerem que tanto a ablação do TNF como a da microglia atenuam a aberrante supressão sinática, prevenindo a perna de sinapses excitatórias, contendo a disrupção da transmissão sinática durante o consumo de álcool.

Palavras-chave: CNS, microglia, álcool, disrupção sináptica, perda de sinapses

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List of Abbreviations

AD – Alzheimer's Disease

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate

ATP – Adenosine Triphosphate

AUD – Alcohol Use Disorder

BBB – Blood Brain Barrier

BDNF – Brain-Derived Neurotrophic Factor

BSA – Bovine Serum Albumin

C1q – Complement component 1q

C3 – Complement component 3

CNS – Central Nervous System

CR3 – Complement Receptor 3

CT - Control

DAM – Disease-associated Microglia

DAMP – Damage-associated Molecular Pattern

DAP12 – DNAX Activation Protein of 12kDa

DGAV – Direção Geral de Alimentação e Veterinária

DMEM – Dulbecco's Modified Eagle Medium

DT – Diphtheria Toxin

DTT - Dithiothretiol

EMPs – Erythromyeloid Percursors

EtOH - Ethanol

EYFP – Enhanced Yellow Fluorescent Protein

FACS – Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

GABA – Gamma-AminoButyric Acid

GABA R - Gamma-AminoButyric Acid Receptor

GFAP – Glial Fibrillary Acidic Protein

H_2O - Water

HD – Huntington's Disease

IFN-ɣ - Interferon Gamma

IGF-1 – Insulin-like Growth Factor

IL-1β – Interleukin 1β

IL-6 – Interleukin 6

IL-10 – Interleukin 10

IL-12 – Interleukin 12

LTP – Long Term Potentiation

MAPK – Mitogen-activated Protein Kinase

NF-kB – Nuclear Factor k B

NLR – Nucleotide-binding Oligomerization Domain-like Recaptors

NMDA – N-Methyl-D-Aspartate

NMDA R - N-Methyl-D-Aspartate Recptor

NPCs – Neural Percursor Cells

P2RY12 – P2Y Purinoreceptor 12

PAMP – Pathogen-associated Molecular Pattern

PBS – Phosphate-buffered Saline

PD – Parkinson's Disease

PFA – Paraformaldehyde

PRR – Pattern Recognition Receptor

RIPA Buffer – Radioimmunoprecipitation Assay Buffer

ROI – Region of Interest

ROS – Reactive Oxygen Species

TGF-β – Transforming Growth Factor β

TLR – Toll Like Receptor

TNF – Tumor Necrosis Factor

TREM2 – Triggering Receptor Expressed on Myeloid Cells

WT – Wild Type

WHO – World Health Organization

YFP – Yellow Fluorescent Protein

YS – Yolk Sac

Chapter 1

Introduction

1. Introduction

1.1. Alcohol

1.1.1. Alcohol addiction and Social impact

For decades, alcohol consumption has been socially acceptable for recreational purposes and as a mood enhancer . Because it is an everyday dietary constituent consumed by thousands of people worldwide, society often undermines alcohol as a drug of abuse (Henriques et al., 2018). However, according to the World Health Organization (WHO), the harmful use of alcohol is responsible for 5,1% of the global disease burden, accounting for 3 million deaths per year, with the most affected group being people between 15-49 years (10% of deaths in this group). The most recent WHO data display high alcohol consumption rates per capita worldwide, with some variation between continents (**Figure 1**) (Hammer et al., 2018).

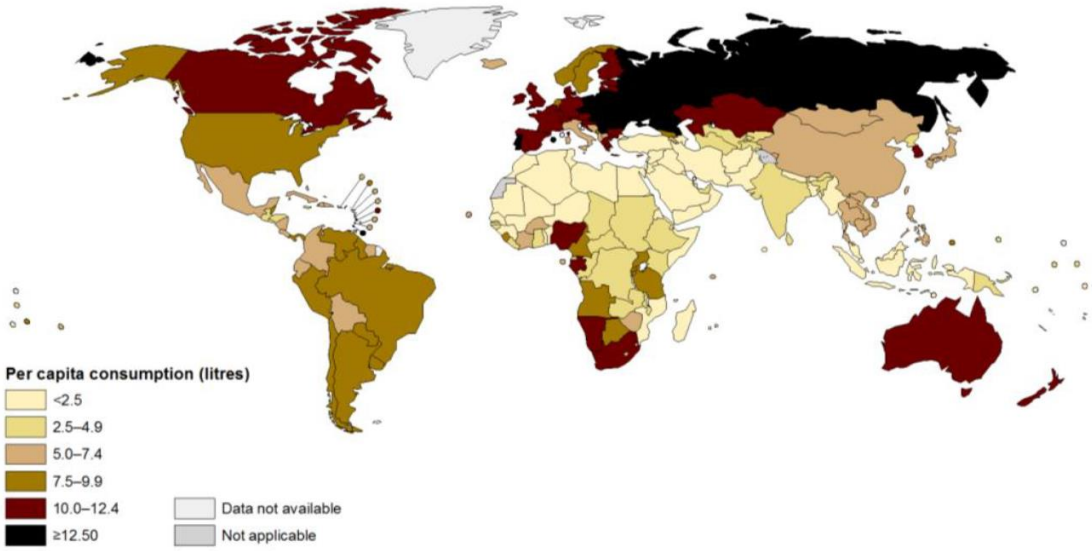

Figure 1 - Total alcohol consumption per capita (15+ years), in liters of pure alcohol. Individuals abouve 15 years of age consume on average 15.3 grams of pure alcohol per day. (Hammer et al., 2018)

Alcohol consumption has been part of many cultures for thousands of years before the moderns era (McGovern, 2009). Outside of a well-established socio-cultural context, alcohol abuse is a public health problem and social concern. The debate whether alcohol addiction is a disease or an intrinsic character flaw continues to differ opinions, despite the unmistakable existence of pieces of evidence demonstrating structural and functional alterations in the

alcoholics' brain, the likes of which dramatically change the neurobiology of the individual (Schomerus et al., 2011).

1.1.2. Alcohol-associated pathologies

Although occasional alcohol consumption might have some beneficial effect on cardiovascular diseases, the continuous consumption of alcohol, with abnormally high binge drinking levels, affects several organs morphologically and functionally, including the brain. Alcohol is a psychoactive substance with the potential to cause addiction when consumed in an abusive manner. Like many other drugs of abuse, alcohol/ethanol (EtOH) can cross the blood-brain barrier (BBB), causing morphological and physiological changes in the Central Nervous System (CNS), especially in the brain (Banerjee, 2014). The injury degree depends on several factors such as genetics, the age of onset, the consumption pattern (regular vs. binge), and the amount per episode (heavy vs. moderate) (Abrahao et al., 2017; Lovinger, 2013).

CNS exposure to EtOH results in prolonged disturbance in brain function, closely associated with anxiety, motor dysfunction, and cognitive decline (Abrahao et al., 2017). According to WHO, the harmfulness of alcohol is present in 6% of patients in primary health care offices suffering from mental illness (Hammer et al., 2018). Nonetheless, due to its preventable nature, alcoholism has not been considered a neurodegenerative disease, although chronic heavy drinking is responsible for the majority (57%) of early-onset dementia. Fortunately, the view that alcohol abuse does not cause brain disease has been changing gradually (Hammer et al., 2018).

Besides being considered one of the primary causes of gastrointestinal, cardiovascular, and infectious diseases morbility, alcoholism-associated symptoms can give rise to Alcohol Use Disorder (AUD). AUD is a chronic relapsing brain disease characterized by compulsive alcohol use and loss of control over alcohol intake. Disproportionate alcohol consumption alters the neuronal transmission and synaptic plasticity and can cause neurodegeneration. These alterations in neuronal function are thought to be caused, at least in part, by microglia activation and resulting neuroinflammation (Marshall et al., 2016).

1.1.3. Alcohol-induced synaptic impairment

A significant hallmark of alcohol abuse is the impairment in synaptic function, which likely underlies some of the behavioral deficits observed in chronic alcohol abusers. Indeed, alcohol adversely impacts neurons due to its direct action on pre and postsynaptic terminals and secretion/recycling of neurotransmitters (Tiwari et al., 2014). Thus, alcohol directly affects the neuronal signaling, disrupting primary excitatory (glutamatergic) and inhibitory (GABAergic) brain circuits. However, the effects on each system may vary according to acute vs. chronic alcohol exposure (Lovinger, 2013).

Additionally, regarding a single EtOH consumption, the damage depends on the concentration and the amount of intake, which in the brain can fluctuate from a few millimolar (one drink) to more than 100 millimolar. As a CNS relaxant, in a range of ≥ 5 ~ 10 mM (approximately three drinks), EtOH produces the sensation of mood elevation (so-called being "high") associated with increased disinhibition and socialization (Olsen & Liang, 2017). On the contrary, when one starts experiencing dependence due to prolonged misuse, other behavioral alterations emerge, e.g., mood disturbances, depressive-like features, and anxiety.

1.1.3.1. Alcohol effect on the GABAergic system

One of the most characterized targets of EtOH in the CNS is the GABAergic neurotransmission. The GABAergic system is part of extensive inhibitory neuronal circuits connecting different brain areas such as the cerebral cortex, the hippocampus, and the limbic system. There are three types of GABA receptors (GABA Rs): two of them are ligand-gated ion channels associated with the fast inhibitory transmission ($GABA_A$; $GABA_C$)) and the third one is a G-protein coupled receptor involved in a slower transmission ($GABA_B$). The neurotransmitter that activates these receptors is gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the CNS.

Regarding the effects of alcohol on this neuronal circuit, the primary binding target is the $GABA_A$ receptors ($GABA_ARs$) (Olsen & Liang, 2017). In adult GABAergic neurons, $GABA_ARs$ are complexes composed of five subunits from several classes, each consisting of distinct isoforms (Harris, 2008). The activation of these receptors results in an influx of chloride ions (Cl^-) along the chloride gradient, leading to the cell's hyperpolarization. EtOH is established as having a GABA-mimetic effect because alcohol interaction with $GABA_ARs$ leads to an aberrant increase in the influx of chloride into the cell, thereby enhancing inhibitory transmission and amplifying the sedative effects of alcohol (Davies, 2003; Grobin et al., 1998;

Harris, 2008). Besides, the enhancement of GABAergic transmission unexpectedly potentiates presynaptic GABA release, further inhibiting neuronal activity.

On the other hand, molecular changes associated with chronic EtOH exposure involve the internalization of $GABA_A$Rs subtypes and altered subunit gene expression, limiting the types of receptors responsible for the transmission by changes in the conformation and the constitution of the receptor. Behavioral responses to chronic alcohol action on these receptors are highly dependent on receptor subunit composition changes (Grobin et al., 1998; Harris, 2008; Lacagnina et al., 2017; Most et al., 2014). As a result of prolonged exposure, there is a decrease in the effects of EtOH on $GABA_A$Rs, restoring the Cl^- flux to below typical values, reducing the inhibitory tone of the neuron (Grobin et al., 1998; Most et al., 2014).

1.1.3.2. Alcohol effect on the Gluatamatergic system

Besides interfering with the inhibitory transmission, EtOH exposure also alters fast excitatory neurotransmission, being the glutamatergic system the primary component affected (Goodwani et al., 2017). Glutamate is the major excitatory neurotransmitter in the CNS, which, along with its receptors, has a ubiquitous distribution throughout the brain (Most et al., 2014). The glutamatergic system comprises ionotropic and metabotropic receptors, being ionotropic N-methyl-D-aspartate receptors (NMDARs), the most sensitive to alcohol exposure. NMDARs gate the flux of cations leading to fast depolarization of the neuronal membrane giving rise to excitatory neurotransmission (Möykkynen & Korpi, 2012). In general, acute exposure to EtOH inhibits all ionotropic glutamate receptors with the most prominent effect in NMDA receptors. Furthermore, during acute alcohol intake, an imbalance in glutamate release and glutamate uptake is responsible for the decreased extracellular concentration of the neurotransmitter (Goodwani et al., 2017).

Glutamatergic transmission is especially crucial in long-term potentiation (LTP) and, therefore, directly implicated in learning and memory. LTP requires robust glutamate release and is dependent on the activation/inhibition of NMDARs. Thus, the inhibition of NMDARs activity and consequent post-stimulus discharge of glutamatergic neurons suppresses LTP induction (Abrahao et al., 2017). Overall, acute EtOH effects on fast excitatory transmission are associated with cognitive impairment.

On the other hand, towards chronic intake, the inverse relationship is proven, with an improvement in the function of NMDARs. Moreover, repeated EtOH exposure elicits an alcohol-induced sensitization of the presynaptic terminal to increase glutamate release. As a result, along with NMDARs, other glutamate receptors respond to intensifying glutamatergic transmission, ending up in a hyperexcitable state – excitotoxicity (Lovinger, 2013). The

hyperstimulation of glutamatergic transmission usually contributes to withdrawal syndrome and relapse.

This detrimental effect of EtOH on both synaptic systems, highlighting an increased GABAergic inhibition accompanied with decreased glutamatergic excitability, is likely to be responsible for the behavioral changes of being "buzzed" followed by a depressive state, impaired motor coordination, and episodic memory loss.

All these changes at the synaptic level will trigger a neuroimmune response to reestablish homeostasis. Consequently, as the central player in this process, microglia may be activated, leading to either a detrimental pro-inflammatory state or a neuroprotective response.

1.2 Microglia

1.2.1. Discovery and Ontogeny

The CNS complexity has always created controversy among scientists, raising many questions about how its various components interact to keep the system's homeostasis. Historically, glial cells, named neuroglia, were described as the "glue" supporting the CNS structure (Kierdorf & Prinz, 2017). Later on, after discovering a new "third element" of the neuroglia, Pío del Rio-Hortega went further to ontogenically differentiate this newly discovered element from non-neural and non-astrocytic populations. The findings enabled the discrimination of a group of cells of mesodermal origin, apart from oligodendrocytes. Furthermore, morphological and functional data reveal these new cells' true nature by deciphering their capability of changing morphology. By this time, these cells were first label microglia by Hortega (Cherry et al., 2014).

Currently, microglia are considered the CNS resident macrophages, accounting for 5-10% of total brain cells. Indeed, microglia are the most abundant resident mononuclear phagocytes in the CNS. However, other macrophages, including meningeal, perivascular, and choroid plexus macrophages, also influence the CNS's immune response. Nevertheless, microglia are the brain's most prominent guards in the parenchyma (Herz et al., 2017).

The ontogeny of microglia was not always consensual. Microglia were primarily considered to be derived from hematopoietic progenitors entering the brain during early embryonic development. Typically, cells with hematopoietic origin are renewed throughout life from adult stem cells, except for the microglia. Microglia genesis starts with erythroid-myeloid progenitors (EMP) that develop into yolk sac (YS) macrophages (Alliot et al., 1999; Ginhoux et al., 2013; Perdiguero et al., 2015). This peculiar group with migratory capacity enters the developing neuroectoderm at embryonic day 9.5 (E9.5) in the mouse embryo – a single embryonic wave of progenitors giving rise to microglia (Kierdorf et al., 2013).

1.2.2. Brain Homeostasis – Housekeeping function

Microglia are the first line of host defense of the CNS, continually searching for pathogens, tissue damage, and infections to guarantee synaptic functionality and tissue homeostasis. Like other tissue-specific macrophages, microglia are immunosurveillant cells integrating the innate immune response, ready to react whenever the system is compromised. For many years, in the healthy, microglia brain was assumed to be in a dormant "resting" state although ready to intervene. However, *in vivo* observations, using intravital imaging rejected this theory revealing that microglia are highly dynamic cells, continuously extending and retracting their refined processes to surveil and scan the microenvironment, searching for any threat damage to the CNS parenchyma (Nimmerjahn et al., 2005). By this time, ATP was also identified as the critical chemotactic signal to the movement of microglia processes, while the cell body remains static (Davalos et al., 2005). Indeed, the findings proving microglia sensing ability were a feature of the concept of microglia "sensome" (**Figure 5**), a cluster of various proteins for recognizing and distinguish self from non-self (Hickman, 2013). For instance, the ATP receptor P2ry12 is a crucial component of the microglial "sensome" controlling microglia migration towards injuries (Haynes et al., 2006).

Allying all the microglia's functional capabilities, one can conclude that they are essential elements for CNS homeostasis both during development and adulthood.

1.2.2.1. Developing CNS - Neurogenesis and Neuronal Cell Death

Although the brain immune system regulation is the primary function of microglia, these are also responsible for many other brain-specific functions. It is during the early CNS development that microglial progenitors acquire these competencies when they contact critical factors released to the CNS microenvironment. Because of the exposure, there is an upregulation of different cell surface receptors and microglia transcriptional signatures, which is pivotal in discriminating microglia from other myeloid cells (Lavin et al., 2015; Mass et al., 2016). A vital process for brain development is neurogenesis. Simultaneously, and before the genesis of oligodendrocytes and astrocytes, myeloid precursors colonize the developing brain. In line with this, microglia are believed to perform a central role since the beginning of the CNS (Frost & Schafer, 2016; Martynoga et al., 2012). The balance between new neuronal cells' genesis and death is crucial for development without structural defects on neuronal architecture during brain development. Neurogenesis is a complex process from neural precursors to functional neurons, being microglia part of the process through the secretion of adjuvant

molecules, such as insulin-like growth factor 1 (IGF-1) for trophic support (**Figure 2**). Other trophic factors, such as brain-derived neurotrophic factor (BDNF), also secreted by microglia, are crucial to promoting neuronal survival during postnatal development (Bathina & Das, 2015; Ueno et al., 2013). Moreover, microglia express two surface receptors, CX_3CR1 and DAP12, relevant to supporting neuronal wiring and establishing neuronal circuits (Squarzoni et al., 2014).

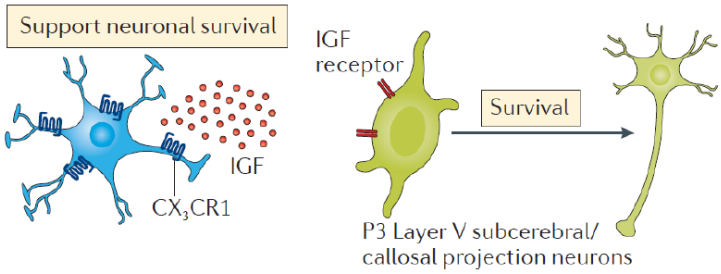

***Figure 2* - Microlgial release of IGF promote neuronal survival**

Additionally, to control neuronal cell pool, microglia *per se* can promote cell death in two ways: engulfing neural precursors cells (NPCs), contributing to the control of embryonic and adult cell pools, and producing ROS (Cunningham et al., 2013; Marín-Teva et al., 2004).

Moreover, as proficient phagocytes, microglia cooperate to eliminate apoptotic neurons in the CNS by recognizing the so-called "eat me" signals, namely phosphatidylserine (PS), disclosed by dying neurons (Ashwell, 1990; Dekkers et al., 2013) and phagocytic receptors on the microglial cell surface (e.g., BAI1, TIM4, or TREM2/DAP12 machinery) mediate this process (Hanayama et al., 2002; Liu et al., 2013; Mazaheri et al., 2014; Takahashi et al., 2005)

1.2.2.2. Developing CNS - Synaptic Pruning

Once the neural networks are established, constant remodeling is necessary to guarantee all connections' correct functioning. As brain "architects", microglia model neural circuits after sensing synaptic inputs or detect weak or desynchronized synapses. Accordingly, microglial

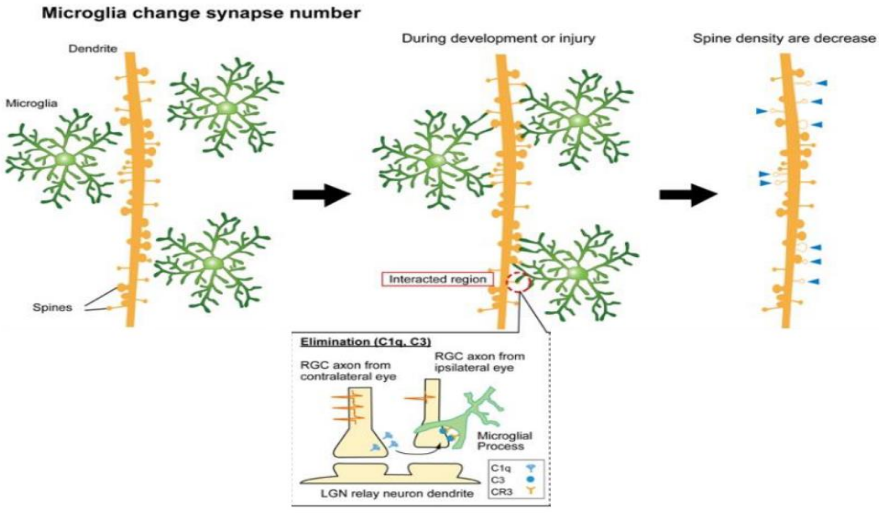

***Figure 3* - Microglia-mediated synaptic pruning.** Microglia use complement system to target and eliminate fragile synapses, resulting in decreased spine density. Adapted from

cells use essential proteins from the complement cascade, exclusive of microglia in the brain (Veerhuis et al., 2011). C3 and C1q, important complement components that localize within synapses, are recognized by complement receptor 3 (CR3), resulting in the microglia-mediated synapse engulfment (Stephan et al., 2012). This synaptic pruning is vital to improve the circuits and avoid possible supernumerary connections established during early development **(Figure 3)** (Paolicelli et al., 2011)**.**

1.2.3. Adult CNS – Synaptic Plasticity

Synaptic strengthening, learning-mediated plasticity, and homeostasis maintenance are also dependent on microglia activity and their interaction with neurons. The most relevant microglia-neuron interaction occurs via CX_3CR1/CX_3CL1 necessary to preserve the microglia homeostatic phenotype (BACHSTETTER, 2011). Furthermore, these cells can decrease synaptic effectiveness by stimulating AMPA receptors' internalization on neurons (Zhang et al., 2014).

Overall, each microglial function is vital to preserving the CNS microenvironment under restrictive conditions and maintaining a fully functional and healthy brain throughout life. The tiniest mistake or simple change in the process might compromise homeostasis or even culminate in a pathology.

1.2.4. Microglial defense function

As stated previously, concurrently with their housekeeping functions, microglia are also known as the "guardians" of the CNS. This immune role is mediated by microglia sensome, being the central role played by pattern-recognition molecular patterns (PRRs), expressed on the cell surface, recognizing both pathogen-associated molecular patterns (PAMPs) and tissue

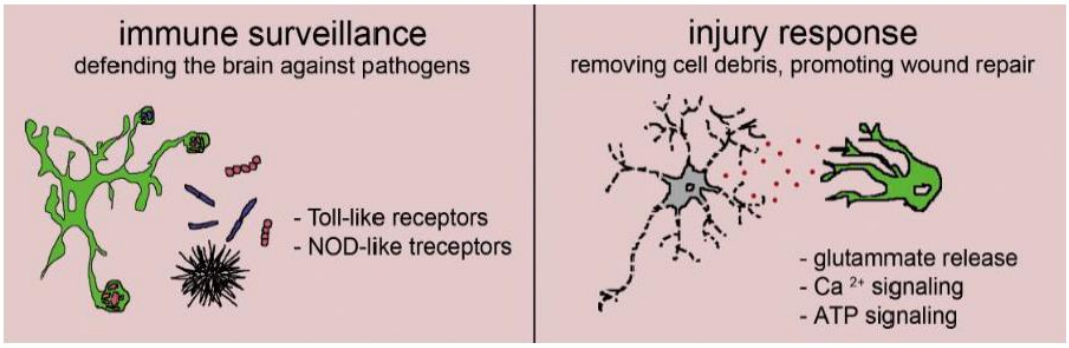

Figure 4 **- Microglia immune-related functions.** The expression of different membrane receptors, microglia are able to recognize and eliminate both possible threats (left panel) and erceive signals form injured cells and repair any damage (right panel) in order to preserve homeostasis. Adapted from (Casano & Peri, 2015)

damage-associated molecular patterns (DAMPs). Whereas the first, namely NOD-like receptors (NLRs), co-work with Toll-like receptors (TLRs), which perceive threatening pathogens over structurally conserved molecules (e.g., lipopolysaccharides)(Olson & Miller, 2004). The removal of cell debris occurs through attractant molecules (e.g., ATP, DNA, glutamate, or Ca^{2+} signaling) delivered by injured cells, hence captivating microglia to repair the damage (Boche et al., 2013; O. Takeuchi et al., 2002).

Indeed, when certain stimuli, either pathogenic or physical trauma, are detected by sensome receptors, they trigger microglial activation. This response involves microglial morphology changes to improve motility, shifting from a ramified to an amoeboid shape (Stence et al., 2001; Torres-Platas et al., 2014; Uhlemann et al., 2016). Moreover, their activation also leads to the synthesis of a batch of cytokines, chemokines, and other molecules that depending on the stimulus will mediate a different response with distinct repercussions in neighboring cells (Block et al., 2007).

Before the current knowledge about microglia activation, this was divided into M1/M2 to differentiate a classical or alternative activation. According to this terminology, the M1 phenotype corresponds to an inflammatory phenotype, induced via triggering TLR, IFN-γ, and NF-kB signaling pathways, leading microglia to produce and release pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α), glutamate, and ROS (Block et al., 2007; Orihuela et al., 2016). On the contrary, during M2 activation, microglia produce anti-inflammatory molecules (e.g., IL-10, IL-12, TGF- β) and BDNF to control inflammation and stimulate repair, therefore being a neuroprotective phenotype (Cherry et al., 2014; Varin & Gordon, 2009). Nonetheless, this categorization is considered an oversimplification of an otherwise multifaceted process. Recent transcriptomic studies and epigenetic analysis indicate that both M1 and M2 phenotypes may co-occur and vary in a region-specific manner within the CNS (Ransohoff, 2016).

Despite the possibility of a pro-inflammatory reaction, a transient microglia-mediated response is essential to eradicate the threat, whether it is a pathogen, injury repair, or dead cell debris, hence reestablishing homeostasis (Aguzzi, 2013). However, if the stimuli persist, an exacerbated or chronic activation of microglia may occur, ultimately leading to synaptic pruning and neuronal damage (Sochocka et al., 2017).

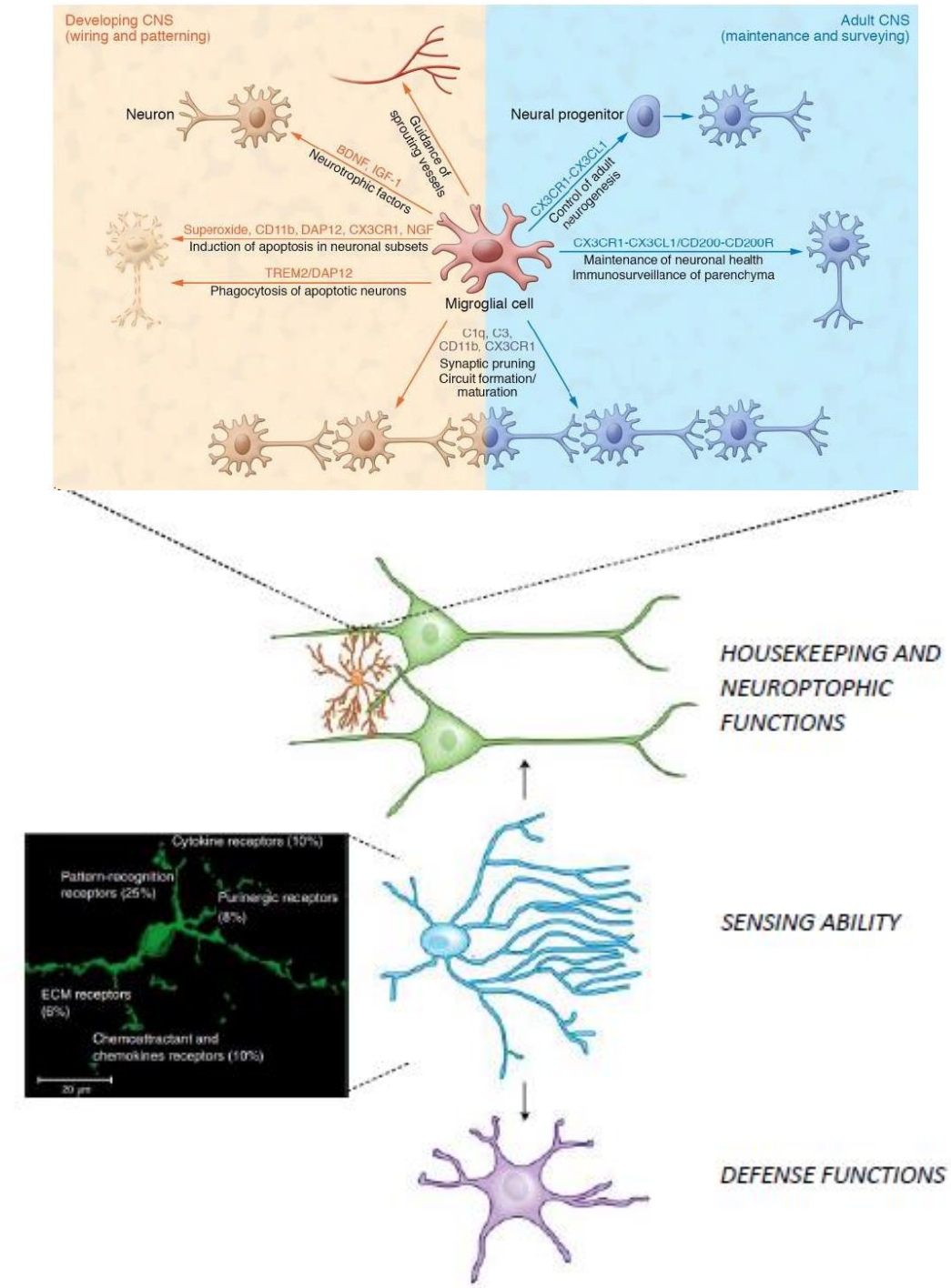

***Figure 5* - Different functional states of microglia and ontology of the cell sensome.** Microglia sensome (dark panel) is the basis for all their functions. From the homeostatic function in the developing and adult CNS (housekeeeing and neurotrophic tasks, upper part), to immune functions, initiating an inflammatory response and playing a defensive role against potential threats (bottom part). Adapted from (Hickman et al., 2018; Kierdorf & Prinz, 2017)

1.2.4.1. Microglial and brain inflammation

The transition from an acute/transitory inflammation to chronic inflammation results from microglia overactivation, which is present in various brain disorders, namely neurodegenerative diseases. Indeed, the shifting to a chronic inflammatory state is accompanied by increased production of pro-inflammatory cytokines, chemokines, ROS, and glutamate by the CNS glial population (Block et al., 2007). Accordingly, high concentrations of these molecules in the brain lead to glutamate excitotoxicity (Barger & Basile, 2001) and ROS-mediated oxidative stress, causing neuronal damage (Harris, 2008). Furthermore, in the most severe cases, pro-inflammatory cytokines and chemokines are involved in BBB impairment and disruption, further worsening inflammation (Shigemoto-Mogami et al., 2018). Consequently, the neuronal damage caused by all the events inherent to chronic inflammation induces reactive microglia to produce neurotoxic factors, in turn, self-perpetuating the neurotoxicity cycle. As a result, neuronal damage may evolve into progressive degeneration **(Figure 6)**.

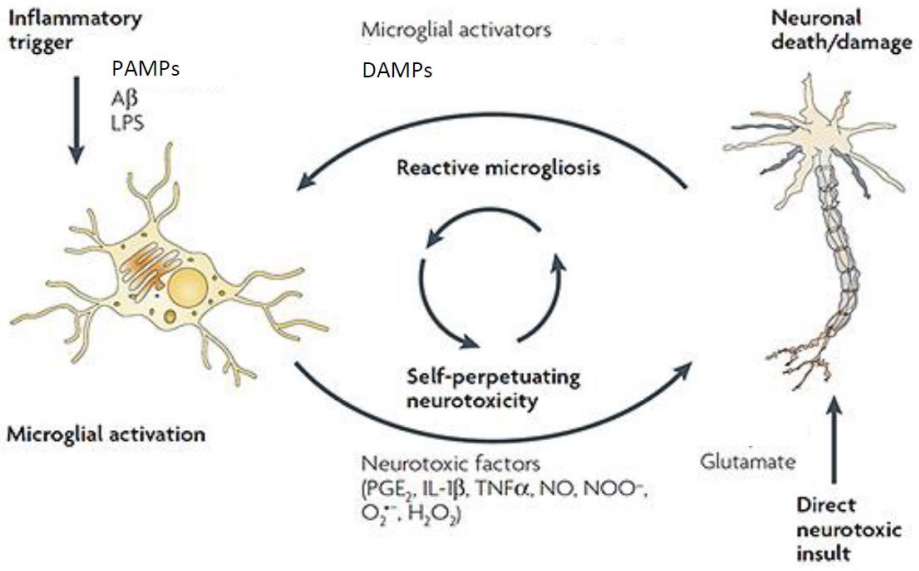

Figure 6 **- Self-perpetuating neurotoxicity cycle induced by exacerbated microglia activation.** Constitutive microglia activation and neurona demage together with the accumulation of different neurotoxic factors is the main feature of chronic inflammation. When perpatuated may result in neurodegeneration. Adapted from (Block et al., 2007)

Subsequently, this harmful inflammatory response is a landmark of well-known neurodegenerative diseases, namely Alzheimer's disease (AD) (Itagaki et al., 1989), Parkinson's disease (PD) (McGeer et al., 1988), and Huntington's disease (HD) (Sapp et al., 2001). Recently, a new terminology to name microglia-mediated inflammation in pathologies was introduced, "degeneration or disease-associated microglia" (DAM) (Deczkowska et al., 2018; Keren-Shaul et al., 2017; Song & Colonna, 2018). Based on single-cell DNA sequencing, DAM merges classic genes from both M1 and M2 phenotypes related to different neurodegenerative diseases. One protein that balances activation and pro-inflammatory responses in myeloid cells is TREM2. DAM's progression was proven to be TREM2-dependent (Wang et al., 2015) and, along with morphological reorganization, genes typically expressed by homeostatic microglia (e.g., P2ry12, Tmem119, Cx3cr1) were downregulated, whereas genes implicated in phagocytic, lysosomal, and lipid metabolism were upregulated (Ajami et al., 2018; Keren-Shaul et al., 2017). Although primarily discovered in AD models, the DAM phenotype has also been observed in other mouse models for different brain pathologies (Song & Colonna, 2018).

Chapter 2

Aims

2. Aims

As the resident immune cells of the CNS, microglia intervention in response to harmful stimuli is crucial for safeguarding brain homeostasis. However, the exacerbation or cessation of microglial response can result in the disruption of brain homeostasis. As previously referred, when activated, microglia release either pro or anti-inflammatory molecules as a response to stimuli. Therefore, TNF, as one of the most prominent factors released by microglia, is a well-positioned candidate for mediating a detrimental increase of microglia engulfment capacity following alcohol intake.

Previous studies from our laboratory revealed a potential role for TNF modulation of microglial function and synaptic impairment (Socodato, 2017). Thus, it is plausible to hypothesize that alcohol intake interferes with TNF releasing by microglia, consequently boosting their engulfment capacity.

In this thesis, we sought to address three main questions using complementary *in vivo* and *in vitro* approaches:

1. Does alcohol intake affect microglia reactivity?
2. Does the release of TNF depend on the reactivity of microglia?
3. Does TNF release impact microglial engulfment capacity?

Chapter 3

Methodology

3. Methodology

3.1. Animals

All mice experiments were reviews by the i3S animal ethical committee and were approved by Direção-Geral de Alimentação e Veterinária (DGAV). All animal experiments considered the Russel and Burch 3R's principles and followed the European guidelines for animal welfare (2010/63/EU Directive). Animals were maintained in standard laboratory conditions with an inverted 12h/12h light/dark cycle and were allowed free access to food and water. The animals used were kept on a C57BL/6 background. Because of the potential behavioral variability related to females' estrous cycle, only male mice (16-20 week-old) were used in the present study. Moreover, using only one gender minimizes intergroup variability and reduced the overall number of animals used.

B6.129P2(Cg)-*Cx3cr1*$^{tm2.1(cre/ERT2)Litt}$/WganJ mice (herein referred to as Cx3cr1$^{EYFP\text{-}CreER/+}$; The Jackson Laboratory Stock) mice were used to study brain microglia in this work. These mice express a Cre-ERT2 fusion protein and enhanced tallow fluorescent protein (EYFP) from the endogenous Cx3cr1 promoter. EYFP fluorescence is observed in more than 95% of Iba1^{+} microglia in the brain.

3.1.1. Microglial Cell Ablation

Microglial ablation mice were generated using two different mice: Cx3cr1$^{EYFP\text{-}CreER/+}$ were intercrossed with R26$^{iDTR/+}$ (The Jackson Laboratory) mice. Genotypes of interest (Cx3cr1$^{EYFP\text{-}CreER/+}$ (control) and Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$ (experimental)) were determined by PCR on genomic DNA. Primers used for R26-iDTR insertion including ROSA26-forward: AAA GTC GCT CTG AGT TGT TAT; ROSA26-reverse: GCG AAG AGT TTG TCCTCA ACC; wild-type (WT) reverse: GGA GCG GGA GAA ATG GAT ATG. Primers for CreER-EYFP insertion were WT forward: AAG ACT GTG GAC CTG CT; WT reverse: AGG ATG TTG ACT TCC GAG TG and mutant reverse: CGG TTA TTC AAC TTG CAC CA.

Tamoxifen was given to adult mice of both control and experimental groups (10mg/kg *per* animal) as a corn oil solution by oral gavage. Mice received two doses of tamoxifen separated by 48h between doses. For microglia ablation, eight weeks after the last tamoxifen pulse, both groups were given diphtheria toxin (DT; 1µg by intraperitoneal injection) for three consecutive days, and sacrifice occurred 24h after the last DT injection.

3.1.2. TNF Knockout mice

This animal model, C57BL/6.TNF knockout (referred to herein as TNK KO) was supplied by Prof. Rui Appleberg (University of Porto) and kept on a C57BL/6 background. TNF KO mice were genotyped by PCR using ATC CGC GAC GTG GAA CTG GCA GAA (forward) and CTG CCC GGA CTC CGC AAA GTC TAA (reverse) primer pair, displaying a single band of 2 kb in the PCR gel. TNF deficient mice were generated from hemizygote progenitors, and WT littermates were used as controls.

3.1.3. SLICK-H Model

Tg(Thy1-cre/ERT2,-EYFP)HGfng/PyngJ, also known as SLICK-H line and herein termed Thy1-YFP (The Jackson Laboratory) mice were maintained as before. These mice have constitutive and exclusive YFP labeling of neurons driven by endogenous Thy1 promoter. In this work, Thy1-YFP mice were used for studying the engulfment of synapses by microglia in vitro.

3.2. Alcohol intake protocol

Mice were habituated for four weeks in experimental rooms at the i3S animal facility. Afterward, mice were randomly assigned to experimental groups. To emulate a moderate alcohol intake pattern, mice were given 1.5 g/kg ethanol (diluted to 25% in sterile tissue culture-grade water) by oral gavage daily for ten consecutive days. Measurements of blood alcohol concentration in adult C57BL/6 mice show that 120 minutes after oral gavage intake, ethanol is detected above 80 mg/dl in their blood, thereby reaching amounts similar to a binge-drinking pattern of ethanol intake. Vehicle control mice were dosed with water in equal amounts to those of ethanol-treated mice. For analyses, mice were sacrificed 120 minutes after the last gavage. Animals were provided with food and water ad libitum throughout the experiments.

For in vitro studies, microglial cultures were exposed to 70 mM (320 mg/dl) ethanol. This concentration of ethanol was used because it simulates the amounts achieved in the blood during binge drinking in addition to being correlated with a dosage inducing neuronal damage.

3.3. Brain tissue preparation and immunohistochemistry

After animal perfusion with ice-cold PBS (15 ml) and fixation by perfusion with 4% PFA, brains were post-fixed by immersion in 4% PFA in PBS, pH 7.2 overnight. After that, brains were washed with PBS and then cryoprotected using sucrose gradient in a row (15% and 30%). After 24h, brains were mounted in OCT embedding medium, frozen, and cryosectioned in the CM3050S Cryostat (Leica Biosystems). Coronal sections from brains (30 µm thickness) were collected non-sequentially on Superfrost ultra plus slides. Tissue sections from controls and experimental mice encompassing identical stereological regions were collected on the same glass slide and stored at -20ºC. Frozen sections were defrosted by at least 1h and hydrated with PBS for 15 minutes. Sections were permeabilized with 0.25% Triton X-100 for 15 min, washed with PBS for 10 min, and blocked in 5% bovine serum albumin (BSA), 5% fetal bovine serum (FBS), and 0.1% Triton X-100 for 1 h. Primary antibodies (ionized calcium-binding adapter molecule 1 (Iba1) (1:500, Wako, Japan); vesicular glutamate transporter 1 (VGLUT-1) (1:1000, Synaptic Systems); post-synaptic density protein 95 (PSD95) (1:600, Cell Signaling); cluster of differentiation 68 (CD68) (1:400, Bio-Rad); NeuN (1:100, Millipore)) were incubated in blocking solution in a humidified chamber overnight at 4°C. Secondary antibodies were incubated for 2 h in blocking solution. After the secondary antibody, sections were washed three times for 10 min with PBS. Slides were coverslipped using glycergel or Immumount and visualized under a Leica TCS SP5 II confocal microscope.

3.4. Confocal imaging and morphometric analysis

Images from tissue sections of the prefrontal cortex and CA1 region of the dorsal hippocampus were acquired using Leica HC PL APO Lbl. Blue 20x /0.70 IMM/CORR or a Leica HC PL APO CS 40x /1.10 CORR water objective in 8-bit sequential mode using standard TCS mode at 400 Hz and the pinhole was kept at one airy in the Leica TCS SP5 II confocal microscope. Images were resolved at 1024 x 1024 pixels format illuminated with 2-5% DPSS561 561 nm wave laser using a HyD detector in the BrightR mode, and entire Z-series were acquired from tissue sections. Equivalent stereological regions were obtained for all tissue sections within a given slide.

Microglia quantification: The number of YFP+ cells was manually scored in stereological identical regions of the prefrontal cortex of stained sections (6 images per section; 5 sections per animal for each experimental group).

GFAP quantification: The number of GFAP+ cells was manually scored in stereological identical regions of the prefrontal cortex of stained sections (6 images per section; 8 sections per animal for each experimental group).

NeuN quantification: The number of NeuN+ neurons was manually scored in stereological identical regions of the prefrontal cortex stained sections (6 images per section; 8 sections per animal for each experimental group).

TNF quantification: Briefly, stereological identical regions of TNF immunostained sections of the prefrontal cortex or the dorsal hippocampal CA1 area (4 images per section; 6 sections per animal for each experimental group) were
imaged, converted into 8-bit grayscale, 3D volume-rendered, and thresholded. Using FIJI software, the percent of TNF immunostained area was calculated for each field, and each section.

Quantification of synapses: Images from stereological identical prefrontal cortex or dorsal hippocampal CA1 region from each experimental group (4 images per section; 6 sections per animal for each experimental group) were acquired using a Leica HC PL APO CS 40x /1.10 CORR water objective at 1024 x 1024 pixels
resolution with 8-bit bidirectional non-sequential scanner mode at 400 Hz and pinhole at one airy in the Leica TCS SP5 II confocal microscope. Z-stacks were converted to maximum projection images using the LAS AF routine. Z-projections were background-subtracted using the rolling ball background subtraction built-in algorithm in FIJI, and then images were upsampled using a bicubic interpolation routine. The number of double-positive PSD-95/vGlut1 puncta per μm^2 was manually scored for each image.

Quantification of PSD-95 engulfment by microglia: Images from stereological identical prefrontal cortex or dorsal hippocampal CA1 region from each experimental group (6 images per section; 4 sections per animal for each experimental group) were acquired using a Leica HC PL APO CS 40x /1.10 CORR water objective at 1024 x 1024 pixels resolution with 8-bit bidirectional scanner mode at 200 Hz in the Leica TCS SP5 II confocal microscope. Using FIJI software, confocal Z stacks were background subtracted and smoothened using a Sigma filter plus. CD68, PSD-95, and Iba1 volumes were reconstructed using 3D surface rendering of confocal Z stacks in Imaris. For quantification of PSD-95 engulfment by microglia, PSD-95 puncta embedded within volume-rendered CD68+ structures in Iba1+ cells were considered to be engulfed by microglia. Unbiased measurements of PSD-95 puncta associated with CD68 were automatically performed using the Imaris co-localization package for each microglia and used for statistical analyses

3.5. Flow Cytometry

To identify the microglial population, the markers used were: CD45-PE (103106 BioLegend) and CD11b-APC (101212 BioLegend, USA). Assessment of microglial viability was done using Zombie Violet Dye (77477 BioLegend, USA) cell death marker.

Mice were anesthetized with sodium pentobarbital (0.2ml *per* 30g of mice body weight) and then perfused (transcardial perfusion) with ice-cold PBS. To obtain single-cell suspensions, neocortices were quickly dissected on ice, placed on ice-cold Gibco® RPMI 1640 (Thermo Fisher, USA), and mechanically homogenized. The obtained cell suspension was passed through a 100µm cell strainer and centrifuged over a discontinuous 70%/30% Percoll (Sigma-Aldrich, USA) gradient for 30 minutes. Cells located on the interface were collected, pelleted, resuspended in FACS buffer (2% BSA; 0.1% Sodium Azide in PBS), and then counted in a Neubauer chamber using trypan blue exclusion to estimate the number of live cells. The single-cell suspensions (1 x 10^6 cells) were incubated with different mixes of FACS antibodies for 30 minutes at 4ºC in the dark. Compensation settings were determined using spleens from wild-type mice. A FACS Canto II analyzer (BD Immunocytometry Systems, USA) was used to evaluate cell suspensions. All data were analyzed by FlowJo X10 software® (TreeStar, USA) using a sequential gating strategy.

3.6. Preparation of lysates

Cultures or mice tissues were lysed using RIPA-DTT buffer (150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS) supplemented with complete-mini protease inhibitor cocktail tablets, 1 mM DTT and phosphatase inhibitor cocktail. Samples were sonicated (6 pulses of 1 sec at 60Hz) and centrifuged at 16,000 g, 4°C for 10 min. The supernatants were collected, and the protein concentration was determined by the BCA method. All samples were denatured with sample buffer (0.5 M Tris-HCl pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, 0.02% bromophenol blue) at 95°C for 5 min and stored at −20°C until use.

3.7. Western Blotting

Samples were separated by SDS-PAGE electrophoresis in a 12% SDS-page gel with a voltage of 120V (adjustments overtime were done). Precision Plus Protein™ Dual Color Standards (1610374; Bio-Rad) was used as a molecular weight marker. Proteins were transferred from gel to the Immun-Blot® PVDF membrane (Bio-Rad) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad). The transference was performed for 10 minutes in diluted Trans-Blot® Turbo™ 5X Transfer Buffer (20% buffer, 20% ethanol, and 60% ultrapure H_2O).

Membranes were blocked for 60 minutes in a blocking solution composed of 5% skimmed milk diluted in tris based saline with 0.1% Tween (TBS-T) pH 7.6 and incubated with the primary antibodies Anti-Iba1 (1:1000; Wako), Anti-GAPDH (1:20000; HyTest Ldt.), Anti-PDS95 (1:2000; Thermo Fisher), Anti-vGlut1 (1:1000; Synaptic Systems) diluted in blocking solution overnight at 4ºC. Membranes were then washed three times, for 10 minutes each, with TBS-T and incubated with peroxidase-conjugated secondary antibodies: HRC conjugated anti-rabbit (1:10000; Promega) and HRC conjugated anti-mouse (1:15000; Promega). Membranes were developed using a Pierce™ ECL Fast Western Kit (Thermo Fisher) and revealed using ChemiDoc™ XRS System (Bio-Rad). Images were finally quantified and processed by FIJI Software®.

3.8. Microglial cell lines

Human microglia clone 3 (HMC3) cell line was obtained through SV40-dependent immortalization of human embryonic microglial cells and authenticated by the American Type Culture Collection (ATCC® CRL-3304™) (Janabi et al., 1995). These cells were cultivated with DMEM + GlutaMAX™-I (supplemented with 10% FBS and 1% Penicillin/Streptomycin) and maintained at 37°C, 95% air and 5% CO2 in a humidified incubator.

3.9. Synaptosomal preparations and microglia engulfment assay

To isolate prefrontal cortex synaptic terminals, synaptosomes were freshly prepared using Syn-PER™ Synaptic Protein Extraction Reagent (catalog no. 87793, ThermoFisher Scientific) precisely as recommended by the manufacturer. Briefly, WT mice or Thy1-YFP were euthanized in a CO2 chamber. Their prefrontal cortex was collected, and homogenization was performed using a Dounce tissue grinder (~10 strokes). The homogenate was centrifuged at 1200 x *g* for 10 min, and the pellet was discarded. The supernatant was centrifuged at 15000 x *g* for 20 min. The resulting supernatant was discarded, and the synaptosomal pellet was resuspended in Syn-Per™ Synaptic Protein Extraction Reagent.

CHME3 microglial cultures were seeded in 12-well culture plates at a density of 2.5 x 104 cells/well in DMEM GlutaMAX™-I supplemented with 10% FBS, 0.1% PenStrep and cultivated for 48 h. Cultures were treated with an FcgR blocking solution (1:100) for 20 min and then refed with fresh medium containing vehicle (water), or EtOH (70 mM), or adalimumab (5 μg/ml), or adalimumab (5 μg/ml) plus EtOH (70 mM). Cells were kept in those conditions for 24 h. Afterward, cells were refed with fresh medium containing vehicle (water) plus synaptosomes (1:100), or EtOH (70 mM) plus synaptosomes (1:100), or adalimumab (5 μg/ml)

plus synaptosomes (1:100), or adalimumab (5 μg/ml) plus EtOH (70 mM) plus synaptosomes (1:100). Cells were cultured under these conditions for an additional 24 h. For analyses, microglial cultures were washed extensively with PBS and either fixed with PFA 4%, immunolabeled for PSD-95 and analyzed in a fluorescence microscope or detached using accutase solution (catalog no. A6964, Sigma), blocked in PBS-BSA 5%, fixed with PFA 2%, resuspended in PBS-BSA 1% and analyzed in a flow cytometer.

3.10. Statistics

A 95% confidence interval was used for statistical evaluation, and $p < 0.05$ was considered a statistically significant difference in all sampled groups. Experimental units in individual replicates were prior evaluated for Gaussian distribution using the D'Agostino & Pearson omnibus normality test with GraphPad Prism. When comparing only two experimental groups Mann-Whitney test for data with non-normal distribution or unpaired Student t-test with equal variance assumption for data with normal distribution was used.

When comparing treatment effects within genotypes/mutants, Two-way ANOVA followed by the Sidak's multiple comparisons test was used. Two-way ANOVA was also used to compare values of microglia intersections retrieved from Sholl analysis. All quantifications were performed blinded.

Chapter 4

Results

4. Results

4.1. Alcohol intake produces a microglia-driven neuroimmune response in the prefrontal cortex

In mice models of alcohol intoxication, alcohol intake leads to microglia activation and oxidative damage (Qin & Crews, 2012). We sought to study the effect of alcohol intake on microglia reactivity and used an EtOH exposure protocol simulating moderate alcohol intake. For this, we administered 1.5 g/kg of EtOH or water (H_2O) for ten consecutive days to a microglia reporter (Cx3cr1$^{EYFP\text{-}CreER/+}$ (Parkhurst et al., 2013)) mouse (**Figure 7A**).

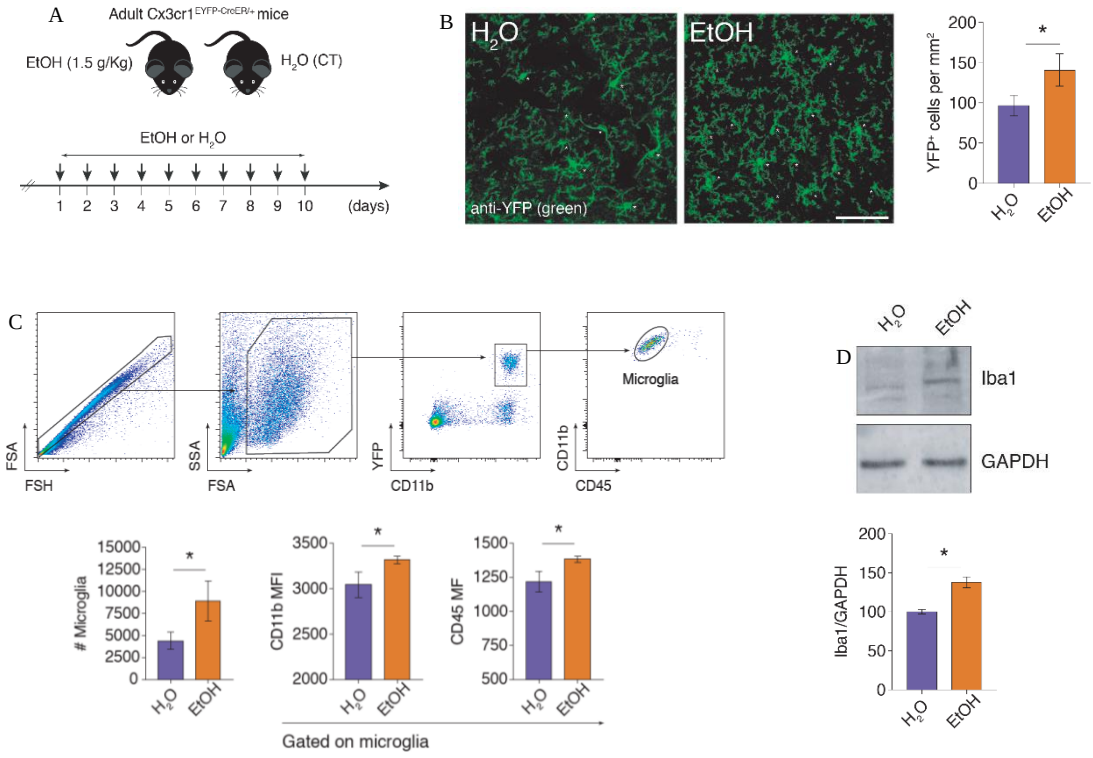

***Figure 7* - Alcohol intake elicits microglia activation in the prefrontal cortex.** Schematic representation of the regimen of repetitive binge.level alcohol (EtOH) intake to adult Cx3cr1$^{EYFP\text{-}CreER/+}$ mice. Mice were given EtOH (1.5 g/kg) or water (H_2O; control) by oral gavage dor 10 consecutive days **(A)**. Histological confocal analysis of microglia (immunostained against YFP) on tissue sections from prefontal cortices of Cx3cr1$^{EYFP\text{-}CreER/+}$ mice exposed to EtOH or H_2O. Asterisks indicate the microglial cell body. Scale bar, 50 µm. Data are means ± SEM from six mice per condition pooled across thress independent experiments. **P*<0.05 by Mann-Whitney test **(B)**. Flow cytimetry analysis of microglial numbers in neocortices of Cx3cr1$^{EYFP\text{-}CreER/+}$ mice exposed to EtOH and H_2O. Microglia were identified as being CD11b$^+$ and Cd45$^{low/mid}$ gated from the double-positive CD11b$^+$ YFP$^+$ population. Data are means ± SEM from eight (H_2O) and nine (EtOH) mice pooled across three independent experiments. **P*<0.05 by Mann-Whitney test **(C)**. Western blot for Iba1 on lysates from prefrontal cortices of Cx3cr1$^{EYFP\text{-}CreER/+}$ mice exposed to EtOH or H_2O. GAPDH (loading control). Data are means ± SEM from six mice per condition pooled across three independent experiments. **P*<0.05 by Mann-Whitney test **(D)**.

Immunohistochemistry on prefrontal (PFC) cortex tissue sections from Cx3cr1$^{EYFP\text{-}CreER/+}$ revealed that this EtOH exposure protocol induced a significant expansion of PFC microglia (YFP^{+} cells, **Figure 7B**). Using flow cytometry, we confirmed this increased microglia number in Cx3cr1$^{EYFP\text{-}CreER/+}$ mice exposed to EtOH (**Figure 7C**). EtOH also increased the expansion of the reactivity markers CD45, integrin alpha M (CD11b), and Iba1 in PFC (**Figure 7D**)

4.2. Microglia ablation model mice

Microglia are the most important producers of TNF after acute binge alcohol intake (23). To test whether microglia were also major TNF producers during our alcohol exposure protocol, we genetically ablated microglia using the microglia iDTR/Cre-lox system (Parkhurst et al., 2013). For this purpose, we used an experimental set up where mice expressing both EYFP and tamoxifen-inducible Cre recombinase (Cre^{ER}) under the endogenous regulation of Cx3cr1 promoter (Cx3cr1$^{EYFP\text{-}CreER/+}$) are crossed with mice expressing R26, a locus used for constitutive and ubiquitous gene expression in mice and inducible diphtheria Toxin receptor (iDTR) (R26^{iDTR}). We first validated the efficiency of this microglia ablation system in the context of our experimental requirements by giving tamoxifen to Cx3cr1$^{EYFP\text{-}CreER/+}$ and Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$ mice at P28 and P30 and DT 2 months later (**Figure 8A**). Indeed, both immunohistochemistry on PFC tissue sections and flow cytometry showed, as expected, that microglia were efficiently eliminated in Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$ mice. (**Figures 8B and 8C, respectively**).

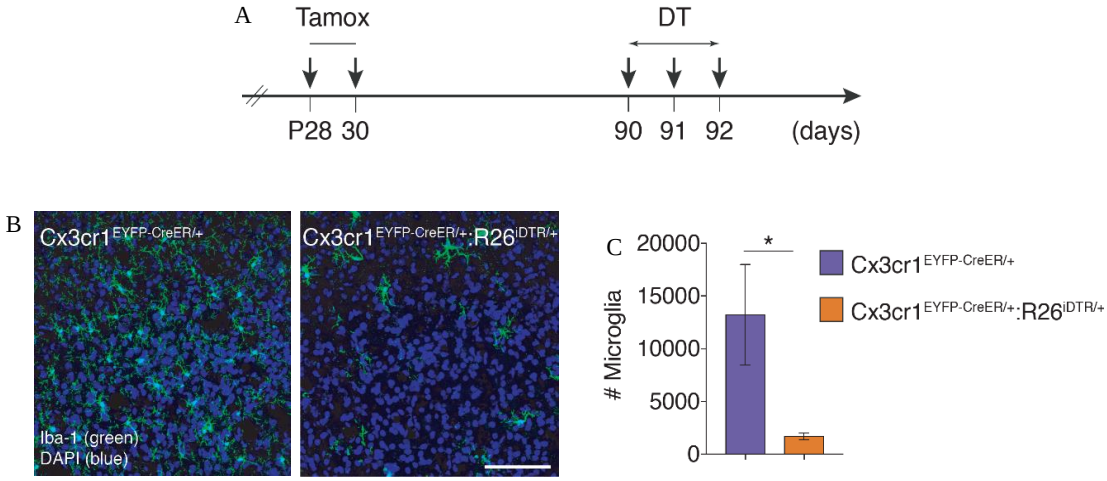

***Figure 8* - Ablation of microlgia in Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$ mice.** Schematic representation of tamoxifen-inducible microglial ablation in the brain from postnatal day 28 (P28). Tamox, tamoxife; DT, dephtheria toxin (A). Histological confocal analysis for Iba1 on tissue sections from prefrontal cortices of Cx3cr1$^{EYFP\text{-}CreER/+}$ and Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$ after exposure to DT (n = 4 animals per genotype). Scale bar, 50 µm **(B)**. Flow cytometry analysis of microglial numbers in neocortices of Cx3cr1$^{EYFP\text{-}CreER/+}$ and Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$ after exposure to DT. Data are means ± SEM from six mice per condition pooled across three independent experiments. *P<0.05 by Mann-Whitney test **(C)**.

4.3. Microglia as the major TNF producers in the PFC during EtOH exposure

After validating the brain's microglia ablation model, we evaluated TNF production in the PFC of Cx3cr1$^{EYFP\text{-}CreER/+}$ and microglia-depleted mice (Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$) following EtOH exposure (**Figure 9A**). Whereas EtOH significantly increased the protein amounts of TNF in Cx3cr1$^{EYFP\text{-}CreER/+}$ mice (**Figure 9B**), it did not increase TNF amounts in microglia-depleted mice (**Figure 9B**), suggesting that microglia are the primary producers of TNF in the PFC during EtOH exposure.

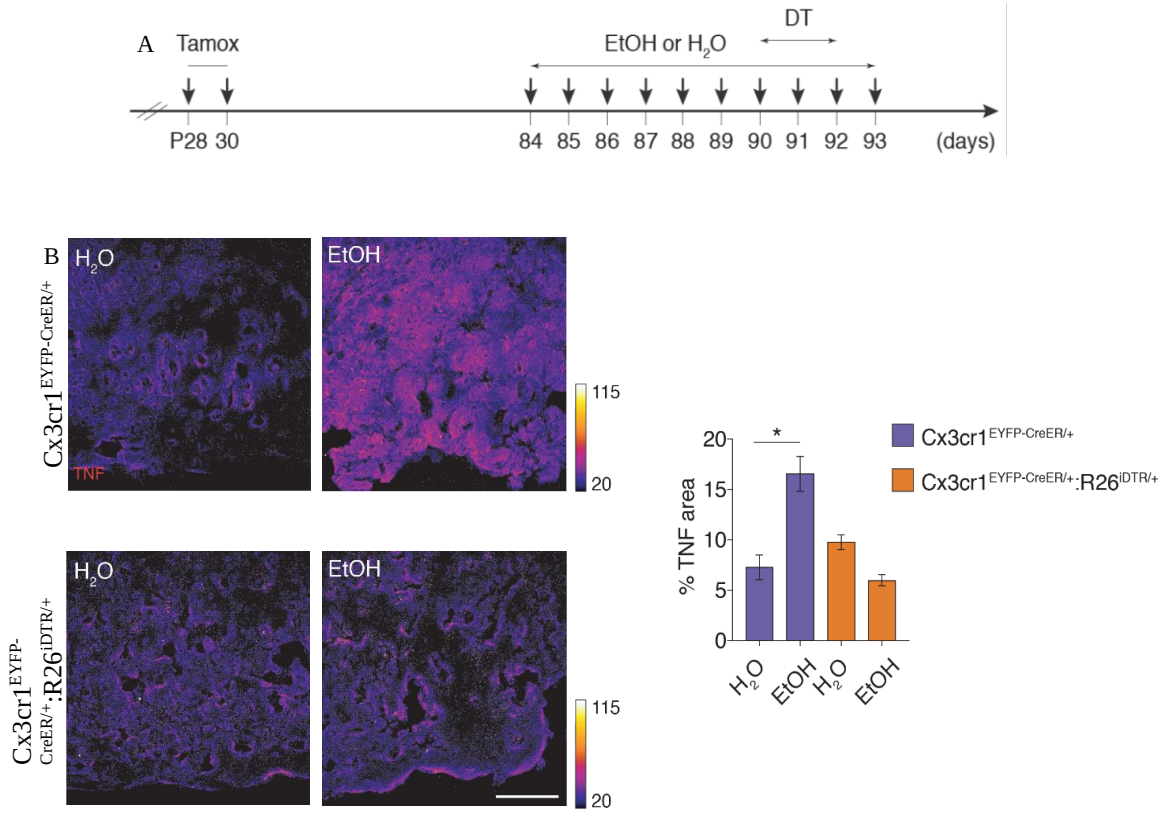

***Figure 9* - TNF production elicited by alcohol intake drives microglia activation in the prefrontal cortex.** Schematic representation of microglial ablation during EtOH exposure **(A).** Histological confocal analysis for TNF on tissue sections from prefrontal cortices of Cx3cr1$^{EYFP\text{-}CreER/+}$ and Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$ after microglia ablation during exposure to EtOH or H_2O. Scale bar, 100 µm. Data are means ± SEM from five mice per condition pooled across three independent experiments. *P<0.05 by two-way ANOVA with Sidak's multiple comparisons **(B).**

4.4. TNF signaling increases microglia phagocytic activity licensing microglia to prune PFC synapses following alcohol intake

Alcohol binge drinking is responsible for commonly cause behavioral alterations, such as increased anxiety, which can be caused by perturbations in excitatory/inhibitory balance in PFC circuits (Froemke, 2015; Tatti et al., 2017). EtOH-elicited microglia activation could alter excitatory synapses in the PFC. The work here presented shows that the amounts of PSD-95 and vGlut1 (major proteins involved in excitatory transmission) along with the number of excitatory synapses (PSD-95^{+}/vGlut1^{+} puncta) were significantly decreased in PFCs of ice exposed to EtOH (**Figure 10**).

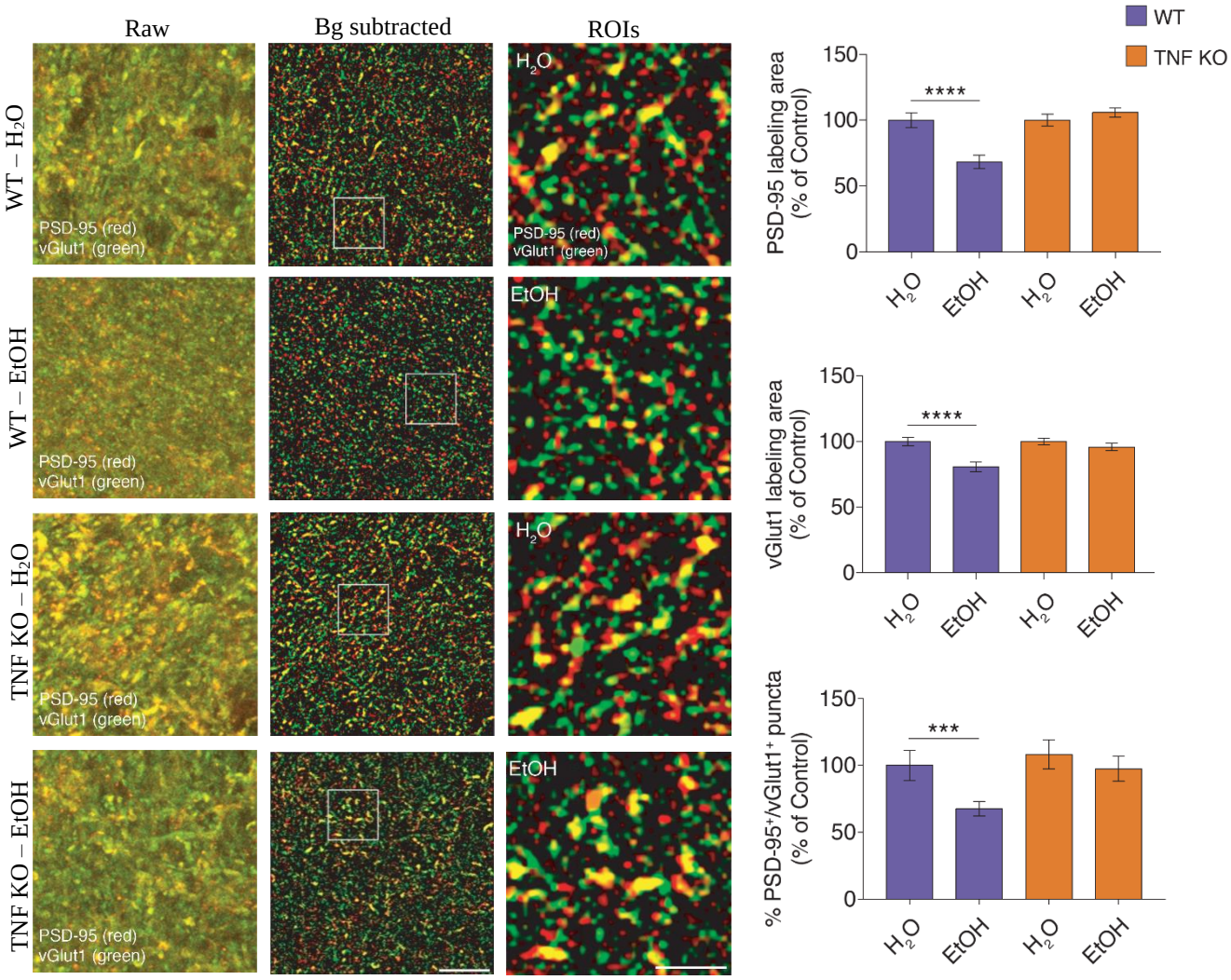

Figure 10 **- Alcohol intake elicits synapse loss in a microglia-dependent manner.** Representative raw and background-subtracted confocal images of PSD-95 (red) and vGlut1 (green) staining on tissue sections from prefrontal cortices of WT and TNF KO mice (genotypes are shown in the boxes) exposed to EtOH or H_2O. Scale bar 10 µm. White squares are the ROIs expanded on the right column. Scale bar 5 µm. Data are means ± SEM from at least six mice per condition pooled across three independent experiments. ****P<0.0001 and ***P<0.001 by two-way ANOVA with Sidak's multiple comparisons.

On the other hand, this decrease of PDS-95 and vGlut puncta and PSD-95^{+}/vGlut1^{+} synapses induced by alcohol intake was prevented entirely in TNF KO mice **(Figure 10)** and microglia-depleted mice **(Figure 11A)**.

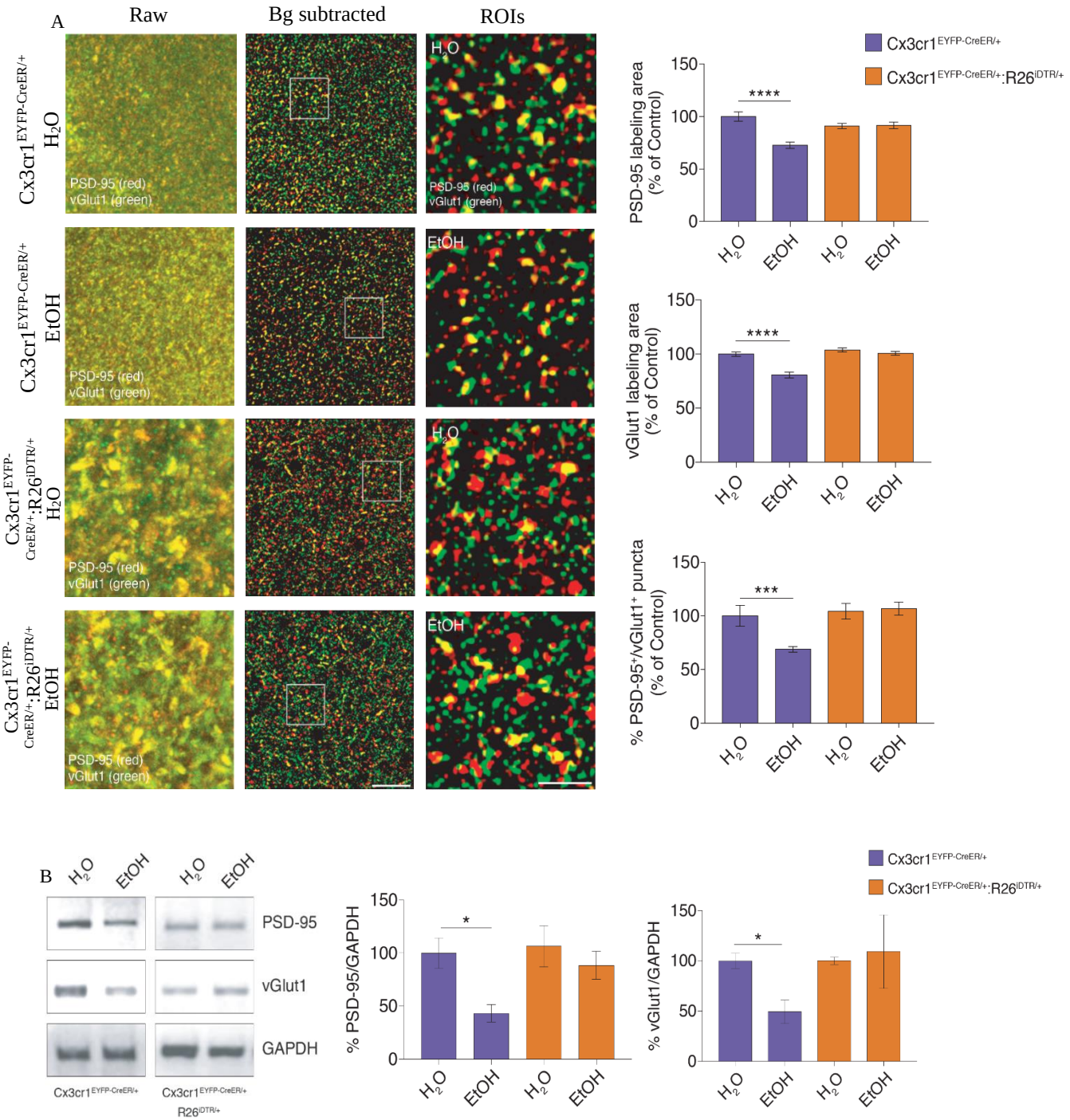

Figure 11 **- Alcohol intake elicits loss in a microglia-dependent manner.** Representative raw and background-subtracted confocal images of PSD-95 (red) and vGlut1 (green) staining on tissue sections from prefrontal cortices of Cx3cr1$^{EYFP\text{-}CreER/+}$ and Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$ mice (genotypes are shown in the boxes) exposed to EtOH or H_2O. Scale bar 10 µm. White squares are the ROIs expanded on the right column. Scale bar 5 µm. Data are means ± SEM from at least six mice per condition pooled across three independent experiments. ****P<0.0001 and ***P<0.001 by two-way ANOVA with Sidak's multiple comparisons **(A).** Western blot analysis for PSD-95 and vGlut1 on lysates from prefrontal cortices of Cx3cr1$^{EYFP\text{-}CreER/+}$ and Cx3cr1$^{EYFP\text{-}CreER/+}$:R26$^{iDTR/+}$ mice after microglia ablation during exposure to EtOH and H_2O. GAPDH (loading control). Data are means ± SEM from five micer per condition pooled acroos three independent experiments. *P<0.05 by two-way ANOVA with Sidak's multiple comparisons **(B).**

As a complement, a biochemical assessment confirmed that EtOH intake decreased, in a microglia-dependent manner, the amounts of PSD-95 and vGlut1 in the PFC **(Figure 11B)**, indicating that TNF production by microglia results in loss of excitatory synapses in the PFC following alcohol intake.

However, the loss of synapses can also be a consequence of increases in neuronal death. Therefore, an histological analysis was performed, showing that the number of neurons (stained with NeuN) in PFC was not significantly different when comparing EtOH-subjected and water-treated mice **(Figure 12).**

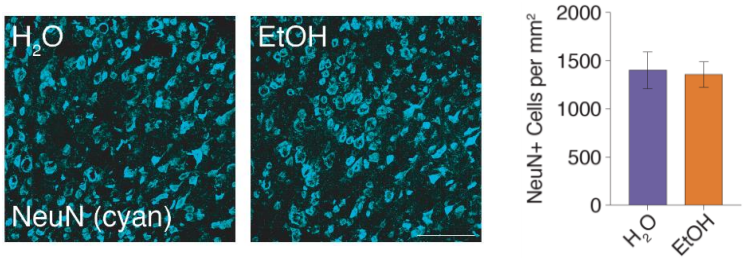

Figure 12 - **Alcohol induces synapse loss without affecting neuronal numbers**. Histological confocal analysis of NeuN on tissue sections from prefrontal cortices of $Cx3cr1^{EYFP\text{-}CreER/+}$ mice exposed to EtOH or H_2O (n = 5 mice per condition). Graph (mean and SEM) shows the number of $NeuN^+$ cells. Statistical comparation was performed using Mann-Whitney test. Scale bar: 50 µm

In pathologies, such as in Alzheimer's disease, microglia can phagocytose and prune healthy synapses, a process named synaptophagy (Vilalta & Brown, 2018). Accordingly, it was hypothesized that the EtOH-mediated loss of excitatory synapses in the PFC could be a direct consequence of excessive engulfment of synaptic structures by microglia. To investigate whether microglia exposed to EtOH engulfed more synapses in vivo, the amount of $PDS\text{-}95^+$ puncta within phagocytic structures (labeled with CD68) in $Iba1^+$ microglia was evaluated by immunofluorescence on PFC tissue sections. Confocal imaging coupled with 3D cell surface rendering revealed that $Iba1^+$ microglia from the PFC of mice exposed to alcohol contained significantly more $PSD\text{-}95^+$ puncta colocalizing with CD68 structures than PFC microglia from mice treated with water **(Figure 13A)**.

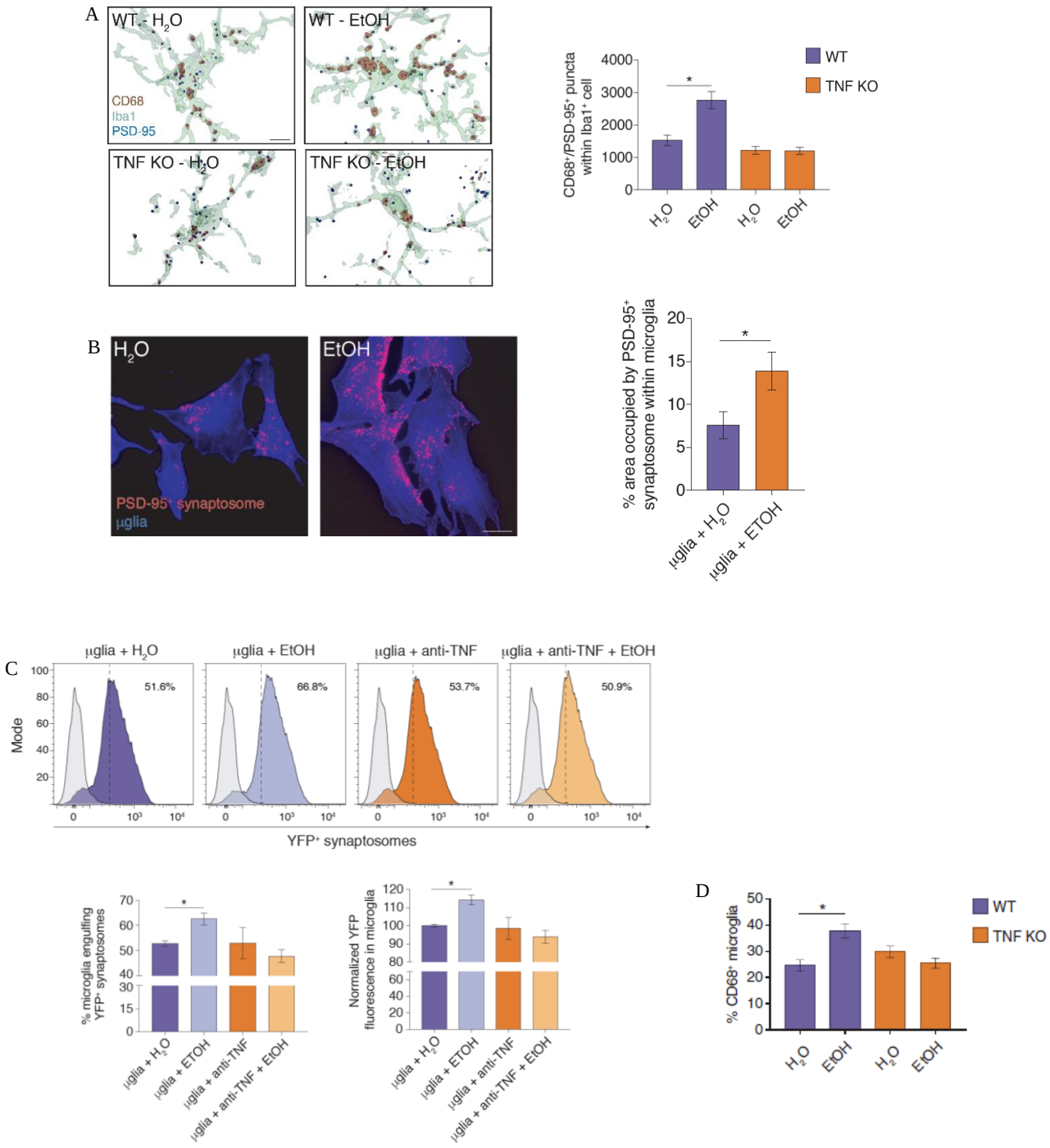

Figure 13 - **Alcohol intake enables microglia to prune synapses.** Representative Imaris 3D surface rendering of confocal maximum projection images showing volume reconstruction of PSD-95 within CD68 structures in microglia (Iba1$^+$ cell) on tissue sections from prefrontal cortices of WT and TNF KO mice after exposure to EtOH or H_2O. Scale bar: 5 µm. Data are means ± SEM from 12 microglia per condition derived from four mice pooled across three independent experiments. **P*<0.05 by two-way ANOVA with Sidak's multiple comparisons **(A)**. Imunnofluorescence images of PSD-95 labeled prefrontal cortex synaptosomes (red) inside cultured CHME3 microglia labeled with CellMask bye (blue). Scale bar, 20 µm. Data are means ± SEM from 62 cells per condition from three independent experiments. **P*<0.05 by unpaired *t* test **(B).** Representative flow cytometry profiles for YFP labeling in CHME3 microglia incubated for 24 hours with synaptosomes isolated from the prefrontal cortex of Thy1-YFP mice. In somes conditions, microglial cultures were pretreated for 24 hours with EtOH (70 mM) or with TNF blocking antibody adalimumab (5 µg/ml). Engulfment ability of microglia was caculated by comparing the percentage (left graph) or the normalized MFIs (right graoh) of microglia expressing high YFP signa. Data are means ± SEM from six independent experiments per condition. **P*<0.05 by two-way ANOVA with Sidak's multiple comparison **(C).** Flow cytometry analysis of CD68 expression in microglia (gated as CD45^{mid}CD11b$^+$ cell population) from prefrontal cortices of Cx3cr1$^{EYFP\text{-}CreER/+}$ mice exposed to EtOH or H_2O. Data are means ± SEM from five mice per condition pooled across three independent experiments. **P*<0.05 by two-way ANOVA with Sidak's multiple comparisons **(D)**.

This microglial engulfment of PFC postsynaptic components required TNF signaling since no significant differences in the engulfment of PSD-95^{+} puncta were observed in the TNF KO mice exposed to EtOH. **(Figure 13A)**.

To further assess synapses' engulfment by microglia, synaptosomes (to isolate synaptic terminals) from the PFC of adult mice were prepared, and microglial cultures were incubated with them. Indeed, immunofluorescence labeling of PSD-95 confirmed that microglia efficiently engulfed synaptosomes prepared from the PFC, further confirming that microglia actively phagocytose synapses in steady-state conditions. Moreover, cultured microglia exposed to EtOH engulfed significantly more PFC synaptosomes than control microglia. **(Figure 13B)**. To further corroborate the excessive EtOH-mediated engulfment of synaptic structures, microglia cultures were incubated with synaptosomes prepared from the PFC of the Thy1-YFP ice. Measuring microglia engulfment capacity by flow cytometry revealed that microglia exposed to alcohol displayed significantly increased engulfment of YFP^{+} synaptosomes elicited by EtOH exposure **(Figure 13C)**.

In line with the view that EtOH intake increases microglia engulfment capacity, mice exposed to EtOH displayed a TNF-dependent increase of PFC microglia expressing the phagocytic marker CD68 **(Figure 13D)**.

Chapter 5

Discussion

5. Discussion

Neuroinflammation is regarded as a major contributing factor in alcohol-induced brain damage (Kane & Drew, 2016; M. Pascual, J. Montesinos, M. Marcos, J.-L. Torres, P. Costa-Alba, F. García-García & Guerri, 2017; Montesinos et al., 2016). Nevertheless, the mechanisms underlying these effects remain uncertain. Although various studies show that alcohol exposure induces microglia activation (Fernandez-Lizarbe et al., 2009), here we demonstrated increased microglial reactivity upon an alcohol exposure protocol simutaling binge drinking, which differ from studies with binge with heavy alcohol intake (Lippai, Bala, Csak, et al., 2013; Lippai, Bala, Petrasek, et al., 2013b).

Microglia activation promote the production of ROS (Nadka I. Boyadjieva and Dipak K. Sarkar, 2018; Qin & Crews, 2012) and consequent oxidative stress. Indeed, microglia activation following acohol intake is typically defined as a proinflammatory response with the release of proinflammatory mediators (mainly TNF), inducing these cells to acquire a neurotoxic profile. In line with that, although our microglia ablation experiments demonstrated that microglia were the major producers of TNF in the prefrontal cortex during alcohol exposure, we cannot exclude that TNF produced in the periphery (namely by Kupffer cells, known as the most representative myeloid cells in the liver and the major producers of cytokines in case of Alcoholic Liver Disease (ALD) (Cubero & Nieto, 2006; Suraweera, 2015)) has reached the brain and also impacted the activation of microglia. Besides, even astrocytes in response to alcohol may also have contributed to the observed microglial phenotype, although we further assessed that in our paradigm of alcohol exposure and found no overt astrocytic reactivity in the prefrontal cortex. TNF, as an inflammatory cytokine, can indirectly promote neurotoxicity by stimulating microglia in an autocrine/paracrine manner (H. Takeuchi & Suzumura, 2014). Moreover, this autocrine activation of TNF receptors triggers the release of glutamate by microglia, causing excitotoxicity, hence demaging neurons (Pickering et al., 2008; H. Takeuchi et al., 2006). After assessing whether the release of TNF was microglia-dependent, we asked whether the TNF release impacted microglial activity and engulfment capacity. Our *in vivo* experiments using TNF deficient mice were important to demosntrate that this cytokine indeed modulated the alcohol effects in the prefrontal cortex.

In this context, the prefrontal cortex contains glutamatergic neurons that synapse locally or project distally to cortical and subcortical nuclei. Therefore, any failure in setting up and sustaining proper excitatory connectivity leads to imbalanced neuronal activity across networks (Haider et al., 2006), possibly explaining at least some of the behavioral impairments found in various neurologic disorders (Li et al., 2013). Accordingly, the loss of prefrontal cortex excitatory synases elicited by alcohol could be sufficient to disrupt the excitatory/inhibitory balance of prefrontal cortex neurons projecting to anxiety-related centers in subcortical regions,

implying that alcohol-induced prefrontal cortex synapse loss might have directly impacted the alcohol-related anxiety-like behavior. Although it is plausible that the microglia-driven synaptic changes in the prefrontal cortex could be causing anxiety-like changes, our data do not formally exclude the possibility that microglia could be directly activated in other brain regions associated to anxiety, such as the amygdala, eliciting the anxiogenic effect of alcohol.

Microglia participate in synaptic remodeling by phagocytic engulfment of synaptic terminals (Weinhard et al., 2018). We found that alcohol exposure increased the engulfment of synaptic structures by microglia likely due to enhancing their phagocytic capacity. On the contrary, alcohol decreases microglia phagocytosis of E. coli (Aroor & Baker, 1998) and of amyloid (Gofman, 2014) and also suppresses microglia phagocytosis stimulated by activation of P2X4 receptors (Kalinin et al., 2018), thereby suggesting that alcohol might alter microglia phagocytic capacity in a context-specific manner. Additionally, microglia clearance activity varies significantly across different brain regions (paper da Ayata and Schaffer na Nat Neurosci.). However, microglia clearance activity may be different depending on the brain reagion, being conseivable that prefrontal crotex microglia were more prone to engulf and prune synapses than their hippocampal counterparts upon exposure to alcohol. Reagrding this, identifying the precise mechanisms underlying microglial regional differences warrant future in depth studies focusing on the characterization of microglia heterogeneity.

The phagocytic engulfment of healthy synapses by microglia can result in synapse loss in the brain (Vilalta & Brown, 2018), and in Alzheimer's disease it can contribute to early disease pathology (Karabiyik et al., 2018). The fact that EtOH licensed microglia to prune prefrontal cortex sunapses in the absence of significant alterations in the number of neurons in the prefrontal cortex also suggests that aberrant synaptic pruning by microglia might be an early hallmark asociated with neurotoxicity during alcohol abuse. This milestone might be important for early detection and possibly prevention of long-term neuronal loss and synaptic dysfunction between different brain regions exposed to alcohol.

Furthermore, by feeding cultured microglia with synaptosomes, we reinforced the increased engulfment capacity of these cells during alcohol exposure. In vivo, using triple staining to label phagocytic stuctures in prefrontal cortex tissue sections, we also demonstrated that microglia exposed to EtOH engulfed more prefrontal crotex postsynaptic elements, which provides important new mechanistics evidence into how the brain immune system might contribute to the impairment of synaptic transmission, a major detrimental consequence of alcohol abuse. Thus, our study opens new possibilities to clarify and further investigate new mechanisms underlying synaptic pruning, from which possible therapeutic targets and preventive strategies may arise for alcohol abuse-induced brain damage.

A caveat of the present study is that the observed phenotypes were only reported for male mice. Sex differences underlie substantial transcriptional differences between female and male

microglia (Villa et al., 2018), which together with the fact that female microglia are more prone to develop alcohol-related inflammation and neuronal damage (Alfonso-Loeches et al., 2013; M. Pascual, 2017), indicate that further studies using female mice are required to understand better the contribution of EtOH exposure to microglia-dependent synaptic pruning and synapse function.

Microglia-mediated neurotoxicity damages the CNS parenchyma (Kempermann & Neumann, 2003) during alcohol abuse and neurologic diseases. Therefore, inhibition of microglial activation has been considered as a putative therapeutic approach to manage neuropathologies. Notwithstanding, microglia are also known for their neuroprotective functions, through the release of neurotrophic factors, clearance of neurotoxic substances and cell debris, and excess glutamate uptake (Kempermann & Neumann, 2003; Kipnis et al., 2004; Schwab & Schluesener, 2004). Although challenging, to be a viable therapeutic strategy, only the detrimental effects of microglia activation should be targeted, without compromising their beneficial functions.

Chapter 6

Concluding Remarks

6. Concluding Remarks

As suggested by the main title, this work aimed at shedding light into some of the mechanisms under the aberrant synaptic pruning by microglia following alcohol intake. The association of two well established *in vivo* models of microglia ablation and TNF sustain, to accurate assess alcohol-elicited influence under both microglial reactivity and TNF expression, respectively. Additionally, the same models with the association of an *in vitro* analysis, allowed the evaluation of the engulfment capacity of microglial cells. Accordingly, in the course of this work, different hypothesis were progressively tested, with our results retrieving the following main conclusions:

1. Moderate ethanol exposure does increase microglia number and reactivity.
2. TNF expression increases following ethanol exposure.
3. TNF increased expression is microglai-dependent.
4. Our ethanol exposure paradigm induces synapse loss without affecting neuronal numbers.
5. Alcohol intake enable microglia to prune synapses.

Overall, alcohol intake enable active microglia to prune synapses.

To the best of our knowledge, this study is pioneer in demonstrating specific microglia engulfment capacity following alcohol misuse, considering that our data pointed out some mechanistic features involved in the immune response involved in synapse loss. Our study further demonstrates clear and robust data showing microglia feeding itself with postsynaptic structures from excitatory synapses. However, a more in-depth analysis of mechanisms underlying synapse prune which remain to be determined. Moreover, this study is focus only on excitatory synapses, so a similar analysis investigating changes in inhibitory synapses would be a great addition in the future. A step further on alcohol influce on neuroimmune response and more realistic in term of future clinical approach would be an in depth-study of a chronic module of alcohol intake along with complement simutation of prolonged withdrawal.

The experiments I performed for this work were included in a scientific paper recently published on Science Signaling.

Title: *Daily alcohol intake triggers aberrant synaptic pruning leading to synapse loss and anxiety-like behavior*

1st author: Renato Socodato

Manuscript number: 10.1126/scisignal.aba5754

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