Mechanisms of Alcohol Modulation of Microglial Function

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Resumo

O álcool é uma das substâncias de abuso mais consumidas a nível mundial. Doenças associadas ao uso do álcool afetam milhões de pessoas em todo o mundo causando enormes danos sociais e económicos na sociedade moderna. O consumo excessivo de álcool ou intoxicação pode provocar danos no organismo induzindo imunossupressão, danos no fígado e doenças neurológicas. No sistema nervoso central (SNC), a exposição ao álcool pode resultar em perda neuronal, défice cognitivo, disfunção motora, inflamação e disfunção das respostas neuroimunes. A microglia, células imunes residentes do SNC, têm um importante papel no dano neuronal causado pela inflamação. Apesar de algumas evidências indicarem que o consumo de álcool promove a inflamação no cérebro levando à ativação da microglia, a verdadeira contribuição destas células na disfunção neuronal e danos comportamentais causados pelo álcool é ainda pouco conhecida. Após um estímulo inflamatório, a microglia torna-se ativada libertando mediadores citotóxicos, como o glutamato, que causam neurodegeneração. Experiências preliminares realizando ensaios com células vivas baseados no mecanismo de Förster Resonance Energy Transfer (FRET) foram efetuadas de forma a compreender os eventos de sinalização associados à libertação de glutamato por parte da microglia induzida por etanol no córtex cerebral. Os resultados permitiram concluir que essa libertação requer a ativação contínua da tirosina cinase c-Src visto que a sua ativação forçada mimetizou o efeito do etanol levando à libertação de glutamato, sendo esta prevenida na ausência de c-Src. Além disto, a libertação de glutamato mediada pela associação etanol/c-Src não se verificou em microglia isolada de murganhos deficientes de *Tumor Necrosis Factor* (TNF). Isto indica que o etanol desencadeia a libertação de glutamato através da produção de TNF induzida pela c-Src. Os inibidores de hemicanais de *gap junctions* GAP-27 e 18α-Glycyrrhetinic acid (GA) foram utilizados de forma a prevenir a libertação de glutamato pela microglia induzida pelo etanol, uma vez que a microglia ativada liberta grandes quantidades de glutamato através de hemicanais. Os nossos resultados exibem uma ligeira diminuição da libertação de glutamato induzida por etanol na presença do GAP-27, mas não do inibidor 18α-

GA. Estudos *in vivo* envolvendo murganhos *wild-type* (WT) ou deficientes em TNF ($TNF^{-/-}$) expostos a dose semi-crónica de etanol foram desenvolvidos com o objetivo de explorar os aspetos moleculares e comportamentais relativos ao efeito do álcool no cérebro. Testes comportamentais, incluindo paradigmas como *elevated plus maze* e *open field*, mostraram um aumento de ansiedade em murganhos WT expostos a etanol, enquanto que este efeito não se verificou em murganhos $TNF^{-/-}$. Análises de RT-qPCR revelaram que o etanol é capaz de induzir alterações em alguns genes relacionados com a imunidade e com a identidade da microglia em cérebros de murganhos WT, mas não em deficientes em TNF. Por outro lado, utilizando as técnicas *Western Blot* e Imunohistoquímica não foi possível observar quaisquer diferenças significativas no número de células da microglia e de astrócitos entre o controlo e cérebros tratados com etanol. Globalmente, os nossos resultados revelam existir um potencial papel desempenhado pelo TNF na modulação da função da microglia e danos comportamentais.

Abstract

Alcohol is one of the most consumed substances of abuse in the world. Alcohol use disorders affect millions of people worldwide causing huge social and economic burden on modern society. Excessive alcohol consumption or intoxication can provoke severe damage to the body inducing immune suppression, liver damage and neurological disorder. In the central nervous system (CNS), alcohol exposure can lead to neuronal loss, cognitive decline, motor dysfunction, inflammation and impairment of neuroimmune responses. Microglia, the immune resident cells of the CNS, are major players in inflammation-induced neuronal damage. Although several lines of evidence indicate that alcohol intake will promote brain inflammation driving microglia activation, the *bona fide* contribution of microglia in alcohol-mediated neuronal dysfunction and/or behavioural impairment is still poorly understood. After inflammatory stimuli, microglia become activated releasing cytotoxic mediators like glutamate, which might cause neurodegeneration. Preliminary experiments using Förster Resonance Energy Transfer (FRET)-based live cell were performed to dissect the signaling events associated with ethanol-induced glutamate release from cortical microglia. We report that such release requires sustained activation of the tyrosine kinase c-Src because forced c-Src activation mimicked the ethanol effect triggering glutamate release and the knockdown of c-Src abrogated it. In addition, the ethanol/c-Src-mediated glutamate release was prevented in microglia isolated from Tumor Necrosis Factor (TNF) deficient mice, indicating that ethanol triggers glutamate release via c-Src-induced TNF production. GAP-27 and 18α-Glycyrrhetinic acid (GA) gap-junction hemichannels inhibitors were used to prevent the ethanol-induced microglial glutamate release, because it was found that activated microglia release large amounts of glutamate through hemichannels. Our results demonstrate a slight decrease in ethanol-triggered glutamate release with GAP-27 but not with 18α-GA. *In vivo* studies comprising semi-chronic ethanol-exposure in wild-type and TNF deficient mice were also performed aiming at explore molecular and behavioural aspects of the alcohol effects on the brain. Behavioural tests including elevated plus maze and open field paradigms showed an

increase in anxiety-like behaviour in wild-type mice exposed to ethanol, whereas this ethanol effect was not found in TNF deficient animals. RT-qPCR analysis revealed that ethanol induced changes in some immune- and microglial signature transcripts in the brains of wild-type but not on those of TNF deficient mice. Besides, western blot and immunohistochemistry showed no significant differences in the levels of microglial and astrocytic cells between control and ethanol-treated brains. Overall, our results reveal a potential role for TNF in alcohol modulation of microglial function and behavioural impairment.

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Abbreviations

CNS = Central Nervous System

FRET = Förster Resonance Energy Transfer

18α-GA = 18α-Glycyrrhetinic acid

AUD = Alcohol Use Disorders

FASD = Fetal Alcohol Spectrum Disorders

EtOH = Ethanol

ROS = Reactive Oxygen Species

GSH = Glutathione

NF-κB = Nuclear Factor-kappa B

IL = Interleukin

NO = Nitric Oxide

TNF = Tumor necrosis factor

TGF-β = Transforming growth factor beta

DAMP = Danger-associated Molecular Patterns

TLR = Toll-like Receptor

RAGE = Advanced Glycation Endproducts

MAPK = Mitogen-activated Protein Kinase

ATP = Adenosine Triphosphate

MCP-1 = Monocyte Chemoattractant Protein-1

IFN-γ = Interferon gama

EAAT = Excitatory Amino Acid Transporter

Cx = Connexin

GLT-1 = Glutamate Transporter 1

$TNF^{-/-}$ mice = TNF knockout mice

EPM = Elevated plus-maze

OF = Open Field

NOR = Novel Object Recognition

LPS = lipopolysaccharide

H_2O_2 = hydrogen peroxide

GFAP = Glial Fibrillary Acidic Protein

CHAPTER 1 | Introduction

Alcohol

Alcohol abuse and addiction

Alcohol is a psychoactive substance that potentially causes dependence upon harmful and abusive consumption (Koob and Volkow, 2010; Marshall et al., 2013; Ofori-Adjei D et al., 2007). In 2012, alcohol contributed to 5.9% of all global deaths (roughly accounting for 3.3 million deaths). Presently, alcohol is considered a causal factor in more than 200 diseases and injury conditions worldwide (WHO, 2014). Consumption of alcohol is part of many cultural contexts for thousands of years, already existing in aboriginal and rural communities prior to the modern era (McGovern, 2009). Outside of well-established cultural contexts, alcohol use usually becomes a health and social problem. In addition, alcohol consumption is an upward trend and socially well-accepted to attenuate negative events and enhance positive mood states. The most recent World Health Organization (WHO) data shows a high rate of alcohol consumption per capita with a huge variation around the world **(Fig. 1)**. Despite the benefits of mild-to-moderate alcohol consumption (Yang et al., 2014a), recurrent drunkenness with

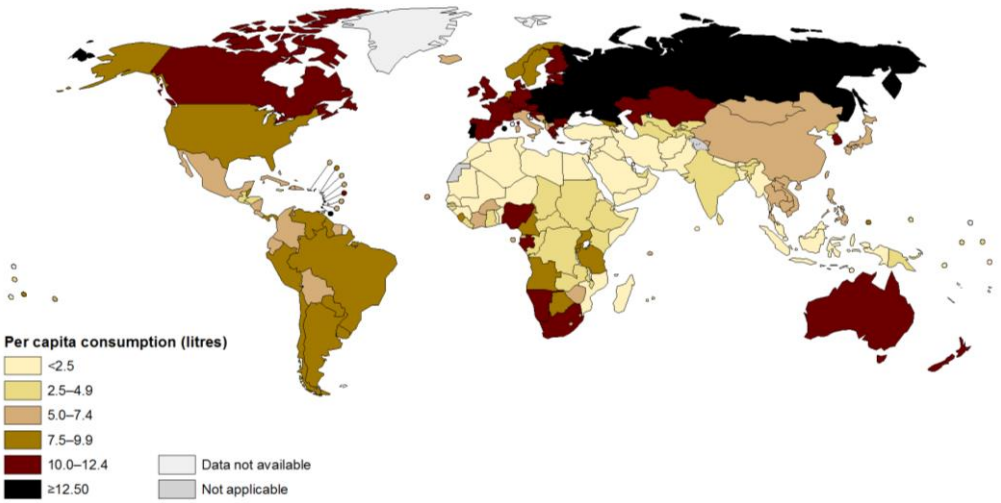


Figure 1. Total alcohol per capita (15+ years) consumption, in liters of pure alcohol, 2010.
Individuals above 15 years of age consume on average 6.2 litres of pure alcohol per year (about 15.3 grams of pure alcohol per day). The highest consumption levels of alcohol are found in the developed world, essentially in European and American regions (WHO, 2014).

abnormal high levels of binge drinking affects the physiology, structure and function of several organs including the liver, stomach, kidney, lungs and brain (Yang et al., 2014a). Therefore, alcohol use and abuse affects several aspects of modern society and is related, but not limited, to public health, to public safety and to the economy in general (Spanagel, 2009).

Due to its preventable nature, for many years alcoholism was not conventionally categorized as a neurodegenerative disorder, but chronic or excessive alcohol consumption causes damage to the temporal lobe similarly to neurodegenerative disorders such as Alzheimer's disease (Marshall et al., 2016). The societal debate whether alcoholism is a curable disease or simply an intrinsic character flaw continues today even with the unquestionable existence of evidence showing alterations in brain structure and function in alcoholics (Schomerus et al., 2011). It was proposed that typical drunkenness or alcoholism deeply changes the neurobiology of individuals (Gunzerath et al., 2011). Fortunately, the perception that alcoholism is not a disease is gradually changing. The understanding of alcoholism as a mental disorder facilitates treatment for individuals who suffer from this condition and encourages clinicians to counsel the use of pharmacotherapies.

Alcohol-associated disorders

Chronic alcohol abuse is intrinsically associated with several primary and secondary disease states, usually starting as an experience and leading to gradual progression of addiction over years of high consumption (Spanagel, 2009). High alcohol consumption produces: i) toxic effects to organs and tissues; ii) intoxication, with physical, behavioural and cognitive impairments; and iii) dependence without drinking self-control (Babor et al., 2003). The main feature of alcohol dependence is the withdrawal symptomatology (occurring from few hours to several days after reducing or interrupting the intake of alcohol), which can lead to hyperactivity, tremor and anxiety, sporadically accompanied by hallucinations and delirium (Cami and Farre, 2003; De Witte et al., 2003; Sullivan and Pfefferbaum, 2005). Under withdrawal, alcoholics often develop coping behaviours to avoid related disturbances, which

eventually leads to restarting the intake of alcohol. Heavy alcohol ingestion and the effective risk of development of alcohol addiction is strongly increased by factors associated with numerous psychiatric manifestations and personality traits such as anxiety-related disorders and depression (Kelley and Dantzer, 2011). In such conditions, patients gradually present different responses to global environmental challenges and display great propensity to seek reward in alcohol, becoming addicted upon excessive exposure (Cloninger, 1987). In fact, according to WHO, harmful use of alcohol or dependence is present in 6% of patients in primary health care offices suffering from mental illness (Blanco and Guerri, 2007). Importantly, mental disorders may promote or aggravate the development of alcoholism and vice versa, due to an existing mutual interaction. In addition to the mental condition interaction, alcohol is thought to be a major cause of gastrointestinal, cardiovascular and infectious diseases, because it impacts directly on hepatic function and weakens the overall “health” of the individual (WHO, 2014).

Problems related to alcohol misuse have been combined giving rise to alcohol use disorders (AUDs). Based on the *DSM-5* criteria, eleven symptoms are used for diagnosing AUDs and their severity levels are classified as mild, moderate, or severe depending on the number of criteria met (Hasin et al., 2013). Similar to other addictive contexts, AUDs’ features include strong desire toward alcohol consumption, impaired drinking control, development of tolerance, development of signs of physiological withdrawal and worry about drug of choice, with possibility of relapse even after several years of abstinence (Grant et al., 2007). In another context, Fetal Alcohol Spectrum Disorders (FASD) are a group of heterogeneous conditions characterized by the development of serious and long-lasting physical, behavioural and learning impairments in the offspring due to maternal alcohol use during pregnancy (Guizzetti et al., 2014). Less severe variations of FASD include birth defects and behavioural disorders related with alcohol (Riley and McGee, 2005). More extreme manifestations are characterized by pre- and post-natal growth retardation, developmental delay, craniofacial anomalies and

intellectual deficiencies (Jones, 2011). Searching for a preventive treatment for these disorders is therefore urgent and of paramount significance.

Alcohol-induced neurodegeneration and cognitive deficits

Alcohol is one of the most well-known neurotoxic agents whose abusive consumption causes neuroinflammation, myelin damage and oxidative stress, contributing for brain damage and neurodegeneration in humans (Alfonso-Loeches et al., 2016; Marshall et al., 2016). Neurotoxicity to the hippocampus and in the cerebral cortex, two main brain regions suffering from the harmful effects of alcohol (Harper and Matsumoto, 2005), is linked to a variety of cognitive and motor deficits in alcoholics, including learning and memory impairment, attention deficits, reduction of motor coordination and impulse-control problems (Alfonso-Loeches and Guerri, 2011; Sullivan et al., 2000; Yang et al., 2014a). The brain is a key target of alcohol effects and even alcoholics with no apparent neurological or hepatic problems reveal signs of regional brain damage and cognitive dysfunction with clear alcohol-dependent effects on nerve cells (Harper and Matsumoto, 2005).

Oxidative stress is proposed as an important mechanism in alcohol-induced brain damage (Qin and Crews, 2012). The formation of reactive oxygen species (ROS) occurs intracellularly in various tissues following ethanol (EtOH) exposure and free radicals can react chemically with key cellular macromolecules, which may lead to cell dysfunction and loss (Reynolds et al., 2007; Sorce and Krause, 2009; Wu and Cederbaum, 2003). Mitochondrial injury, which will further disturb components of the antioxidant pathway such as glutathione (GSH), also increases the susceptibility of the brain to oxidative damage upon alcohol exposure (Boyadjieva and Sarkar, 2013).

Several studies describe the manifestations of alcoholism on brain structure and function, which include loss of dendrites, atrophy and degeneration of cortical neurons (Alfonso-Loeches and Guerri, 2011). These events lead to the enlargement of the lateral ventricles, cortical thinning in the temporal lobes, and decrease of glial cell numbers in both the temporal

and frontal cortices (Blanco and Guerri, 2007; Crews, 1999). In fact, heavy alcohol consumption results in loss of brain mass and function apparently due to a reduction of white matter, primarily in the frontal lobe, being the degree of brain atrophy highly correlated with the amount of alcohol consumed over the lifetime (de la Monte, 1988; Kril and Halliday, 1999).

Neuroinflammation is an important mechanism in the response to pathogenic events, traumatic brain injury and environmental toxins, but it is also known as a major contributor in various neurological and neurodegenerative disorders (Glass et al., 2010; Lucas et al., 2006; Ransohoff et al., 2015). Alcohol increases the host vulnerability to infections by drastically altering the immune response (Crews et al., 2015; Kelley and Dantzer, 2011). Neuroinflammation emerges from the imbalance in the expression of a variety of inflammation-associated genes and cytokines involved in innate immune responses (Crews et al., 2011). For example, chronic EtOH exposure increases innate immune signaling through the activation of the pro-inflammatory transcription factor nuclear factor-kappa B (NF-κB) (Crews et al., 2015; Crews et al., 2011). Whereas a variety of pro-inflammatory signals are associated with increased EtOH drinking and preference (Blednov et al., 2012), peripheral inflammation increases voluntary ethanol intake and administration of anti-inflammatory agents reduces EtOH consumption (Blednov et al., 2012; Marshall et al., 2013). In this context, close interactions occur between different CNS cell types – neurons, astrocytes, microglia and oligodendrocytes – and microglia and astrocytes appear to be pivotal in CNS immunity because they are the primary mediators of neuroinflammatory responses in the brain (Hughes, 2012; Lucas et al., 2006; Salter and Beggs, 2014; Yang et al., 2014a). Although neuroinflammation is thought to be a key factor in alcohol-induced neurodegeneration, remarkably little is known about the direct effects of EtOH on these immune cells, particularly in microglia. Understanding how excessive alcohol consumption results in microglial dysfunction and/or microglia overactivation, and consequent immune cell dysregulation and chronic degeneration, is crucial for the development of strategies aiming at preventing or attenuating neuronal loss and brain damage.

Microglia

Microglia in the central nervous system

Microglia are a type of non-neuronal cell within the CNS, being the brain's myeloid resident cells and representing approximately 10-15% of the total cell population in the adult CNS (Benarroch, 2013). These immune-like cells participate in the maintenance of neuronal homeostasis, monitoring their microenvironment for potential hazards and protecting the brain from trauma and diseases (Azevedo et al., 2013; Kim and de Vellis, 2005; Salter and Beggs, 2014). Microglia are also considered to be an important cellular component involved in the inflammatory response, and may also contribute to the development of chronic inflammation associated with various neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, among others (Neumann et al., 2009; Salter and Beggs, 2014). By becoming phagocytic and contributing to the elimination of toxic cellular debris, microglia also play major roles in resolving brain infections and in the progression of brain diseases (Hong and Stevens, 2016). During CNS development, these cells are involved in refining brain wiring and synaptic circuits (Paolicelli et al., 2011; Schafer and Stevens, 2015; Wu et al., 2015). In the immature brain, microglia are intensively involved in the phagocytosis of apoptotic neurons, stimulation of programmed cell death and synaptic pruning (Guizzetti et al., 2014).

To carry out such diverse functions, microglia respond to almost all types of CNS injury by switching from a surveilling to an activated state, which leads to the acquisition of macrophage-like markers and effector properties varying according to the type of insult and the inflammatory or immune context (Hughes, 2012; Salter and Beggs, 2014). Sustained, uncontrolled and/or chronically activated microglia may initiate inflammatory processes that lead to excessive secretion of various molecules (Graeber, 2010). Excess of these mediators contributes to neuronal damage by triggering oxidative damage and potentiating the activation of cell-death pathways (Azevedo et al., 2013; Thomas et al., 2004). Because microglia activation is a graded

process, there are morphological differences displayed by these cells accompanied by changes in cell surface proteins that result in corresponding alterations in cytokine and growth factor secretion (Benarroch, 2013). Altogether, variations in these features can be used to categorize microglia in an effort to understand their function in the CNS under pathological conditions. Thus, close monitoring of microglial activation in the CNS is essential to maintain the balance between homeostasis and pathophysiology.

Microglia morphology

In normal conditions (at the steady state), microglia are in a resting, quiescent state (Nimmerjahn et al., 2005). In this non-active mode, they have thin, highly ramified processes that extend from a compact cell body and are highly motile, allowing them to constantly survey their environment and modulate neuronal activity (Boche et al., 2013). Microglia activation by exogenous stimulus alters their resting morphology into a bushy-like shape characterized by the thickening and shortening of their processes with concomitant enlargement of the cell body (Ábrahám and Lázár, 2000; Nimmerjahn et al., 2005). Upon more intense activation, microglia might lose their thickened processes, becoming rounded, which is usually called amoeboid microglia **(Fig. 2)**. However, morphology alone is not synonym for functional microglia activation because alterations in microglial gene expression profile can occur in the absence of obvious morphological transitions (Marshall et al., 2013). Mere observation of the ramified or amoeboid morphology therefore does not to tell everything about the real nature of microglia activation phenotype (Kettenmann et al., 2011).

Microglia activation

Microglia become activated in response to various stimuli including neuronal damage, noxious agents, astrocytic reactivity, and even slight changes in environmental signals like fluctuations on ionic concentrations (Lai and Todd, 2008). However, microglia activation varies with the extent, type and duration of an insult, ultimately rendering microglia in a completely

different phenotype, which will be consistent with their state of activation (Boche et al., 2013; Kettenmann et al., 2011; Salter and Beggs, 2014).

The terms used to describe distinct microglia activation phenotypes are different within each classification system, including M1 versus M2, classical versus alternative and pro-inflammatory versus anti-inflammatory (Marshall et al., 2013). Classical activation (M1 state) consists of phagocytic phenotype in response to pathogens, damaged neurons or lymphocytes, and corresponds to amoeboid microglia that secretes pro-inflammatory cytokines (such as interleukins (IL)-1β, IL-6, IL-23, and Tumor necrosis factor (TNF)) in addition to cytotoxic substances (such as ROS and nitric oxide (NO)) (Raivich et al., 1999). Alternative activation (M2 polarization) are used to describe bushier ramified microglia responding to apoptotic cells or to lymphocytes, triggering an anti-inflammatory response via the release of IL-10 and Transforming growth factor (TGF)-β or, otherwise, promoting tissue repair by producing, for example, extracellular matrix proteins **(Fig. 2)** (Benarroch, 2013; Boche et al., 2013; Kettenmann et al., 2011).

Pro-inflammatory microglia are generally thought to be associated with neuroinflammation-induced neurodegeneration but such phenotype does not always result in excessive damage and can also be associated with recovery (Hanisch and Kettenmann, 2007). However, chronic activation of microglia is associated with neuronal loss and has been proposed to contribute for the progression of neurodegenerative diseases (Guizzetti et al., 2014). Because microglia advance stepwise through their various phenotypes, it is necessary to carefully analyze phenotypic hallmarks within a given disease state before attaining their specific role either in repair processes or neurodegeneration (Marshall et al., 2013).

Regulators and effectors of microglial functions

Surveillance and effector function of microglia are regulated by a multitude of receptors, ion channels, adhesion molecules, transporters and intracellular enzymes present in these

cells (Salter and Beggs, 2014). Besides, microglia also secrete various effector molecules that will further modulate their function.

Different types of channels and transporters, including potassium, calcium and chloride channels, control microglia activation, migration and function through events such as calcium release from the endoplasmic reticulum in response to, for instance, G protein–coupled receptors activation, or external influx through Ca^{2+} channels (Skaper, 2011).

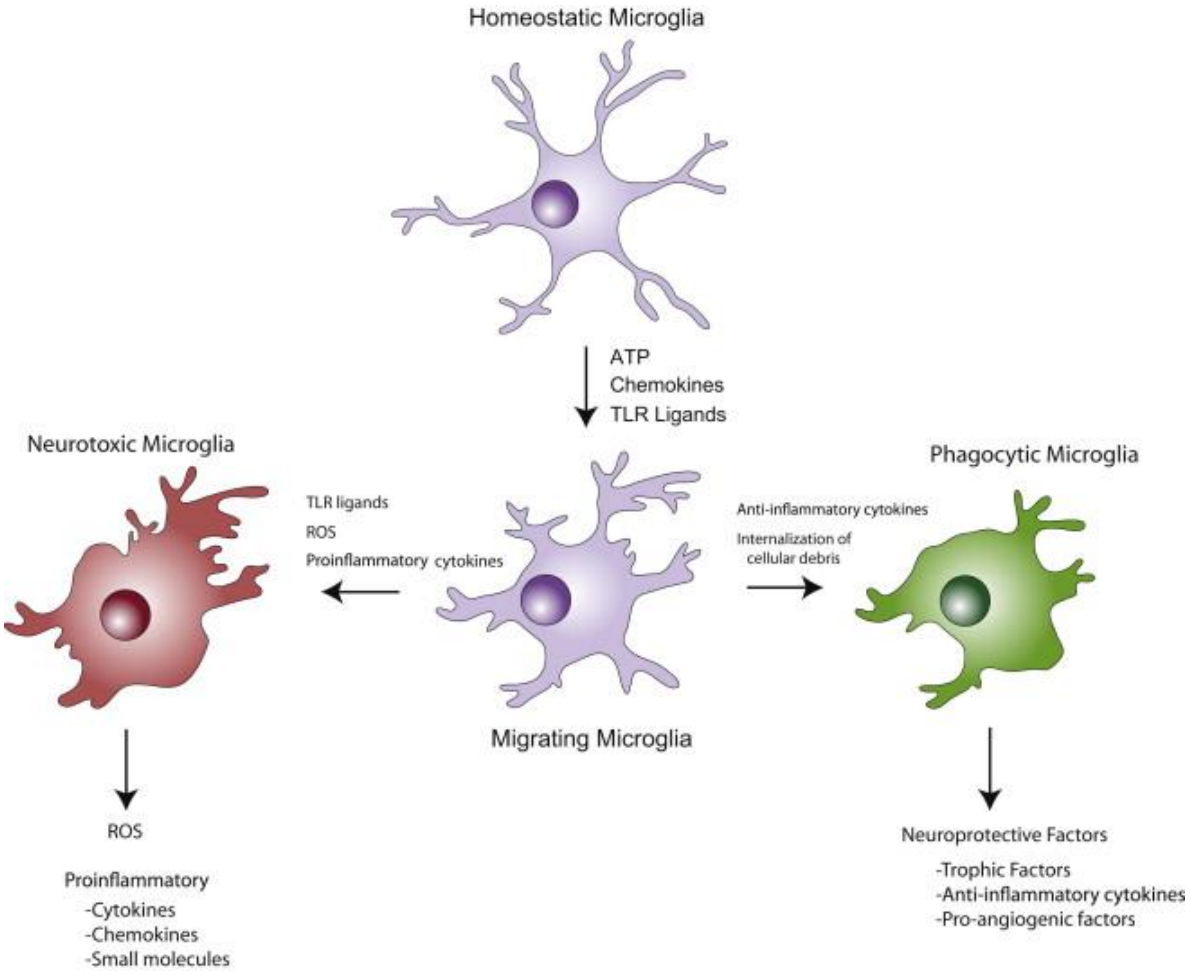


Figure 2. Distinct roles of microglia in the CNS.
In the healthy CNS, microglia in a "resting state" survey the surrounding environment and express little-to-none inflammatory mediators. After exposure to a number of extracellular signals (damaged neurons for instance), microglia rapidly migrate to the injury site. Activated microglia may assume a pro-inflammatory state (shown in red), releasing molecules such as pro-inflammatory cytokines and ROS aimed at protecting the milieu against pathogens. Alternatively, microglia may also exhibit an anti-inflammatory behaviour (shown in green), secreting molecules that promote tissue repair and internalizing cellular debris through phagocytosis. Adapted from (Garden and La Spada, 2012).

Microglial receptors for factors including neurotransmitters, cytokines, chemokines and danger-associated molecular patterns (DAMP), directly or indirectly control the different activation states of microglia (Saijo and Glass, 2011; Salter and Beggs, 2014). DAMPs are typical signals recognized by microglia through innate immune response receptors such as toll-like receptors (TLR), receptors for advanced glycosylated end-products (RAGE) and scavenger receptors (Takeuchi, 2010). Some of these signals activate downstream signaling cascades in microglia, including NF-κB and mitogen-activated protein kinase (MAPK) pathways, which can induce the transcription of various pro-inflammatory mediators like cytokines, chemokines and adhesion molecules (Saijo and Glass, 2011). Moreover, neuron- or astrocyte-derived adenosine triphosphate (ATP) activates microglia through P2X and P2Y receptors, which further contributes for these cells to exert their surveillance function (Benarroch, 2013).

Activation of microglia leads to the production of numerous effector molecules that include pro-inflammatory cytokines (*e.g.* IL-1β, IL-6, IL-23 and TNF); anti-inflammatory cytokines (*e.g.* IL-10 and TGF-β); chemokines (*e.g.:* monocyte chemoattractant protein-1 (MCP-1)); growth factors; cytotoxic substances (*e.g.* oxygen free radicals, NO); enzymes (*e.g.* matrix metalloproteinases), among others **(Fig. 2)** (Kettenmann et al., 2011).

Alcohol, Microglia and Neuroimmune System

Ethanol-induced activation of microglia

A role for microglia in alcohol-induced brain damage has been proposed since the 1990s but direct evidence of the relationship between alcohol consumption and microglia activation and the mechanisms involved are poorly established (He and Crews, 2008; Marshall et al., 2013; Streit, 1994). Initially, some investigators suggested that the alcoholism-induced damage is “too chronic” or “too low level” to affect microglia due to the lack of activation evidences (Marshall et al., 2013). However, a significant decrease of glial cells in alcoholic

hippocampus demonstrates that pathological glia-mediated alterations might be associated with alcohol exposure (Korbo, 1999). Other authors claimed that some degree of microglia activation occurs both in animal models of alcohol exposure and in the brains of alcoholics (Ahlers et al., 2015; Fernandez-Lizarbe et al., 2009; Marshall et al., 2016). Although glial cells are critical brain elements, interest in their function only gained relevance recently. In accordance, the effects of EtOH over glial cells were also neglected (Aronne et al., 2011; Yang et al., 2014b; Zhao et al., 2013).

Several studies focused on the morphological alterations and abnormal activation of microglia induced by binge EtOH exposure (McClain et al., 2011; Nixon et al., 2008; Qin and Crews, 2012; Ward et al., 2009). Few days after a binge event, morphological changes in microglia are observed (Nixon et al., 2008). In response to low EtOH exposure, newly formed microglia can preserve their changed morphological features into young adulthood (McClain et al., 2011). Adult rats exposed to high alcohol concentrations following a period of abstinence display strong increase in activated microglia (Ward et al., 2009), while binge alcohol exposure in adolescent rats induces morphological changes in microglia but does not trigger their full activation in the hippocampus (McClain et al., 2011). All these different results indicate that how alcohol affects microglia is not well understood and appears to vary depending on the EtOH-exposure model used.

An earlier study showed that microglial-derived NO provokes delayed neuronal death following acute EtOH injury in the striatum, with neurons gradually disappearing and microglial cells increasing (Takeuchi et al., 1998). In vitro, conditioned medium from EtOH-treated microglia cultures induced neuronal apoptosis depending on the EtOH concentration, which suggests that microglial activation can cause neural impairment (Boyadjieva & Sarkar, 2013b). Taken together, these studies suggest that there is a tight connection between microglia-induced neuronal apoptosis and EtOH-induced neurodegeneration (Yang et al., 2014a).

Neuroinflammation

Neuroinflammation is assumed as one of the mechanisms of alcoholism-induced neuropathology because ethanol-induced neurodegeneration appears to be associated with glial activation and neuroinflammation (Crews et al., 2015). The high possibility of infection occurrence in alcoholics has been proved clinically and experimentally, despite the difficulty in understanding the connection between alcohol consumption, brain infections and dysregulation of immune-resident cells within the brain. In this context, TLRs appear as important host molecules in innate and adaptive immune responses during infections and CNS damage (Alfonso-Loeches et al., 2010; Montesinos et al., 2016). When TLRs activation occurs, there is the release of inflammation-related molecules and cytokines induced by complex intracellular signaling cascades classically associated with inflammation such as the NF-κB pathway (Akira and Takeda, 2004). In addition to the functions in the response to pathogens and clearance of damaged tissues by TLRs, there is evidence for a role for these molecules in neuroinflammation and neurodegeneration (Jin et al., 2008; Okun et al., 2009). In fact, microglial cells can sense invading organisms through various receptors, including TLRs, which seem to be related with microglial activation and neurotoxicity (Block et al., 2007). Among the twelve members of the TLR family, microglia are described to express TLRs 1-9, being reported that TLR4 is critical for microglial response in LPS-induced neurodegeneration (Lehnardt et al., 2003). Moreover, EtOH increases the host vulnerability to infections through disturbance of TLRs signaling responses (Szabo et al., 2005).

In context of EtOH-induced microglial activation, microglial TLR4 appears as a necessary factor in alcohol-induced activation of these cells (Montesinos et al., 2016). EtOH can activate microglia directly, acting as a ligand of TLRs, or by an indirect way, following neuronal damage, with the activation of TLR4 receptors by DAMPs (Alfonso-Loeches and Guerri, 2011; Alfonso-Loeches et al., 2010). The latter event triggers a cyclic EtOH-induced microglial inflammatory response leading to amplification of brain injury (Alfonso-Loeches and Guerri, 2011). There is also evidence suggesting that the TLR4 response could be an important mechanism in EtOH-

induced neuroinflammation (Alfonso-Loeches and Guerri, 2011; Montesinos et al., 2016). In fact, a study aiming at clarifying the role of TLR4 in chronic ethanol-induced brain damage reported that TLR4 depletion, using small interfering RNA or cells obtained from TLR4-deficient mice, abolished the microglial and astrocytic activation and consequent overproduction of inflammatory mediators (Alfonso-Loeches et al., 2010). Therefore, TLR4 seems to be critical for EtOH-induced inflammatory signaling in glial cells. In a previous *in vitro* study, alcohol activates microglia with the stimulation of TLR4 signaling through MyD88-dependent and independent pathways, subsequently producing inflammatory mediators and leading to neuronal loss (Fernandez-Lizarbe et al., 2009). The importance of these receptors was confirmed in the same study when inflammatory response was obliterated in microglial cells from TLR4-deficient mice, which protected neurons from the damage caused by EtOH-activated microglia (Fernandez-Lizarbe et al., 2009). Other studies clarify that the response of TLRs depends on EtOH concentrations (low, moderate or high), treatment duration (acute or chronic) and type of cells and pathogens (Blanco et al., 2005; Fernandez-Lizarbe et al., 2008; Fernandez-Lizarbe et al., 2009).

As already said, after microglial activation induced by EtOH, these cells release a range of factors related to inflammation, such as TNF and other cytokines, that exacerbate neuroinflammation (Crews et al., 2015). Although astrocytes and neurons can also produce TNF, microglia are recognized as the major source of this cytokine during neuroinflammation (Olmos et al., 2014). Excessive amounts of TNF are associated with demyelination and neurodegeneration (Sriram et al., 2006). In fact, in an *in vitro* study, the apoptotic effect of EtOH in cultured hypothalamic neurons is enhanced in the presence of microglia-conditioned media (Boyadjieva and Sarkar, 2010). However, this effect is obliterated with TNF immunoneutralization, indicating the potential involvement of glial NF-κB expression associated with release of TNF, which could lead to neuronal apoptosis. Another report revealed that LPS-activated microglia produce enough amounts of TNF to cause apoptosis of

mouse neural precursor cells involving a mitochondrial pathway regulated by the Bcl-2 family protein Bax (Guadagno et al., 2013).

Neurotoxicity

CNS inflammation, including microglial activation, likely contributes to the neurotoxicity observed in neurodegenerative diseases and excitotoxicity may play an important role in this context (Block et al., 2007; Takeuchi, 2010). Several studies shown that microglia not only are markers of potential neuroinflammation but also might have roles in the other suggested mechanisms of ethanol-induced brain damage such as reduction in neurogenesis, and induction of oxidative damage and glutamate excitotoxicity (Marshall et al., 2013).

The effects of alcohol consumption on the function of the immune system through secretion of multiple pro-inflammatory cytokines have been described (Boyadjieva and Sarkar, 2013). Particularly, the type of cytokines produced by microglia affects neurogenesis and upon microglial pro-inflammatory activation these cells are generally associated with reduced neurogenesis (Monje et al., 2003). For example, when the pro-inflammatory cytokine IL-6 is produced, it results in decreased proliferation (Vallieres et al., 2002) while other pro-inflammatory cytokines, such as interferon (IFN)-γ can dysregulate differentiation, changing the fate of newborn neuronal precursor cells (Walter et al., 2011). On the other hand, it is also known that microglial activation is essential for reactive neurogenesis in response to neuronal damage (DeBoy et al., 2006; Wainwright et al., 2009).

Under chronic or binge alcohol, oxidative stress appears as an important mechanism associated with brain damage (Crews and Nixon, 2009; Thakur et al., 2006). Chronic EtOH intake not only induces the increase of cytokines and inflammatory mediators, but also leads to excessive production of toxic compounds, such as ROS and nitric oxide (Wu and Cederbaum, 2003). The loss of natural counter-balance on ROS production and elimination by cellular antioxidants mechanisms results in mitochondrial dysfunction and increased oxidative and nitrosative stress (Song et al., 2014). Therefore, alcohol-induced neurotoxicity appears to

be associated with a mechanism involving ROS generation after alcohol exposure (Alfonso-Loeches and Guerri, 2011). The so-called by-products of alcohol metabolism lead to a further increase in oxidative stress and neuronal cell death, potentiated by the alcohol-induced decrease of endogenous antioxidant levels **(Fig. 3)** (Alfonso-Loeches and Guerri, 2011). Activated microglia generates ROS extracellulary and intracellulary (Block et al., 2007). Extracellular neurotoxic molecules directly induce neuronal damage while intracellular ones exert their influence in pro-inflammatory processes and cellular homeostasis as second messengers (Reynolds et al., 2007; Terada, 2006). In fact, intracellular ROS have a great influence on survival and pro-inflammatory responses of microglia, being able to affect kinase cascades and activation of transcription factors, consequently influencing the inflammatory response through alteration of gene expression (Rojo et al., 2014). In this context, activated microglia can be a source of ROS by releasing superoxide, hydrogen peroxide and hydroxyl free radicals and such ROS production, like so many other facets of microglia activation, is also directed by pro-inflammatory cytokines such as TNF **(Fig. 3)** (Boyadjieva and Sarkar, 2013). The view that microglia are relevant players in oxidative stress further implicates

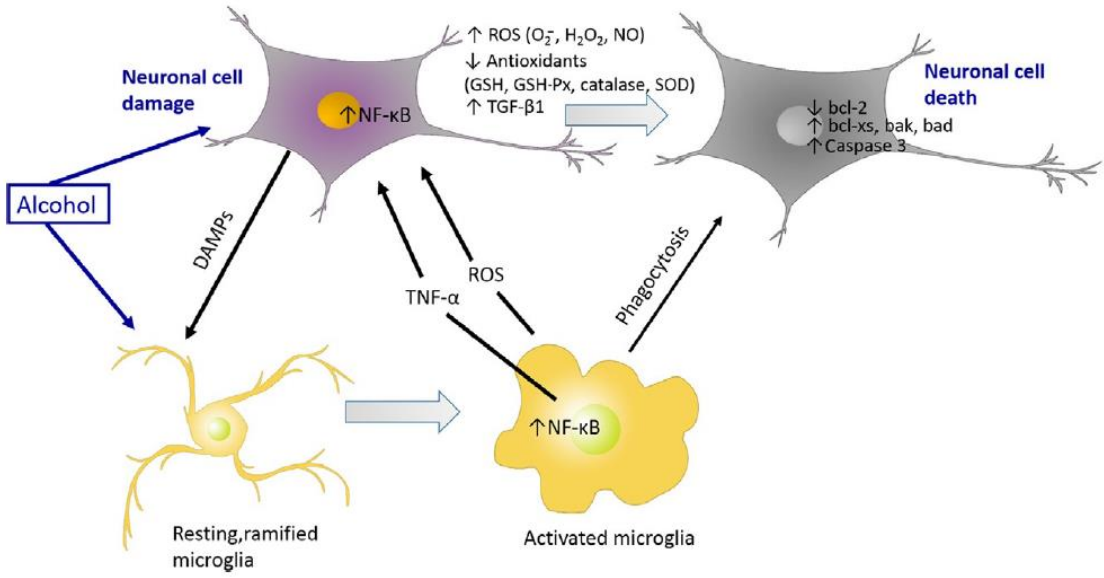


Figure 3. Mechanisms of alcohol-induced neurodegeneration facilitated by microglia.
Alcohol has direct negative effect in developing neurons which leads to the increase of ROS levels and decrease of levels of antioxidants. On the other hand, alcohol activates microglia, in a direct way or via neuronal-released DAMPs, worsening neuroinflammation and oxidative stress due to the increased TNF-α and ROS microglial release. All these events culminate in amplified TGF-β1 apoptotic signaling leading to increase in pro-apoptotic protein levels, augmented phagocytosis of dead neurons by microglia and increased neuronal cell death (Chastain and Sarkar, 2014).

microglia activation as a potential source for neurodegeneration. These glial cells, although equipped with efficient antioxidant defence mechanisms, are highly responsive to changes in the redox balance, producing and releasing a broad spectrum of inflammatory mediators leading to additional neuronal damage (Song et al., 2014; von Bernhardi et al., 2015).

Glutamate is recognized as a crucial excitatory neurotransmitter in CNS, being strongly related with several neurological diseases (Barger and Basile, 2001; Takeuchi et al., 2008; Takeuchi et al., 2006). In higher concentrations, glutamate leads to severe excitotoxicity, which may result in another mechanism of neurotoxicity due to excessive or prolonged stimulation of glutamate receptors (Olmos et al., 2014). Two pathways for glutamate production have been described: (1) dehydrogenase pathway – α-ketoglutarate conversion to glutamate mediated by dehydrogenase – which is used to maintain physiological glutamate levels; and (2) glutaminase pathway with glutaminase-mediated conversion of extracellular glutamine into glutamate **(Fig. 4)** (Takeuchi and Suzumura, 2014). Glutamate release might occur through ion channels, reverse efflux through excitatory amino acid transporters (EAATs), astrocytic vesicular glutamate release, by the xC^- exchanger and through hemichannels (Haroon et al., 2017). Microglia maintain intracellular glutamate levels via the glutamate dehydrogenase pathway **(Fig. 4)** (Takeuchi, 2010). Once activated, these cells secrete inflammatory cytokines, which are thought to be responsible for neuronal damage. However, even deleterious inflammatory cytokines, such as TNF and IFN-γ, have a relatively weak direct neurotoxic effect due to their concomitant neuroprotective role, eliciting indirect neurotoxicity through microglial glutamate release (Ghezzi and Mennini, 2001; Kamata et al., 2005). Some authors suggest that these cytokines induce glutaminase upregulation – and subsequent glutamate production – and also enhance hemichannels expression on the microglial cell surface, increasing glutamate release (Eugenin et al., 2001; Takeuchi et al., 2006). In fact, Takeuchi et al. described that TNF stimulates extensive microglial glutamate release in an autocrine manner, promoting further microglial TNF production and perpetuating microglial neurotoxicity through TNFR1 signaling **(Fig. 4)** (Takeuchi et al., 2006). Moreover, it was demonstrated that such

glutamate release occurs preferentially through the microglial connexin (Cx) 32 hemichannel and not glutamate transporters because this response was substantially repressed by a mimetic peptide directed against Cx32 (Takeuchi et al., 2006).

The excessive glutamate levels that mediate glutamate excitotoxicity occur because of both increased release as well as decreased uptake and microglia have the capacity to affect both processes that control glutamate excitotoxicity (Takeuchi, 2010). Although microglial release of glutamate could contribute to glutamate excitotoxicity in alcohol-induced neurodegeneration, activated microglial cells also upregulate the expression of the glutamate transporter 1 (GLT-1), which is responsible for taking up glutamate into microglia where it can

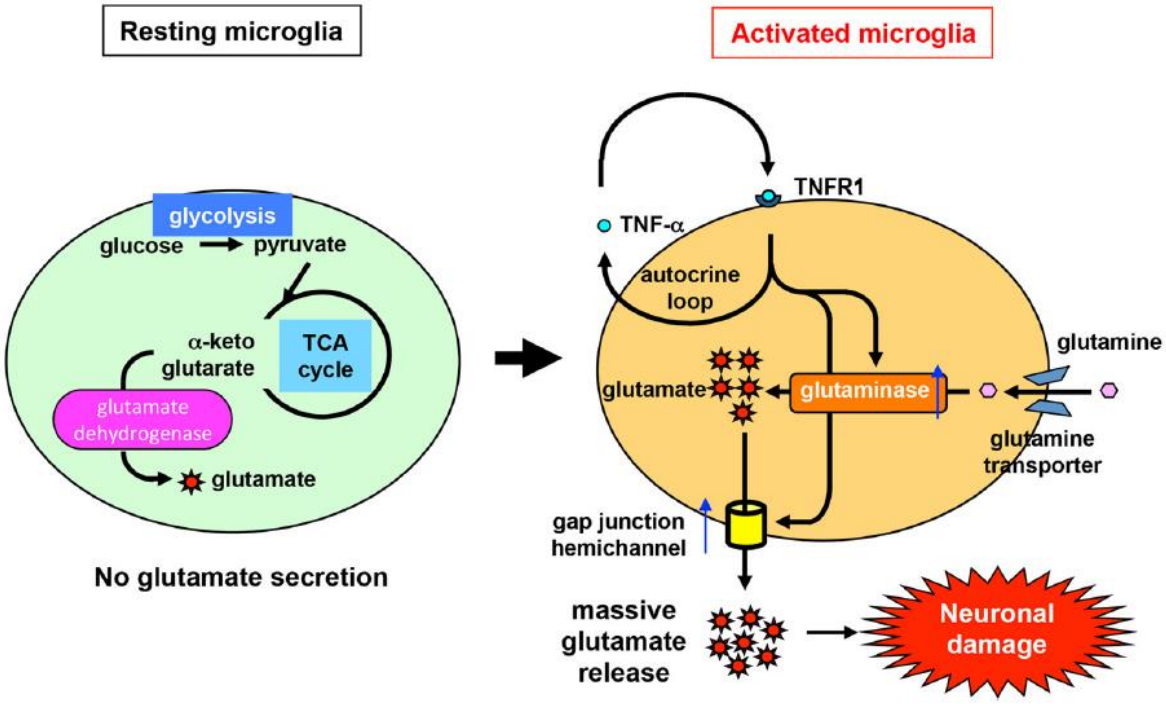


Figure 4. Mechanisms associated with glutamate production and release by activated microglia.
In a "resting" state, microglia produce glutamate by glutamate dehydrogenase pathway to the physiological normal levels maintenance of this neurotransmitter. Upon activation, microglial up-regulated glutaminase synthesizes large amounts of glutamate which are released through up-regulated gap-junction hemichannels of microglia, leading to eventual neuronal damage (Takeuchi and Suzumura, 2014).

be recycled (Persson et al., 2005). In this context, glutamate uptake and metabolism by glia would be neuroprotective by reducing the levels of glutamate in the neuronal parenchyma. Therefore, inhibition of microglial activation appears not to be a promising therapeutic strategy because microglia also exert neuroprotective roles, which should not be affected during

inhibition of microglial neurotoxic effects (Takeuchi, 2010). On the other hand, blockade of glutamate receptors has been proven unsuccessfully in therapeutic terms because perturbing physiological glutamate signals leads to severe collateral damage on other CNS cells (Parsons et al., 2007). Experimental evidence demonstrated that glutaminase inhibitors or hemichannel blockers might be used as new therapeutic approaches in order to reduce the microglial glutamate release, possibly rescuing some of toxic effects on neuronal cells (Takeuchi et al., 2006). Inhibitors directed against glutaminase and/or gap junctions, therefore, would likely be more specific and effective in suppressing microglial glutamate release with minimum adverse side effects.

Preliminary results

Ethanol activates c-Src to promote glutamate release from microglia

The cytotoxic role of glutamate released by overactivated microglia, which promotes neuronal cell death, has been demonstrated previously (Barger and Basile, 2001; Chen et al., 2012; Socodato et al., 2015a; Takeuchi et al., 2008; Takeuchi et al., 2006). There are several reported modulators of microglial glutamate release, such as lipopolysaccharide (LPS), hypoxia, hydrogen peroxide (H_2O_2) and TNF (Barger et al., 2007; Socodato et al., 2015a; Socodato et al., 2015b; Takeuchi et al., 2006). Therefore, preliminary studies performed by Socodato *et al.* from the Glial Cell Biology research group (i3S) focused on understanding whether ethanol can also regulate glutamate release from microglia and the pathways associated with this event.

Glutamate release was studied using an intramolecular FRET biosensor (Okumoto et al., 2005) by measuring the changes in CFP to FRET emission ratios **(Annex 1A)** in living rat primary cortical microglia. Ethanol exposure resulted in glutamate release from microglia **(Annex 1B)** with fast and sustained kinetics **(Annex 1B.1, black line)**. Knocking down c-Src

in rat primary cortical microglia abrogated the ethanol-triggered glutamate release **(Annex 1B.1, red line and 1B.2)**.

Src plays a major role in regulating cortical microglial function (Socodato et al., 2014; Socodato et al., 2015b) and we tested whether ethanol exposure would affect c-Src activation in microglia. We evaluated c-Src activity in living rat primary cortical microglia by FRET (Ouyang et al., 2008) **(Annex 1C)**. Ethanol activated c-Src consistently **(Annex 1D)** with fast, sustained **(Annex 1D.1)** and robust **(Annex 1D.2)** activation signal.

To further support the role of c-Src in ethanol-induced glutamate release in microglia we acutely inhibited c-Src with a pharmacological inhibitor (SKI) during the sustained phase of glutamate release (after 28 minutes of ethanol exposure; **Annex 1E and 1E.1**). FRET measurements in rat primary cortical microglia showed that inhibition of c-Src with SKI led to a blockade of glutamate release **(Annex 1E)**, which was upheld at baseline values even in the continuous presence of ethanol **(Annex 1E.1)**. In addition, time-controlled c-Src activation using a rapamycin-inducible c-Src allosteric construct (RapR-Src (Karginov et al., 2010)) was sufficient to induce a large increase in glutamate release in rat primary cortical microglia **(Annex 1F-F.2)**. From these data, we concluded that ethanol induces glutamate release from microglia in a c-Src-dependent manner.

Ethanol-induced c-Src activation in microglia triggers glutamate release through TNF

TNF has been shown to promote the release of glutamate through hemichannels from activated microglia (Takeuchi et al., 2006). N9 microglia overexpressing a constitutively active c-Src mutant (Src^{Y527F}) released more TNF than control N9 cells **(Annex 2A)**, corroborating that c-Src induces TNF production in microglia. Accordingly, microglia exposed to TNF released glutamate similarly when treated with ethanol **(Annex 2B-B.2)**. TNF production is necessary for ethanol-induced glutamate release **(Annex 2C-C.2, black line and whisker)** because such release was prevented in microglia isolated from TNF deficient mice **(**$TNF^{-/-}$ mice; **Annex 2C-C.2, red line and whisker)**. Using the RapR-Src construct, to force c-Src

activation in a time-controlled manner, we found that rapamycin-mediated c-Src activation led to glutamate release from wild-type **(Annex 2D-D.2, blue line and whisker)** but not from $TNF^{-/-}$ microglia **(Annex 2D-D.2, blue line and whisker)**. We concluded that in microglia TNF is a downstream target of the ethanol/c-Src pathway to trigger glutamate release.

CHAPTER 2 | Aims

Microglia might be involved in several mechanisms of alcohol-induced neurodegeneration and behavioural deficits. The main goal of this study is to investigate both functional and morphological characteristics of microglia after semi-chronic exposure to ethanol. Gaining mechanistic insight into these processes will be relevant to better analyze the effects of alcohol that lead to neurodegeneration, and find new therapeutic targets.

The main goals of this work are:

In vitro:

1) Quantification of glutamate release after ethanol exposure in the different experimental conditions by FRET experiments.

In vivo:

1) Evaluation of the effect of alcohol in microglial and astrocytic cells in the brain:
    - Determination of microglial and astrocytic cell numbers after semi-chronic ethanol exposure by immunohistochemistry;
    - Protein expression analyses of microglial and astrocytic markers by Western blotting.
2) Measurement of mRNA transcripts of: a) the antioxidant-related neuroimmune response; and b) microglia signature and homeostasis-related genes, by Quantitative Real-Time PCR (RT-qPCR), that might be involved in alcohol-induced dysfunction and behavioural deficits.
3) Evaluation of alcohol influence in mice behaviour by comparing the performance of control mice and mice subjected to ethanol exposure in different behavioural paradigms.

CHAPTER 3 | Methods

IN VITRO STUDIES

Drugs

Ethanol absolute was from Millipore; 18α-Glycyrrhetinic acid (GA) (G8503) and GAP-27 (G1794) were from Sigma-Aldrich.

Plasmids

pDisplay FLIPE-600n (Plasmid 13545) was from Addgene. Transfection of human microglial cells was performed using 1 μg of plasmid with jetPRIME® (Polyplus Transfection) according to the manufacturer's protocol.

Cells

Human microglial cell line

CHME3 microglial cells (Janabi et al., 1995) were cultured in DMEM GlutaMAX™-I (Life Technologies) supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin (Life Technologies), and were maintained at 37 °C, 95% air and 5% CO2 in a humidified incubator. CHME3 cells were used in experiments for determining the nuclear accumulation of p65 NF-κB subunit or glutamate release.

FRET-based live cell imaging and quantification of FRET images

Human microglial cells (CHME3) were plated on plastic-bottom culture dishes (μ-Dish 35 mm, iBidi). Imaging was performed using the Leica DMI6000B inverted microscope as

previously described (Socodato et al., 2015a; Socodato et al., 2015b). The excitation light source was a mercury metal halide bulb integrated with an EL6000 light attenuator. High-speed low vibration external excitation/emission filter wheels (equipped with CFP/YFP excitation and emission filters) were mounted on the microscope (Fast Filter Wheels, Leica Microsystems). A 440-520 nm dichroic mirror (CG1, Leica Microsystems) and a PlanApo 63X 1.3NA glycerol immersion objective were used for CFP and FRET images which were acquired with 4x4 binning using a digital CMOS camera (ORCA-Flash4.0 V2, Hamamatsu Photonics). Shading illumination was online corrected for CFP and FRET channels using a shading correction routine implemented by Leica for the LAS X software. At each time-point, CFP and FRET images were sequentially acquired using different filter combinations (CFP excitation plus CFP emission, and CFP excitation plus YFP emission, respectively).

Images were processed in FIJI software as 16-bit tiff files. Background was dynamically subtracted from all slices. Segmentation was achieved on a pixel-by-pixel basis using a modification of the Phansalkar algorithm. After background subtraction and thresholding, binary masks were generated for the CFP and FRET channels. Original CFP and FRET images were masked and ratiometric images (CFP/FRET for glutamate release probe) were generated as 32-bit float tiff images. Photobleaching was corrected using bi-exponential decay curve fitting based on the histogram distribution for the whole time-series. Mean gray intensity values were generated using the multi calculation function in FIJI and exported for statistical evaluation in the GraphPad software.

IN VIVO STUDIES

Animals

TNF knockout mice (referred herein as $TNF^{-/-}$) were originally supplied by Prof. Rui Applelberg (University of Porto) and were kept on a C57BL/6 background. $TNF^{-/-}$ 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. $TNF^{-/-}$ mice display a single band of 2000 bp in the PCR gel. Such mice were bred at i3S animal facility and maintained at heterozygosity. 150 days old male WT and $TNF^{-/-}$ littermates were used in this study. Animals were kept under stable conditions (20-22ºC, 60% humidity in 12-hour light/dark cycle), with water and appropriate food supplied ad libitum. Cylindrical tubes and soft paper for nest construction were made available. All procedures used were approved by local ethical committee and by the Portuguese Agency for Animal Welfare, general board of Veterinary Medicine (ref. 11769/2014-05-15), in compliance with the 2010 European Community Council Directive (2010/63/UE). All procedures involving animals were conducted by FELASA C or B graded researchers and all efforts were made to ensure minimal animal stress and discomfort.

Ethanol exposure to mice

Mice were subjected to a ten-day model of alcohol binge (semi-chronic exposure). Divided into two experimental groups (WT and $TNF^{-/-}$ mice) of similar weights, animals received either 200 µL of 25% ethanol diluted in dH_2O or 200 µL of dH_2O (control) through intragastric administration performed via oral gavage undertaken using a flexible catheter 22G. After ten successive days of administration and 3-5 days of ethanol withdrawal period, males were subjected to behavioural analysis. Then, perfusion was performed with PBS and some animals also had the brains fixed by perfusion with 4% paraformaldehyde (PFA). Extracted brains were sliced and used for histological analysis, mRNA was harvested for PCR or tissue was homogenized to obtain protein lysates. At the end, it was obtained four experimental distinct groups for data analysis: WT Saline, WT EtOH, $TNF^{-/-}$ Saline, and $TNF^{-/-}$ EtOH.

Behavioural tests

All testing procedures were conducted in the dark phase of the light/dark cycle. Before each session, mice were removed from their home cage in the colony room and brought into adjacent testing rooms (illuminated with 100 lux and attenuated noise). All behavioural tests were recorded with a camera placed above the apparatus. In the elevated plus-maze and open-field tests, movement and location of mice were analyzed by an automated tracking system equipped with an infrared-sensitive camera (Smart Video Tracking Software v 2.5, Panlab, Harvard Apparatus). Data from the object recognition test were analyzed using the software Observer 5 (Noldus Information Technology, Wageningen, The Netherlands). All apparatus were thoroughly cleaned with neutral soap after each test session. Only male mice were used in the behavioural tests.

Behavioural analysis were performed during this project by Ana Magalhães from the Addiction Biology group.

Elevated plus-maze (EPM)

The maze is made of opaque grey PVC and consists of four arms arranged in a plus-shaped format; two arms have surrounding walls (closed arms, 37x6 cm x18 cm-high), the other two opposing arms have no walls (open arms, 37x6 cm). The apparatus was elevated at a height of 50 cm above the ground. Mice were placed on the central platform facing an open arm and were allowed to explore the maze for 5 minutes. Open arms entries and time spent in open arms were measured automatically (video tracking) to assess anxiety-like behaviour. Protected head dipping (the mouse points its head to the floor but its body is still in the centre or in a closed arm of the maze), head dipping (the mouse points its head to the floor with its body in open arm) and stretched attended postures (animal stretching forward into an open arm and retracting to its original position without moving its hind paws) were scored manually to assess risk-taking behaviour.

Open field (OF)

Mice were placed in the centre of an OF apparatus (40 x 40 x 40 cm) and then allowed to move freely for 10 min. The distance travelled, peripheral activity and center activity (locomotion in the central section of the OF) were counted automatically (video tracking).

Novel object recognition (NOR)

The NOR test was performed as previously described (Leger et al., 2013). Briefly, the test apparatus consists of an open box and the objects used were made of plastic, glass or metal in three different shapes: cubes, pyramids and cylinders. The test consisted of three phases. During habituation phase, mice were allowed to explore the apparatus for 10 min (time used to perform OF test). In the following day, the acquisition/sample phase started by placing each mouse in the apparatus with two identical objects (familiar) for 10 min and then the mouse was removed back to its home cage. After 4 hours (inter-trial interval, ITI), the retention/choice session was performed. In this phase, the apparatus contained a novel object and a copy of the previously seen familiar object, and animals were allowed to explore the objects for 3 min. The time spent exploring the novel object served as the measure of recognition memory for the familiar object. Exploration was defined as follows: mouse touched the object with its nose or the mouse's nose was directed toward the object at a distance shorter than 2 cm (Ennaceur et al., 2005). Circling or sitting on the object was not considered exploratory behaviour. The product of the difference (subtraction) between exploration time for novel and familiar objects was measure as indicator of object recognition translated into a condition score: a) =+1, preference for spend more time with new object; b) =0, null preference; c) =-1, preference to spend time with familiar object (Ennaceur et al., 2005). The index of discrimination (DI) was calculated as index of memory function, DI = time exploring the novel object / (total time spent exploring both objects).

Immunohistochemistry

Tissue preparation

After perfusion with 4% PFA in 0.16 M phosphate buffer (PB), the brains were removed from the skull and post-fixed for 4 h in the same medium. Then, the tissue was rinsed with PB and cryoprotected using two sucrose solution gradient in a row (15 and 30%). After at least 24h, each brain was mounted in OCT (ThermoScientific) medium, frozen and cryosectioned in the CM3050S Cryostat (Leica Biosystems) at a thickness of 30µm. Coronal and non-sequential tissue sections were collected on Superfrost glass slides. The slides were stored at -20ºC until processed for immunolabeling using.

Immunohistochemistry and analysis

Slides with sections including hippocampus and cortex were washed 3 x 10 min with PBS and incubated for 60 min with blocking solution (3% bovine serum albumin, 3% FBS and 1% Triton X-100 in PBS). Then, incubation with primary antibodies (anti-Iba-1 1:500, 016-20001, WAKO; anti-GFAP 1:500, ab7260, Abcam) was performed and the slides were maintained in a humidified chamber during two overnights. Afterwards, sections were washed 3 x 10 min with PBS and incubated with secondary antibodies (Alexa488 1:500, Life Technologies) for 90 min. To stain the nuclei, cells were incubated with Hoechst 33342 0,5µg/mL (B2261, Sigma-Aldrich) or NucRed® Dead 647 (R37113, Life Technologies) for 10 min at room temperature. After 3 x 10 min in PBS, slides were cover slipped using glycergel or Immumount and visualized using a Leica TCS SP5 II confocal microscope. Images were processed and quantified using the LAS AF software (Leica Microsystems).

Cortical protein extraction and Western Blot analysis

Total protein of cerebral cortex samples was extracted with RIPA buffer (150mM NaCl, 50mM Tris–HCl, pH 7.4, 5mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5),

supplemented with 50mM sodium fluoride (NaF), 1.5mM sodium ortovanadate (Na_3VO_4), 1mM dithiothreitol (DTT) and a cocktail of protease inhibitors (0.1mM phenylmethylsulfonyl fluoride (PMSF), CLAP (1µg/ml chymostatin, 1µg/ml leupeptin, 1µg/ml antipain, 1µg/ml pepstatin; Sigma). Protein concentration was determined using the Bicinchoninic Acid (BCA) method, and denaturant solution 5X (buffer (625mM Tris, pH 6.8, 10% SDS, 500mM DTT, 50% glycerol and 0.01% bromophenol blue) was added to a final concentration of 1X. Samples were heated to 60ºC for 10 minutes, and stored at -20ºC for further use.

SDS-page electrophoresis was performed, where 120µg of sample protein was loaded in a 12% SDS-page gel and let run at 80V for 2 hours, using PageRuler™ Plus Prestained Protein (Thermo Fisher Scientific) as a molecular weight marker. PVDF membrane was activated by submerging it in methanol for 15 seconds (until transparent), washed in distilled water with agitation, and incubated for 30 minutes in transfer buffer. Proteins were transferred from gel to the PDVF membrane using a Trans-Blot® Turbo™ Transfer System (Bio-Rad). The transference was performed for 15 minutes in diluted Trans-Blot® Turbo™ 5X Transfer Buffer (20% buffer, 20% ethanol and 60% ultrapure H_2O). Membranes were blocked for 60 min in blocking solution composed of 5% skimmed milk diluted in tris based saline with 0.1% Tween (TBS-T) and incubated with primary antibody (anti-GFAP 1:100, ab7260, Abcam; anti-Iba-1 1:1000, 016-20001, WAKO; anti-GAPDH 1:10000, G7121, Promega) diluted in blocking solution overnight at 4ºC. Membranes were then washed 5 times, for 5 minutes each, with TBS-T and incubated with the secondary antibody (HRC conjugated anti-rabbit 1:10000, A0545, Sigma-Aldrich; HRC conjugated anti-mouse 1:3000, 31432, Thermo Fisher Scientific). Membranes were incubated with Clarity™ Western ECL Substrate (Bio-Rad) for 5 min, and revealed using ChemiDoc (ChemiDoc™ MP System, Bio-Rad). Results were quantified and processed with ImageLab software (Image Lab™ Software, Bio-Rad).

Membranes were reprobed using other primary antibodies according to the following steps: washed for 5 min in distilled water, incubated for 20-30 min in NaOH 0,2M, and finally

washed for 5 min in distilled water. The procedure was repeated from the blocking step forward.

Quantitative Real-Time PCR (RT-qPCR)

RNA extraction using modified RNA Kit

Total RNA was extracted from the cortical tissue of WT and TNF -/- brains using TRIzol™ (Ambion by Life Technologies) reagent combined with PureLink RNA Mini Kit (Ambion by Life Technologies), according to the manufacturers' instructions. To obtain single cell suspensions, tissue samples were mechanical disaggregated in TRIzol™, homogenized and centrifuged with chloroform. Ethanol 70% in RNase-Free water was added to RNA fraction in a 1:1 proportion and mixed vigorously avoiding precipitate formation. The lysate was subjected to a silica-membrane column RNA purification and concentrated RNA was eluted in 30 µL RNase free water **(Fig. 5)**. The total amount of RNA was assessed with NanoDrop® 1000 Spectrophotometer (Thermo Scientific), and RNA quality and integrity with Experion Automated Electrophoresis System (BioRad).

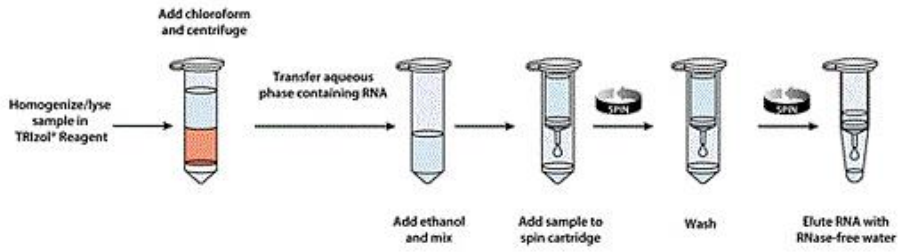


Figure 5. Scheme of silica-based purification protocol using Purelink® RNA Mini Kit in conjunction with TRIzol® Reagent.
Adapted from (https://www.thermofisher.com/pt/en/home/life-science/dna-rna-purification-analysis/rna-extraction/rna-types/total-rna-extraction/purelink-rna-mini-kit.html).

cDNA synthesis

To proceed to the RT-qPCR analysis, cDNA synthesis was conducted using SuperScript® III First-Strand Synthesis System (Invitrogen) following manufacturer's instructions. Briefly, to

1µg of total RNA of samples, it was added 10µL RT Reaction Mix, 2µL RT Enzyme Mix and RNA/Nuclease free water, reaching a final volume of 20 µL. Samples were then incubated in a thermocycler (Bio-Rad) at 25ºC for 10 min, heated until 50ºC for 30 min and then heated until 85ºC for 5 min. Afterwards, 1µL of E. Coli RNase was added to each sample and incubated in thermocycler at 37ºC for 20 min. cDNA was stored at -20ºC until further use.

RT-qPCR analysis

In order to evaluate mRNA expression, RT-qPCR analysis was performed. Primers were designed using OligoPerfect™ Designer (ThermoFisher Scientific), specifically to the selected genes, accordingly with the specified requirements (75-200 base pairs long, GC content between 50-60%, minimized single-bases repetition, minimal to inexistent secondary structure formation and melting point between 57-63ºC).

Table 1. Specific primers used on RT-qPCR.

	Sequences (5' - 3')	
	Forward	**Reverse**
IL-1B	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG
TNF	GCCACCACGCTCTTCCTGTCT	TGAGGGTCTGGGCCATAGAAC
IL-6	CACAAGTCCGGAGAGGAGAC	CAGAATTGCCATTGCACAAC
IFNB	CCCTATGGAGATGACGGAGA	CTGTCTGCTGGTGGAGTTCA
C1QA	GTGTGCTGACCATGACCCTA	ATTCCCCTGGGTCTCCTTTA
C1QB	AGACACAGTGGGGTGAGGTC	GGTCCCCTTTCTCTCCAAAC
C1QC	GAGGACCCAAGGGTCAGAAG	TGTATCGGCCCTCCACAC
COX-2	GCTGTACAAGCAGTGGCAAA	CCCCAAAGATAGCATCTGGA
TREM2	AACTTCAGATCCTCACTGGACC	CCTGGCTGGACTTAAGCTGT
TSPO	TGGGAGGTTTCACAGAGGAC	GCCAGGTAAGGGTACAGCAA
MHC-II (CD74)	ATGACCCAGGACCATGTGAT	ATCTTCCAGTTCACGCCATC
GCLC	CACAGACCCAACCCAGAG	TGGCACATTGATGACAACCT
GSR	CACGACCATGATTCCAGATG	CAGCATAGACGCCTTTGACA

TXNRD1	CAGGGTGACTGCTCAATCCACAAAC	CTCTTCCTACCGCCAGCAACACTG
HMOX1	GAAGGGTCAGGTGTCCAGAGAAGG	CGCTCTATCTCCTCTTCCAGGGC
CXCL1	GCTGGGATTCACCTCAAGAA	TGGGGACACCTTTTAGCATC
ICAM-1	CGAAGGTGGTTCTTCTGAGC	GTCTGCTGAGACCCCTCTTG
CCL2	ATCCCAATGAGTAGGCTGGA	TCTGGACCCATTCCTTCTTG
CCL5	GTGCCCACGTCAAGGAGTA	CCCACTTCTTCTCTGGGTTG
P2RY12	CACCTCAGCCAATACCACCT	CAGGACGGTGTACAGCAATG
MERTK	GCCCACAATGACAAAGGACT	GGGAGTAGCCATCAAAACCA
PROS1	GATTCTCGCTCTGGAACGTC	GGTGTGGCACTGAAGGAAAT
CSF1R	CCCTAGGACAAAGCAAGCAG	GATGTCCCTAGCCAGTCCAA
GPR34	GGTTGCTCTTGCTGGATTTC	CCGGGCTGTTGTAGCATATT
PU.1	CAGTTCTCGTCCAAGCACAA	TTTCTTCACCTCGCCTGTCT
TGFBR1	AAATTGCTCGACGCTGTTCT	TTCCTGTTGGCTGAGTTGTG
ARG-1	GTGAAGAACCCACGGTCTGT	CTGGTTGTCAGGGGAGTGTT
MRP14	TCATCGACACCTTCCATCAA	TCAACTTTGCCATCAGCATC
MRP8	GGAAATCACCATGCCCTCTA	TGCCACACCCACTTTTATCA
SOCS3	AGCTCCAAAAGCGAGTACCA	AGCTGTCGCGGATAAGAAAG
NLRP3	ATGCTGCTTCGACATCTCCT	AACCAATGCGAGATCCTGAC
IL-18	CAGACAACTTTGGCCGACTT	GGGTTCACTGGCACTTTGAT
IRAK3	GGACCTCCTCATGGAACTGA	CCAGAGAGGACAGGACTTCG
SVCT2	CACTGATAGAAGTGGTCAT	AACACTAGGAAAATCGTCAG
TLR2	TTGCTCCTGCGAACTCCTAT	GCTTTCTTGGGCTTCCTCTT
TLR4	GCTTTCACCTCTGCCTTCAC	GCGATACAATTCCACCTGCT
TLR7	TGGAAATTTTGGACCTCAGC	TTGCAAAGAAAGCGATTGTG
CX3CL1	TTCAACCCCAGAGGAAAATG	TCGGGGACAGGAGTGATAAG
CD163	TGGTGTGCAGGGAATTACAA	AGCTCCACTCTTCCCTCACA
CD14	GCTCAACTTTTCCTGCGAAC	CCCGCAGTGAATTGTGACTA
YWHAZ	GATGAAGCCATTGCTGAACTTG	GTCTCCTTGGGTATCCGATGTC

PCR was performed using iTaq™ Universal SYBR® Green Supermix. Template PCR reactions were performed and run in duplicate (see table 2 for reaction setup) and the PCR cycling profile was 3 min at 94ºC for polymerase activation, followed by 40 cycles of 15 sec at 94ºC (denaturation), 20 sec at 60ºC (annealing) and 81 cycles of 30 sec between 55ºC and 95ºC (final extension period).

Table 2. qPCR Reaction Setup (adapted from iTaq™ Universal SYBR® Green Supermix).

Component	Volume per 20µL reaction	Final Concentration
iTaq™ Universal SYBR® Green Supermix (2x)	10µL	1x
Forward and Reverse Primers	Variable	300nM
DNA Template	1µL	1µg
H_2O	Variable	-
Total reaction mix volume	20µL	-

Gene expression was extrapolated from standard curves generated concurrently for each gene using a control cDNA dilution series. qPCR was carried out in iQ5 Multicolor Real-Time PCR Detection System (BioRad) and the results were analyzed using iQ™5 Optical System software version 2.1 (BioRad). All values were normalized to levels of the reference gene (Ywhaz) and expressed compared to control samples. Gene expression was calculated using the 2^(-delta C_T) (Schmittgen and Livak, 2008).

Statistical Analysis

The results obtained in each experiment were normalized by the control mean. Data was presented as mean ± SEM of at least three different experiments, performed in independent preparations. Statistical analysis of the results was performed using GraphPad Prism7 and data were evaluated by unpaired Student t test, One-Way Analysis of Variance (ANOVA) followed by the Fisher's LSD post-hoc test or two-way ANOVA followed by the Fisher's LSD

post-hoc test. In cases where homogeneity of the variances in the One-Way ANOVA was not met (evaluated by the Brown-Forsythe test), multiple t tests were used instead. 95% confidence interval was used for statistical evaluation and $P<0.05$ was considered statistically significant difference in all sampled groups.

Chapter 4 | Results

Ethanol promotes glutamate release from microglia through gap-junction hemichannels

Take into account the preliminary results, we studied whether glutamate release by CHME3 human microglial cells was affected by ethanol. In fact, measuring the changes in CFP to FRET emission ratios we observed a considerable increase in glutamate release from ethanol-stimulated CHME3 microglia **(Fig. 6, black circles)**. This result further validated the hypothesis that ethanol can induce the release of glutamate from human microglia.

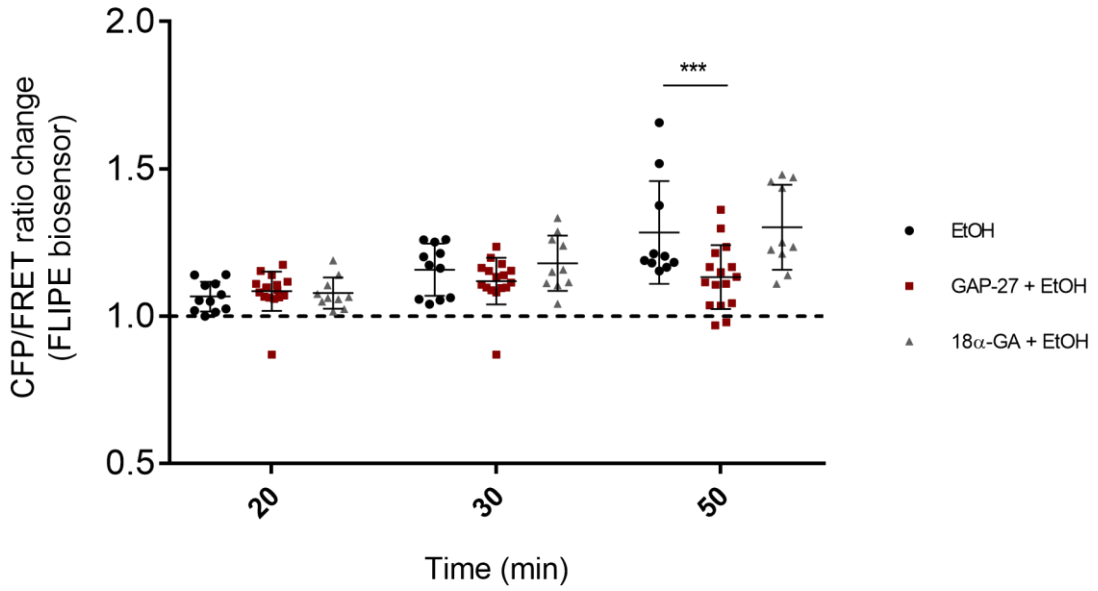


Figure 6. FRET quantification of glutamate release by CHME3.
Human microglial cell line (CHME3) was transfected with the FLIPE 600n^{SURFACE} probe. Cells were treated with EtOH (70mM) or pre-treated with GAP-27 [100μM] or 18α-GA [100μM]. CFP/FRET emission ratios of the chimera were normalized at 0 min. Bars represent the means ± SEM of ten to sixteen cells from one experiment. ***P < 0.001, Two-way ANOVA (Fisher's LSD post-hoc test).

In order to prevent or block the ethanol effect, two gap-junction inhibitors were used because it was found that activated microglia release large amounts of glutamate through gap junction hemichannels (Takeuchi et al., 2006). GAP-27 peptide and 18α-Glycyrrhetinic acid (GA) inhibit intercellular communication through gap junctions and have been used primarily

to block gap junctions composed of Cx43 (Boengler et al., 2012; Ilvesaro et al., 2001), which is described to be present in microglia (Eugenin et al., 2001; Garg et al., 2005; Kielian, 2008). Our results demonstrated a significant but delayed decrease of the ethanol-triggered glutamate release in the presence of GAP-27 peptide **(Fig. 6, red squares)**, but not in microglia treated with 18α-GA gap junction blocker **(Fig. 6, grey triangles)**.

Ethanol exposure to adult mice induces no overt changes on the number of brain microglia and astrocytes

The expansion of the brain resident glial populations is a hallmark of several neuropathologies (Perry and Holmes, 2014) and accumulating evidence suggest that acute and chronic alcohol exposure might impact on microglial and astrocytic viability, activation and proliferation (Alfonso-Loeches et al., 2010; Franke, 1995; Guerri et al., 1990; He and Crews, 2008; Miguel-Hidalgo et al., 2002). Therefore, we analysed by Western blotting the amounts of Iba-1 (a microglial marker) and glial fibrillary acidic protein (GFAP; astrocytes) in protein lysates obtained from the brains of ethanol-exposed and control mice. Our results showed no significant differences in the total amounts of Iba-1 **(Fig. 7A)** or GFAP **(Fig. 7B)** between ethanol-treated and control brains.

In addition, immunohistochemistry for Iba-1 and GFAP on tissue sections obtained from the cerebral cortices revealed that the numbers of Iba1-positive microglia **(Fig. 8A)** and GFAP-positive astrocytes **(Fig. 8B)** were comparable between mice exposed to ethanol and the controls. Here we show that in adult mice, our semi-chronic protocol of ethanol exposure seems do not induce microgliosis or astrocytosis.

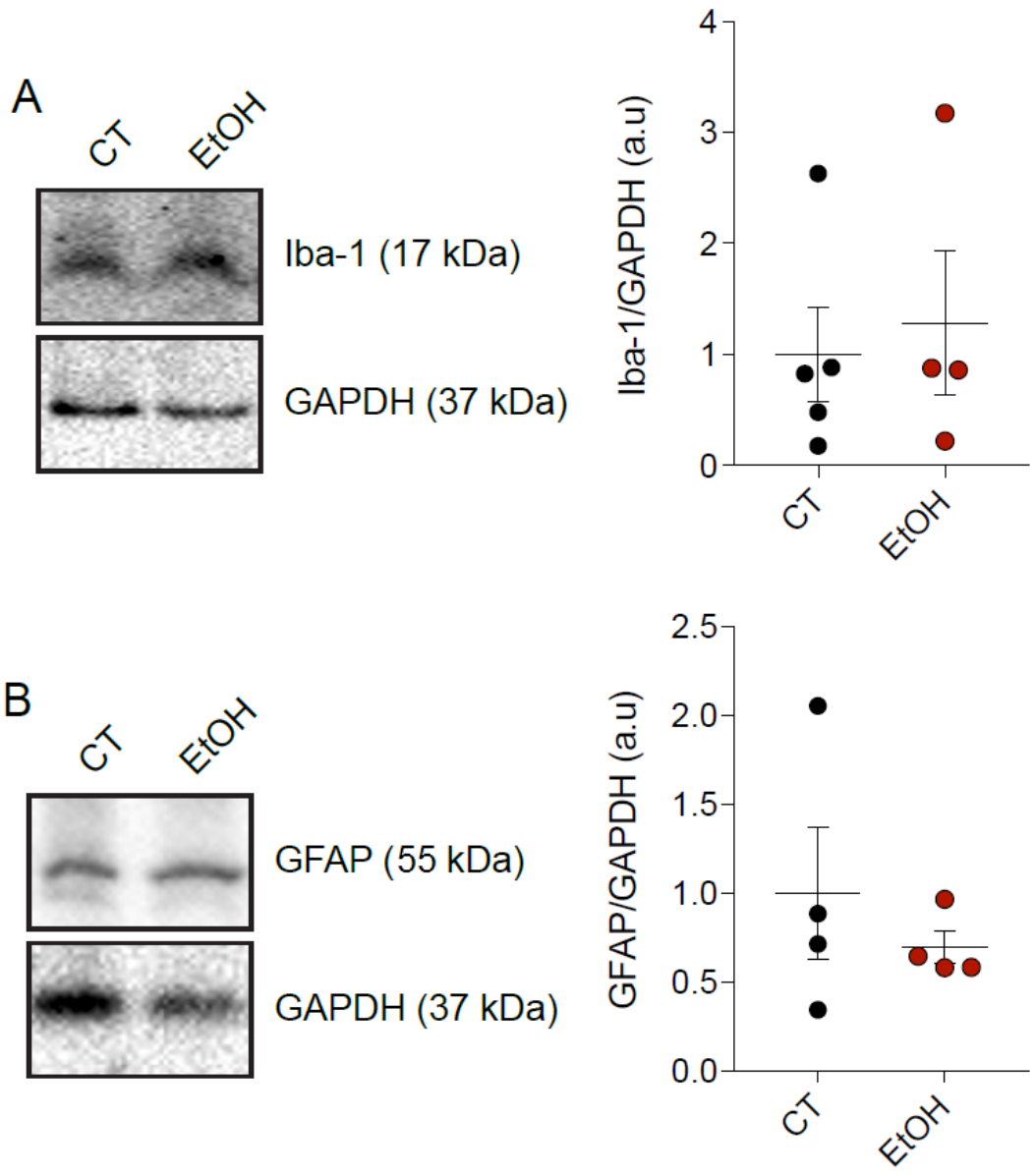


Figure 7. Iba-1 and GFAP protein expression levels on lysates from the cortical tissue of ethanol-treated and untreated wild-type mice.
Iba-1 **(A)** and GFAP **(B)** protein levels were determined by Western Blot. GAPDH was the loading control. A representative immunoblot of each protein is shown. Histograms are the mean ± SEM. N=4-5 CT and EtOH WT mice. Mann-Whitney test. Values displayed were normalized to the control group.

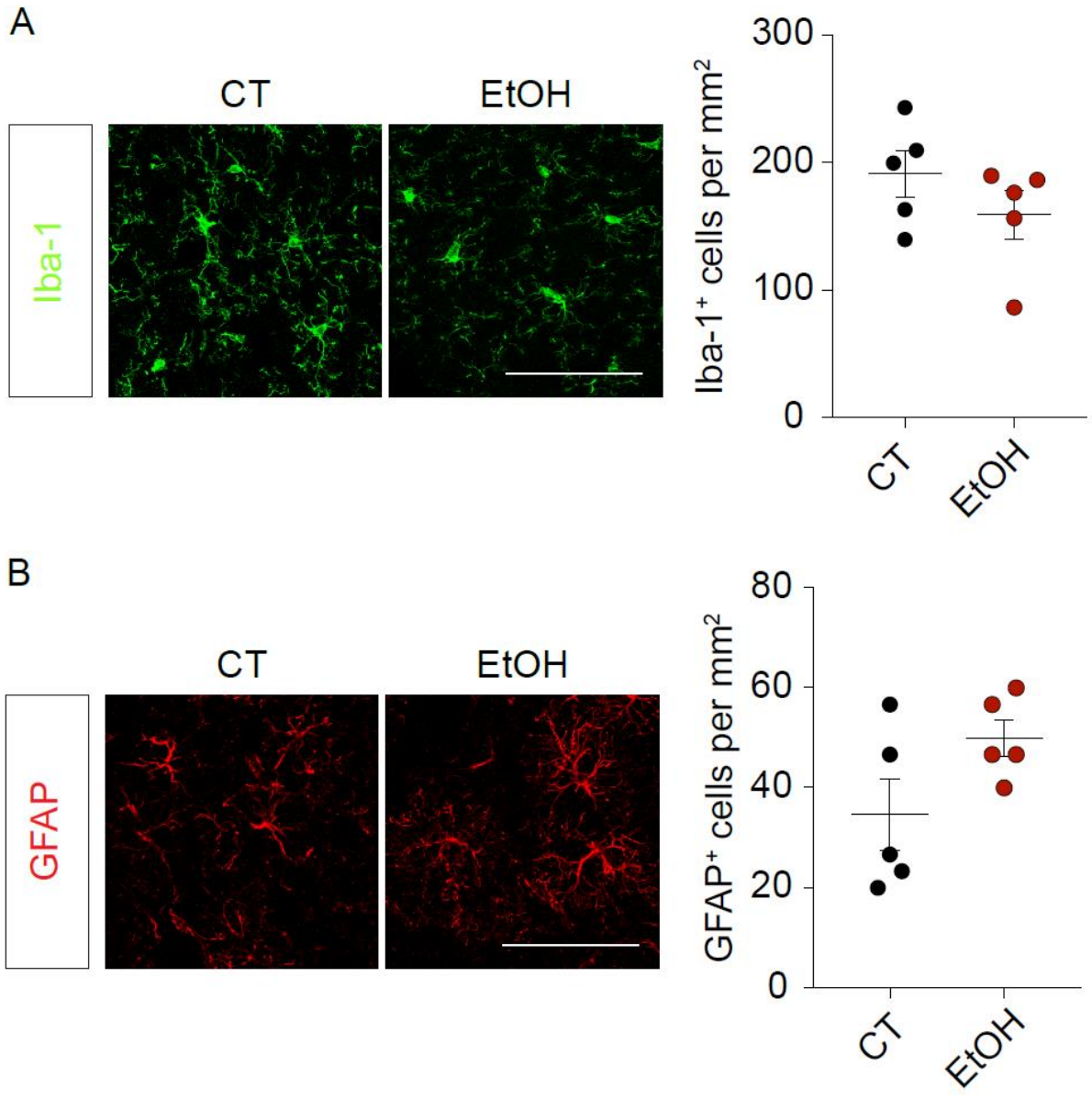


Figure 8. Numbers of Iba-1- and GFAP-positive cells in the brains of ethanol-treated and untreated wild-type mice.

Histological confocal analysis was also performed. Iba-1 **(A)** and GFAP **(B)** immunolabeling in the cerebral cortex from WT mice. Graphs represent (mean ± SEM) positive cell counts per mm^2. Values displayed were normalized to the control group. N=6 CT and 6 EtOH WT mice. Unpaired t test. Scale bar, 10 µm.

Role of TNF in ethanol-induced alteration of mRNA transcripts of immune response genes in the brain

Ethanol alters the mRNA transcripts of immune response genes in the brain of wild-type mice

Data based on studies in brains of human alcoholics and animal models demonstrate that neuroimmune and microglial gene expression are increased by alcohol abuse, emphasizing that ethanol effect on the CNS might be influenced by the innate immune system (Crews et al., 2015; Kelley and Dantzer, 2011; Mayfield et al., 2013; Robinson et al., 2014). We investigated by RT-qPCR the transcripts of several genes related with neuroimmune activation, immune cell recruitment, microglia homeostasis and antioxidant response. Our results show a significant increase in *TNF* and *TLR2* mRNA transcripts **(Table 3; Annex 3A and 3B)** and reduced abundance of *IL-6*, *TLR4*, *TLR7*, *CCL2*, *CCL5*, *TREM2* and *COX-2* mRNA transcripts **(Table 3; Annex 3C-I)** in ethanol-exposed mice compared to the controls. No significant changes in the mRNA transcripts for *ICAM-1*, *C1qA*, *C1qB*, *C1qC*, *MHC-II*, *Arg-1*, *CXCL1*, *CX3CL1*, *MRP14*, *MRP8*, *SOCS3*, *NLRP3, IL-18, IL-1β, IRAK3*, *IFN-β*, *CD14*, *CD163* and *TSPO* **(Table 3; Annex 4A-S)** were found between ethanol-exposed and control animals. Concerning gene expression related with microglia signature and homeostasis, we found higher abundance of the mRNA transcripts for *P2RY12* **(Table 3; Annex 5A)**, reduced abundance of *MERTK* and *GPR34* **(Table 3; Annex 5B and 5C)**, and no changes in *PROS1*, *CSF1R*, *PU.1* and *TGFβR1* **(Table 3; Annex 5D-G)** in the brains of ethanol-exposed animals when compared to control littermates.

Table 3. mRNA expression levels of immune response genes in the brain of wild-type mice.
Data compilation of the mRNA transcripts of several genes related with neuroimmune activation, immune cell recruitment, microglia homeostasis and antioxidant response between ethanol-treated and untreated brains of wild-type mice. Gene expression was calculated using the 2^(-ΔCT) (Schmittgen and Livak, 2008). *P < 0.05, **P < 0.01, Unpaired t test.

	Gene	H_2O Mean($2^{-\Delta CT}$) ± SEM	EtOH Mean($2^{-\Delta CT}$) ± SEM	P value	Ethanol effect
INFLAMMATION-RELATED	**TNF**	0.00008 ± 0.00002	0.00013 ± 0.00001	**0.0490***	Increase
	IL-1β	0.0046 ± 0.0007	0.0046 ± 0.0005	**0.4881**	-
	IL-6	0.00026 ± 0.00002	0.00017 ± 0.00004	**0.0397***	Decrease
	IL-18	0.26 ± 0.02	0.25 ± 0.04	**0.4352**	-
	IFN-β	0.0026 ± 0.0004	0.0024 ± 0.0003	**0.3398**	-
	C1qA	0.052 ± 0.007	0.046 ± 0.003	**0.1821**	-
	C1qB	0.15 ± 0.02	0.13 ± 0.02	**0.1623**	-
	C1qC	0.027 ± 0.003	0.024 ± 0.002	**0.2784**	-
	TLR2	0.0015 ± 0.0001	0.0020 ± 0.0002	**0.0377***	Increase
	TLR4	0.022 ± 0.003	0.016 ± 0.001	**0.0231***	Decrease
	TLR7	0.0014 ± 0.0001	0.0011 ± 0.0009	**0.0112***	Decrease
	ICAM-1	0.0021 ± 0.0002	0.0022 ± 0.0001	**0.3250**	-
	MHC-II	0.0032 ± 0.0004	0.0027 ± 0.0002	**0.1577**	-
	CD163	0.0033 ± 0.0006	0.0028 ± 0.0005	**0.2520**	-
	CD14	0.006 ± 0.001	0.006 ± 0.001	**0.4961**	-
	COX-2	0.14 ± 0.01	0.110 ± 0.004	**0.0050****	Decrease
	TREM2	0.11 ± 0.01	0.083 ± 0.007	**0.0246***	Decrease
	TSPO	0.11 ± 0.01	0.130 ± 0.005	**0.0636**	-
	SOCS3	0.010 ± 0.001	0.011 ± 0.001	**0.1853**	-
	IRAK3	0.0021 ± 0.0002	0.0016 ± 0.0003	**0.0628**	-
	NLRP3	0.0009 ± 0.0001	0.0008 ± 0.0001	**0.3306**	-
	MRP8	0.055 ± 0.007	0.07 ± 0.02	**0.2816**	-
	MRP14	0.0052 ± 0.0007	0.007 ± 0.003	**0.2625**	-
	Arg-1	0.0007 ± 0.0002	0.0007 ± 0.0002	**0.4208**	-
IMMUNE-CELL RECRUITMENT	**CCL2**	0.0034 ± 0.0006	0.0016 ± 0.0002	**0.0158***	Decrease
	CCL5	0.00042 ± 0.00007	0.00024 ± 0.00003	**0.0165***	Decrease
	CXCL1	0.00053 ± 0.00009	0.00061 ± 0.00012	**0.2906**	-
	CX3CL1	0.18 ± 0.02	0.22 ± 0.03	**0.1373**	-

	Gene	H_2O Mean($2^{-\Delta CT}$) ± SEM	EtOH Mean($2^{-\Delta CT}$) ± SEM	P value	EtOH effect
ANTIOXIDANT-RELATED	**GCLC**	0.49 ± 0.02	0.41 ± 0.01	**0.0057****	Decrease
	TXNRD1	0.023 ± 0.002	0.022 ± 0.002	**0.4318**	-
	GSR	0.026 ± 0.003	0.022 ± 0.002	**0.1869**	-
	HMOX1	0.013 ± 0.001	0.009 ± 0.001	**0.0383***	Decrease
	SVCT-2	0.0007 ± 0.0001	0.0009 ± 0.0002	**0.2473**	-
MICROGLIA SIGNATURE AND HOMEOSTASIS	**P2RY12**	0.48 ± 0.03	0.60 ± 0.04	**0.0273***	Increase
	MERTK	0.22 ± 0.02	0.15 ± 0.03	**0.0340***	Decrease
	PROS1	0.024 ± 0.003	0.025 ± 0.002	**0.3270**	-
	GPR34	0.25 ± 0.01	0.20 ± 0.01	**0.0131***	Decrease
	PU.1	0.037 ± 0.004	0.048 ± 0.007	**0.1099**	-
	CSF1R	0.0012 ± 0.0002	0.0011 ± 0.0002	**0.4215**	-
	TGFβR1	0.0075 ± 0.0005	0.0072 ± 0.0007	**0.3597**	-

As previously mentioned, the increase of oxidative stress has harmful effects to the surrounding brain tissue, contributing to neuronal damage in neurodegenerative diseases (Block et al., 2007). Apart from the ethanol-induced oxidative stress through ROS generation (Wu and Cederbaum, 2003), ethanol can also interfere with intrinsic brain defence mechanisms against these compounds by decreasing, for instance, the endogenous antioxidant balance (Henderson et al., 1995; Wu and Cederbaum, 2003). Therefore, we compared the abundance of mRNA transcripts of antioxidant-related genes between ethanol-treated and control brains. We found significantly lower mRNA transcripts for *GCLC* and *HMOX1* **(Table 3; Annex 6A and 6B)** in the ethanol-treated group, whereas the abundance of the transcripts for *GSR*, *SVCT2* and *TXNRD1* were similar **(Table 3; Annex 6C-E)** between the two experimental groups.

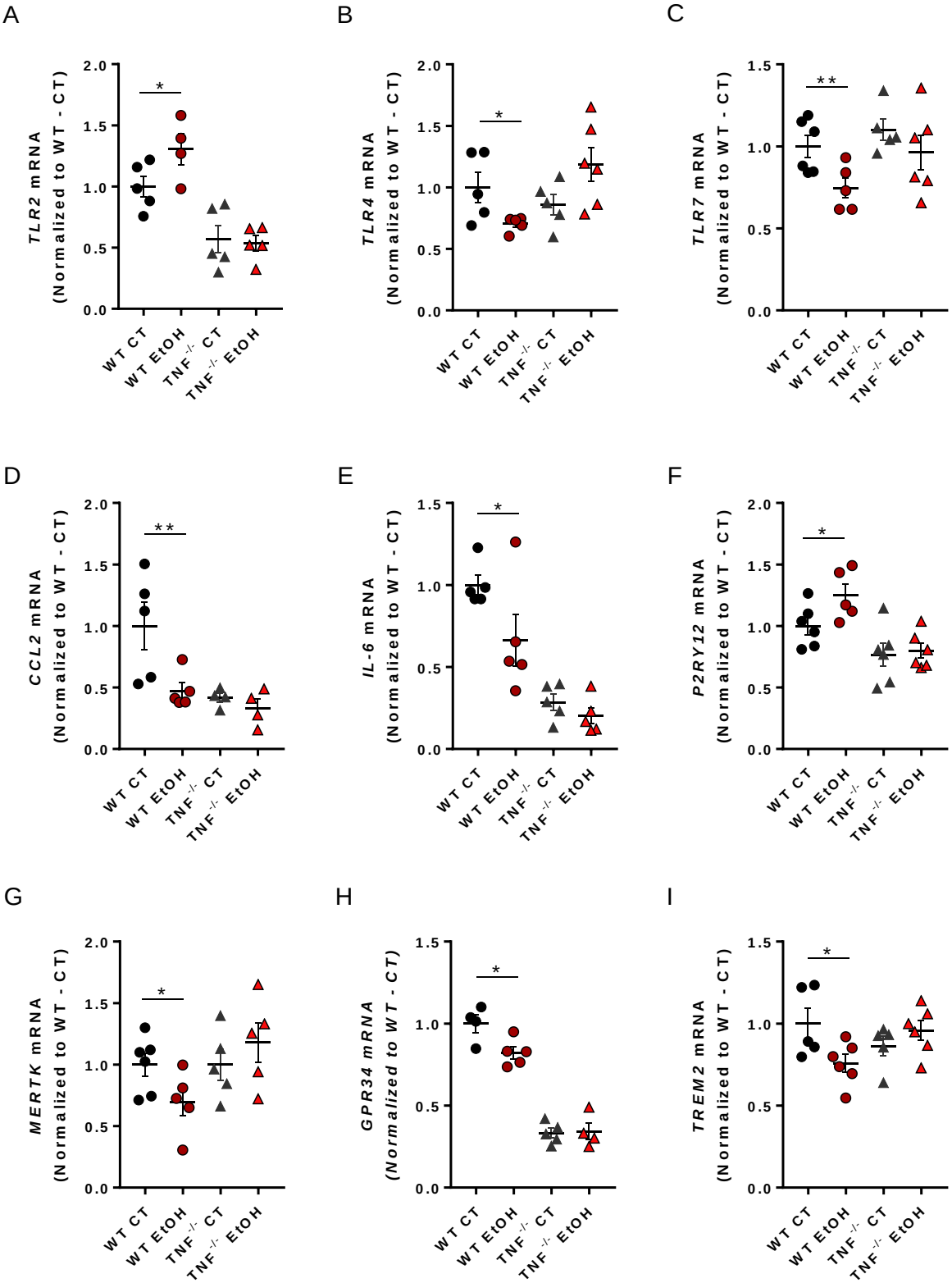
Ethanol-induced alterations in the transcripts of neuroimmune response genes is abrogated in TNF deficient mice

Because ethanol altered the mRNA transcripts of several genes evaluated in wild-type mice we asked at which extent those changes would occur in TNF deficient animals. Our results showed that the ethanol effect in altering the mRNA transcripts for *TLR2*, *TLR4*, *TLR7, CCL2*, *IL-6*, *P2RY12*, *MERTK, GPR34*, *TREM2*, *COX-2, GCLC* and *HMOX1* **(Figs. 9A-L)** was prevented in the brains of $TNF^{-/-}$ animals. On the other hand, the decrease in the transcripts for *CCL5* induced by ethanol in wild-type mice was not prevented in TNF deficient animals **(Fig. 9M)**. From these data, we concluded that TNF may modulate the ethanol-induced modifications of genes involved in the neuroimmune response, showing a potential role for TNF in shaping the ethanol effects in the adult brain.

TNF may influence anxiety-like behaviour in mice exposed to ethanol

Ethanol-induced microglia activation might modify neuronal function adversely influencing cognition and anxiety-related behaviour (Pascual et al., 2011; Sullivan and Pfefferbaum, 2005; Zhao et al., 2013). In order to assess whether ethanol exposure to mice is capable of inducing behavioural impairments, and whether these behavioural alterations could be associated with TNF production, we exposed adult wild-type and TNF deficient mice to ethanol and then tested these animals in different behavioural paradigms. The battery of tests included open field (OF) and elevated plus maze (EPM) to evaluate general locomotor function as well as exploratory and anxiety-like behaviours and novel object recognition (NOR) to test recognition memory. Classical parameters for evaluating anxiety-like behaviour in the EPM showed significant differences between ethanol-exposed and control mice inasmuch as animals treated with ethanol took more time to enter the open arms and displayed decreased frequency of open arms entries **(Figs. 10A and 10B)**, which was also associated with a higher percentage of time

spent in the closed arms **(Fig. 10C)**. The increase of this anxiety-like phenotype induced in wild-type mice by exposure to ethanol did not occur in TNF deficient animals **(Figs. 10A-C)**. In addition to this ethanol-induced increase in anxiety-like behaviour observed in the EPM, OF test indicated reduced exploratory behaviour and higher anxiety levels in wild-type mice exposed to ethanol because they spent lesser time in the central square of the OF arena **(Fig. 10D)** and displayed lower frequency of rearing **(Fig. 10E)** when compared with control littermates. Differently from wild-type mice, these same parameters of the OF test were comparable between $TNF^{-/-}$ animals exposed to water and ethanol **(Figs. 10D and 10E)**. The NOR test did not reveal in wild-type mice any significant differences between the ethanol-exposed and the control group **(Fig. 10F)**, indicating that our ethanol administration protocol caused no overt alterations in recognition memory in wild-type mice.


A
B
C
D
E
F
G
H
I
TLR2 mRNA (Normalized to WT - CT)
TLR4 mRNA (Normalized to WT - CT)
TLR7 mRNA (Normalized to WT - CT)
CCL2 mRNA (Normalized to WT - CT)
IL-6 mRNA (Normalized to WT - CT)
P2RY12 mRNA (Normalized to WT - CT)
MERTK mRNA (Normalized to WT - CT)
GPR34 mRNA (Normalized to WT - CT)
TREM2 mRNA (Normalized to WT - CT)
WT CT
WT EtOH
TNF-/- CT
TNF-/- EtOH
*
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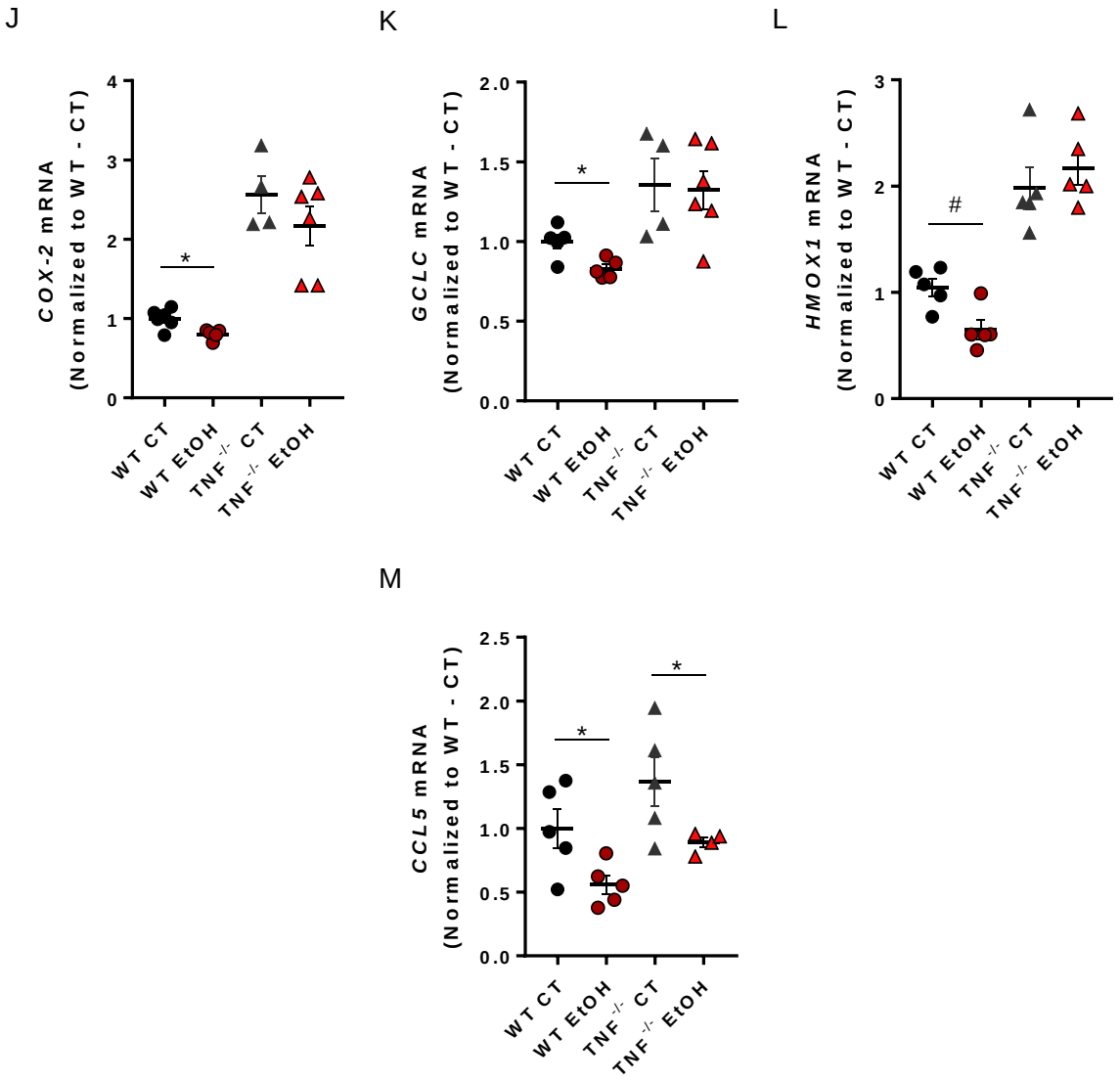

Figure 9. mRNA expression levels of immune-related genes in the cerebral cortex of wild-type and TNF$^{-/-}$ mice.

mRNA was harvested from the cerebral cortex of ethanol-treated and untreated WT and TNF$^{-/-}$ mice and the mRNA expression levels of the immune-related genes were determined by RT-qPCR. RT-qPCR was executed using *YWHAZ* reference gene. Results are expressed as mean ± SEM; N = 4-6 CT and EtOH WT and TNF$^{-/-}$ mice. *P < 0.05, **P < 0.01, One-way ANOVA and multiple t test. # means a P value nearby from 0.05 (P = 0.0766). Values displayed were normalized to the control group.

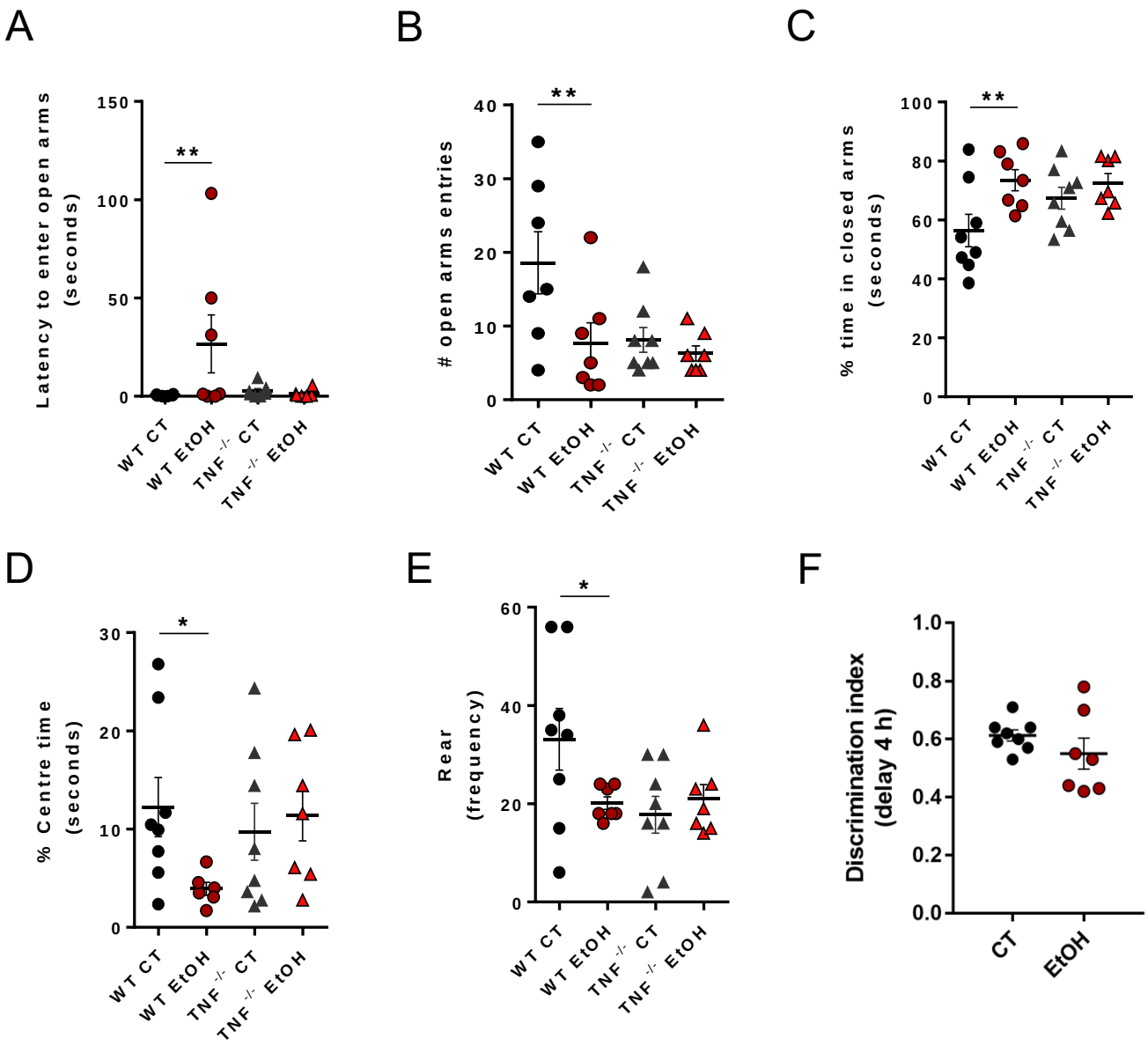

Figure 10. Behavioural tests in WT and TNF$^{-/-}$ mice upon ethanol exposure.
(A-C), Control and EtOH-treated WT and TNF$^{-/-}$ mice were evaluated in the EPM. Some alterations in anxiety-related behaviour were observed between CT and EtOH-treated WT mice, displaying significant difference in the time spent to enter open arms **(A)**, in the number of open arms entries **(B)** and in the time spent in the closed arms **(C)**. **(D-E)**, Control and EtOH-treated WT and TNF$^{-/-}$ mice were evaluated in the OF and show significant decrease in time spent in the centre **(D)** and in the frequency of rearing **(E)**. Animals were also evaluated in the NOR displaying no significant differences in the descrimination index between WT CT and EtOH **(F)**. Data are shown as mean ± SEM. N = 8 CT and 7 EtOH-treated mice. *P < 0.05, **P < 0.01, Two-way ANOVA (Fisher's LSD post-hoc test).

Chapter 5 | Discussion

Alcohol induces brain damage (Harper and Matsumoto, 2005; Pfefferbaum, 2004) and can lead to neuroinflammation and neurodegeneration (Alfonso-Loeches et al., 2010; Crews, 1999; Crews et al., 2004; Kreutzberg, 1996). Nevertheless, the mechanisms underlying these ethanol effects are uncertain. Previous studies found that ethanol exposure induces microglial activation (Fernandez-Lizarbe et al., 2009), which is typically defined as proinflammatory and cytotoxic (Kreutzberg, 1996). In fact, activated microglia reveals a proinflammatory signature in a TLR4-dependent manner upon ethanol exposure (Fernandez-Lizarbe et al., 2013). Moreover, ethanol induces the production of ROS in microglia (Boyadjieva and Sarkar, 2013; Qin and Crews, 2012) promoting apoptosis in developing hypothalamic neurons (Boyadjieva and Sarkar, 2010). Altogether, these evidences suggest that ethanol might induce microglia to acquire a neurotoxic role. Another identified mediator of microglial neurotoxicity is glutamate (Takeuchi, 2010). In this context, this excitatory neurotransmitter appears as an important factor to take in account because excessive amounts of glutamate can cause excitotoxic neuronal damage (Barger and Basile, 2001; Piani et al., 1992; Takeuchi et al., 2008). Although microglia has been established as a major source of this excitotoxic glutamate (Barger and Basile, 2001; Chen et al., 2012; Maezawa and Jin, 2010; Takeuchi et al., 2008; Takeuchi et al., 2006), the regulation of glutamate release by ethanol in microglia was not explored. In order to understand the mechanisms involved in ethanol-induced microglial glutamate release, we first performed in vitro experiments using primary microglial cultures from rats. We demonstrated that ethanol increased c-Src tyrosine kinase activity inducing the production and release of TNF, similar to what has been previously described for LPS and hypoxia (Socodato et al., 2015b). Furthermore, the autocrine activation of TNF receptors, engaged by TNF, leads to the release of microglial glutamate and consequent excitotoxic damage in neurons **(Fig. 11)**. In fact, our *in vitro* data showed that the robust c-Src activation in microglia induced by ethanol was necessary and sufficient for TNF release and that the glutamate release induced by c-Src

was abrogated in microglia from TNF deficient mice. These data suggest that targeting the c-Src/TNF pathway in microglia could be used as a potential strategy to alleviate ethanol-induced neurotoxicity.

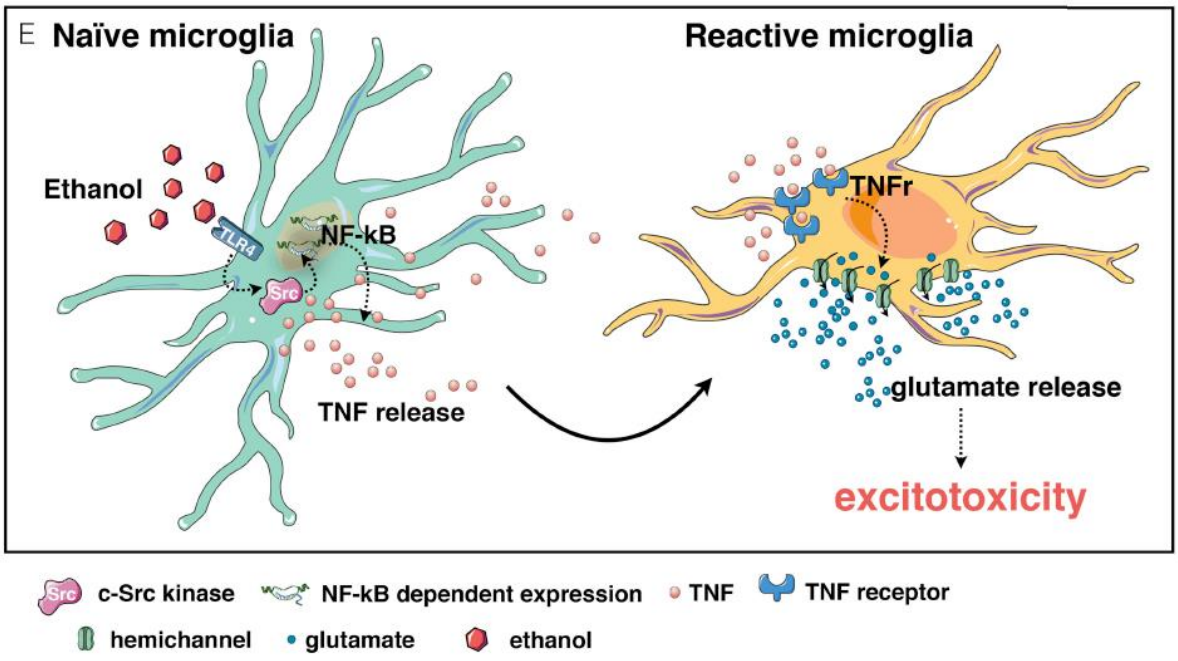

Figure 11. Signaling pathway activated by ethanol in cortical microglia to promote the release of glutamate.

As previously mentioned, microglia have been proved to act as effector cells that can damage the CNS parenchyma (Kempermann and Neumann, 2003). Inhibition of microglial activation has been faced as a therapeutic approach in the context of several neurodegenerative disorders. However, microglia also play neuroprotective roles by mediating the release of neurotrophic factors, clearance of neurotoxic substances and also glutamate uptake (Kempermann and Neumann, 2003; Kipnis et al., 2004; Schwab and Schluesener, 2004; Zietlow et al., 1999). Thus, therapeutic strategies targeting microglia could be feasible if on could inhibit sole the detrimental effects of activated microglia without lessening their beneficial roles (Takeuchi, 2010). On the other hand, inflammatory cytokines produced by activated microglia, such as TNF, can directly induce neuronal damage (Greig et al., 2004; Taylor et al., 2005; Venters et al., 2000). However, direct neurotoxic effects of these cytokines are relatively weak since they also activate neuroprotective factors such as MAPK and

expression of NF-κB (Ghezzi and Mennini, 2001; Kamata et al., 2005). Actually, inflammatory cytokines promote neurotoxicity through an indirect route by stimulating microglia in an autocrine/paracrine manner (Takeuchi and Suzumura, 2014).

TNF signaling plays a crucial role in inducing glutamate release from cultured microglia in an autocrine manner via Cx32 hemichannel (Takeuchi et al., 2006). This microglial glutamate release through gap-junction is more pronounced than by glutamate transporters and by the X_c^- system (Takeuchi and Suzumura, 2014). For this reason, hemichannel blockers appear as a potential therapeutic intervention to diminish microglial glutamate release without disturbing physiological levels of this neurotransmitter. We used two gap-junction inhibitors, GAP-27 and 18α-GA, to prevent the ethanol-induced glutamate release in cultured microglia. These compounds inhibit intercellular communication through gap-junctions involving Cx32 and Cx43 (Boengler et al., 2012; Ilvesaro et al., 2001), which are expressed by microglia (Eugenin et al., 2001; Garg et al., 2005; Kielian, 2008; Takeuchi et al., 2006). Connexin-mimetic peptide GAP-27 decreased the amount of glutamate released by activated microglia upon ethanol exposure. These results are in consistent with previous studies showing that blockade of Cx32 with ^{32}gap27 reduces microglial glutamate release and subsequent neurotoxicity (Takeuchi et al., 2008). 18α-GA gap-junction blocker did not induce significant changes in microglial glutamate release upon ethanol exposure, contradicting reported data showing that 18α-GA reduces TNF-induced microglial glutamate release and subsequent neurotoxicity (Takeuchi et al., 2006). We are planning to investigate more deeply this issue, using for instance different concentrations of 18α-GA gap-junction inhibitor seeking to attenuate or decrease the amount of glutamate released by microglia exposed to ethanol.

In general, in pathological conditions microglia release large amounts of TNF and this inflammatory cytokine is an essential element of the brain neuroinflammatory response (Kathryn et al., 2011; Montgomery and Bowers, 2012; Wyss-Coray and Mucke, 2002). Besides, and as previously discussed, TNF can potentiate glutamate-mediated cytotoxicity (Pickering et al., 2005; Takeuchi et al., 2006). TNF has also been associated with several

neurological disorders since neuroinflammation and excitotoxicity are hallmarks of neurodegenerative processes (Feuerstein et al., 1994; Olmos et al., 2014; Wyss-Coray and Mucke, 2002). Therefore, *in vivo* experiments involving TNF deficient mice seem to be an interesting approach to understand whether this cytokine modulates the ethanol effects in the brain.

Alcohol-mediated neurodegeneration is associated with relevant cognitive deficits, and increased impulsivity, impaired learning, memory and decision-making are some of the symptoms associated with alcohol consumption (Spanagel, 2009; Zahr et al., 2014). The hippocampus and the brain cortex are particularly vulnerable to the alcohol effects, and damage to these regions is deeply implicated in impaired frontal functioning and motor control (Marshall et al., 2016). Upon chronic ethanol-exposure in mice or rats several studies point toward diminished associative and spatial memory, decreased exploratory and locomotor activity and somewhat induction of anxiety-like behaviour (Pascual et al., 2011; Pascual et al., 2015; Zhao et al., 2013). Those evidences corroborate the changes in behaviour seen in ethanol-exposed mice in the EPM and OF tests. The changes found in the locomotor and anxiety-like behaviour, induced by ethanol, were largely attenuated in TNF deficient mice, suggesting the potential involvement of this cytokine in behavioural impairments induced by ethanol.

A key hallmark of the alteration of the brain neuroimmune response is the activation of glial cells, including microglia and astrocytes, which, upon activation, can produce and secrete a plethora of pro-inflammatory mediators and neurotoxic factors, such as cytokines, chemokines, ROS and glutamate (Block et al., 2007). Alcohol-dependent neuroimmune responses emerge from the alteration of inflammation-related and immunoregulatory genes/molecules involved in the innate immune system of the brain (Crews et al., 2015; Mayfield et al., 2013). We demonstrated that the mRNA transcripts of classical inflammatory genes were either unaltered or downregulated upon ethanol exposure, contradicting the literature describing that ethanol increases the expression of inflammatory cytokines,

chemokines, TLRs and COX-2 (Ahlers et al., 2015; Alfonso-Loeches et al., 2010; Drew et al., 2015; Fernandez-Lizarbe et al., 2013; Marshall et al., 2013; Pascual et al., 2015). Our data suggest that ethanol triggers only a mild inflammatory response in our semi-chronic exposure model. On the other hand, ethanol reduced the mRNA transcripts of some antioxidant-related genes, demonstrating its contribution for oxidative stress by interfering with the intrinsic brain defence mechanisms against ROS, as previously reported (Henderson et al., 1995; Wu and Cederbaum, 2003).

As previously mentioned, microglia have an important role both in CNS homeostasis and in disease progression and recovery. Distinguish microglia from peripheral myeloid cells that infiltrate the nervous system has piqued the interest of researchers recently. In fact, the identification of an exclusive genetic signature for microglial cells enables the better understanding and modulation of unique biological features of these cells in the context of CNS diseases (Butovsky et al., 2014). Our findings suggested that ethanol impacts on microglial homeostasis because the mRNA transcripts of different microglia signature genes were altered by ethanol exposure. These results, together with a previously reported ethanol-dependent effect on microglia viability, activation and proliferation (Alfonso-Loeches et al., 2010; Franke, 1995; Guerri et al., 1990; He and Crews, 2008; Miguel-Hidalgo et al., 2002) encouraged us to evaluate whether microglial numbers in the brain were also influenced by ethanol. Chronic alcohol exposure induces microglial activation in the cerebral cortex denounced by the upregulation in CD11b immunoreactivity (Alfonso-Loeches et al., 2010). Likewise, microglial Iba-1 show increased immunoreactivity in the cingulate cortex of alcohol-dependent humans abusers (He and Crews, 2008). We however observed that semi-chronic ethanol treatment did not exert any effect in increasing microglial Iba-1 protein content or in the cell density of microglia in cortical tissues.

In addition to microglia, astrocytes have also been implicated in neurodegeneration and inflammation and their activation (astrogliosis) can be triggered by different brain insults (Jha et al., 2016). A previous study using astrocytic progenitor cells from the fetal brains of rats

show that acute alcohol reduces GFAP expression and diminishes astrocyte cell proliferation (Guerri et al., 1990; Renau-Piqueras et al., 1989). On the other hand, post-mortem human tissue analysis shows reduced GFAP immunoreactivity in dorsolateral prefrontal cortex (Miguel-Hidalgo et al., 2002) and a great reduction of astrocytes in the hippocampus of alcoholics (Korbo, 1999). Similarly to microglia, semi-chronic ethanol exposure did not affect GFAP protein content and numbers of GFAP immunoreactive astrocytes in cortical tissues.

Chapter 6 | Conclusions and Future Perspectives

Our work not only reveals that TNF is essential for ethanol-induced microglial glutamate release, which is dependent on the activation of the tyrosine kinase c-Src, but also shows that TNF might to be involved in alcohol-induced behavioural changes and alterations of immunoregulatory genes/molecules involved in the neuroimmune responses of the brain.

Although ethanol did not induce effects in glial cell numbers, more specific studies are needed to dissect the relevance of microglial activation and inflammatory function upon alcohol exposure. Thus, assessment of microglial immunoreactivity upon semi-chronic ethanol exposure will give us another insight about the effect of this substance in microglia. Moreover, beyond the cerebral cortex, other brain regions, such as hypothalamus, hippocampus, striatum, cerebellum and amygdala, should be investigated to obtain an overall picture of different ethanol actions in CNS in this model system. Because semi-chronic ethanol treatment is capable of inducing changes in some neuroimmune- and antioxidant-related genes, it would be interesting to analyze both co-localization with neuronal and glial cell markers and protein expression levels of important molecules that could be implicated in alcohol-induced neurotoxicity and dysregulation of brain innate immunity. In another context, the inhibition of ethanol-induced microglial glutamate release through gap-junction inhibitors should be faced as a promising therapeutic approach to diminish glutamate excitotoxicity and behavioural deficits induced by ethanol exposure. We predict that a better comprehension of the mechanisms underlying microglial dysfunction upon ethanol exposure would be of paramount importance for understanding neuronal cell damage and the associated behavioural impairments caused by alcohol abuse.

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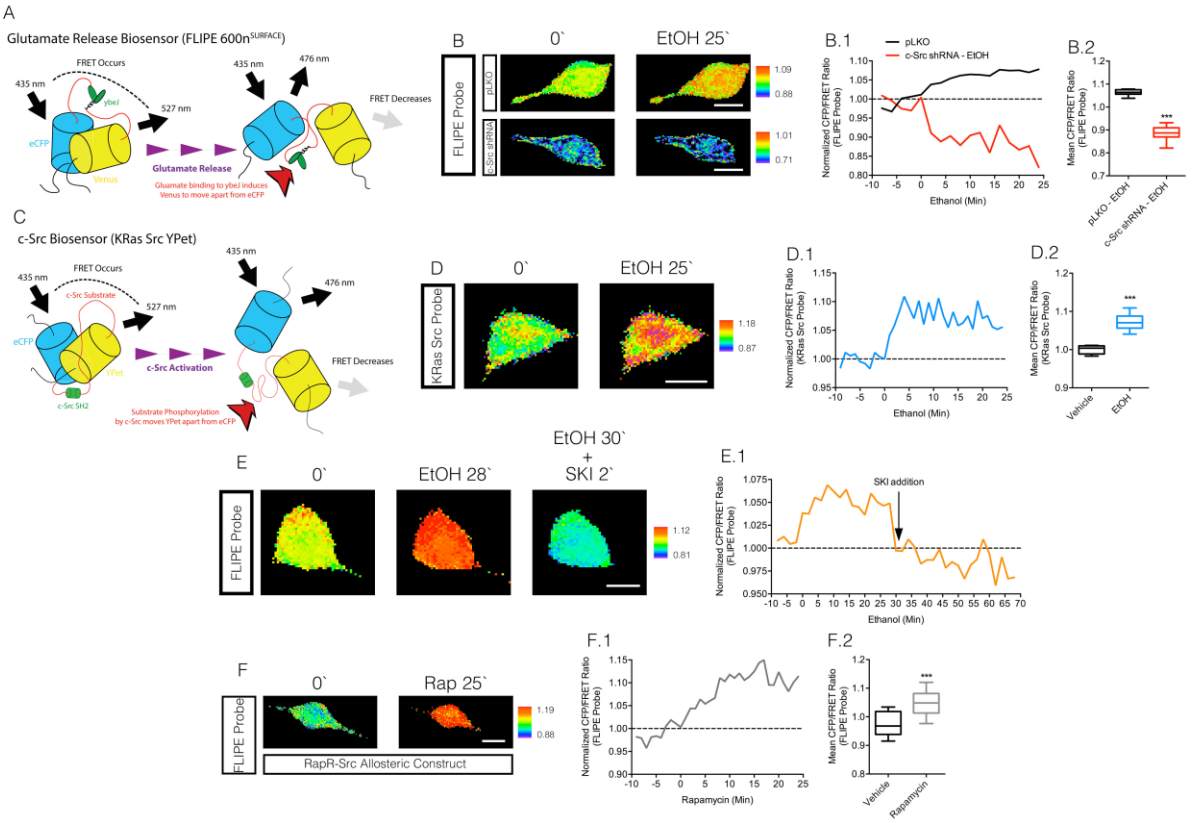
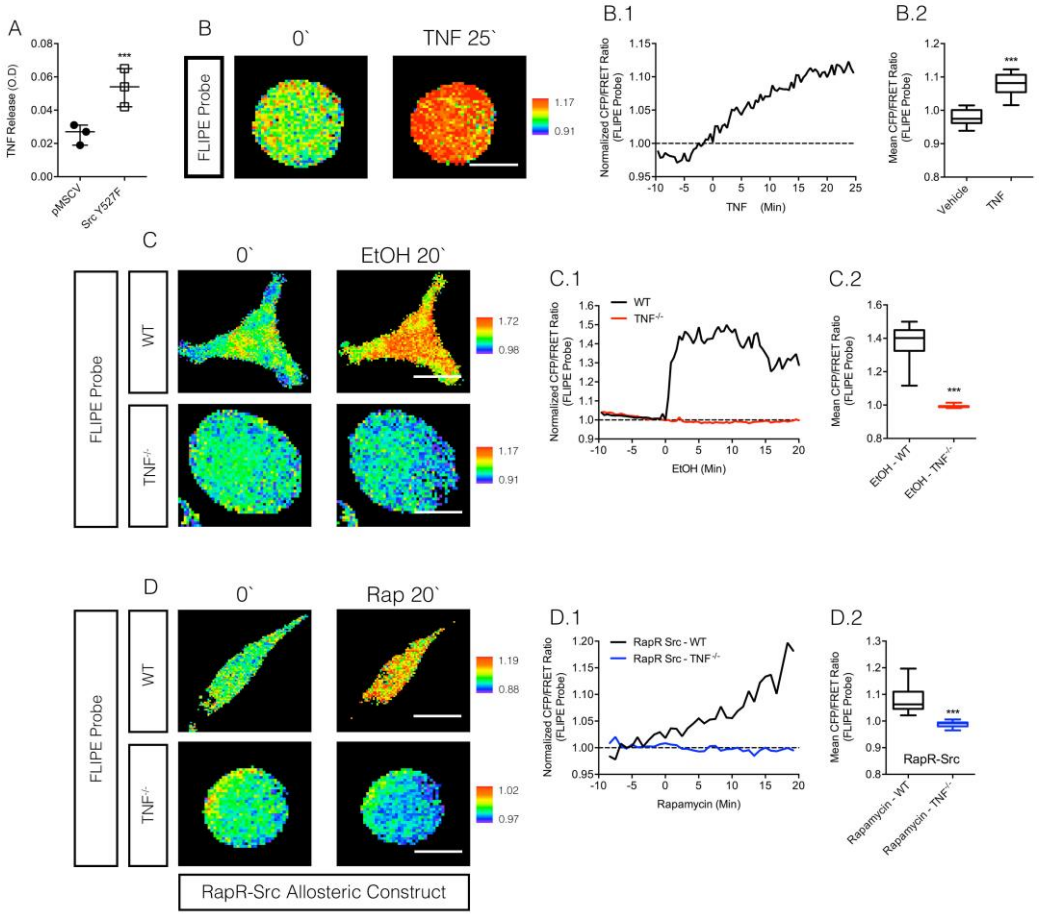
Annexes

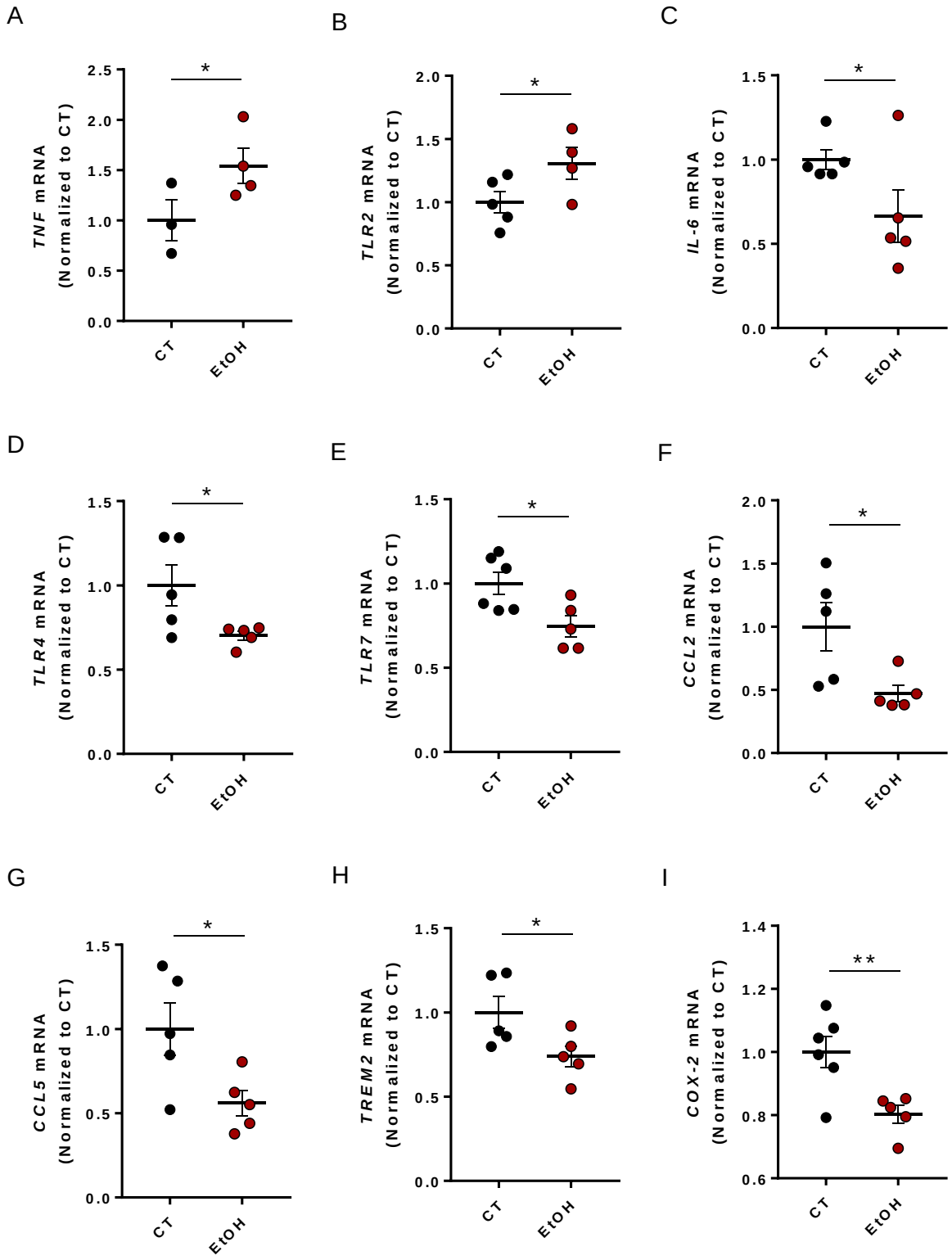


Annex 1. Ethanol triggers microglial glutamate release via c-Src.
(A) Diagram displaying the functioning of glutamate release FRET probe (FLIPE 600nSURFACE). (B) Cortical microglia were infected with the empty vector pLKO or c-Src shRNA and then transfected with the FLIPE 600n^{SURFACE} probe. Cells were treated with vehicle (MiliQ-water 10 µl; black) and EtOH (70 mM; red). The CFP/FRET emission ratios of the chimera were normalized at 0 min. Lines in B.1 represent the mean of 4 cells (pLKO) or 5 cells (c-Src shRNA). Box whiskers in B.2 display the median and min to max amplitude variation in pLKO + EtOH vs. c-Src shRNA + EtOH. ***P < 0.001, N=4, Mann-Whitney test. The panels in B show time-lapse CFP/FRET images color-coded according to the pseudocolor ramp. Calibration bar = 10 µm. (C) Diagram displaying the functioning of c-Src FRET probe. (D) Primary cortical microglia cells expressing a c-Src FRET probe (KRas Src YPet) were challenged with 70 mM ethanol (EtOH). CFP/FRET emission ratios of the chimera were normalized at 0 min. Line in D.1 represents the mean of 4 cells. Box whiskers in D.2 display the median and min to max amplitude variation in vehicle (HBSS) vs. EtOH. ***P < 0.001, N=4, Mann-Whitney test. The panels show time-lapse CFP/FRET images, coded according to the indicated pseudocolor scale, of representative cells transfected with the c-Src probe. Calibration bar = 10 µm. (E) Cortical microglia expressing the FLIPE probe were treated with EtOH (70 mM) and then, after 28 min, co-treated with EtOH + SKI (70 mM and 200 nM, respectively). CFP/FRET emission ratios of the chimera were normalized at 0 min, plotted and color-coded as in B. Line represents the mean of 5 cells. Calibration bar = 10 µm. (F) Cells were co-transfected with the FLIPE probe and a rapamycin-inducible c-Src construct (RapR-Src; E). Rapamycin (50 nM) was used to activate the c-Src construct. Line in F.1 represents the mean of 6 cells. Box whiskers in F.2 display the median and min to max amplitude variation in vehicle (DMSO) vs. Rapamycin. ***P < 0.001, N=6, t test. Calibration bar = 10 µm.

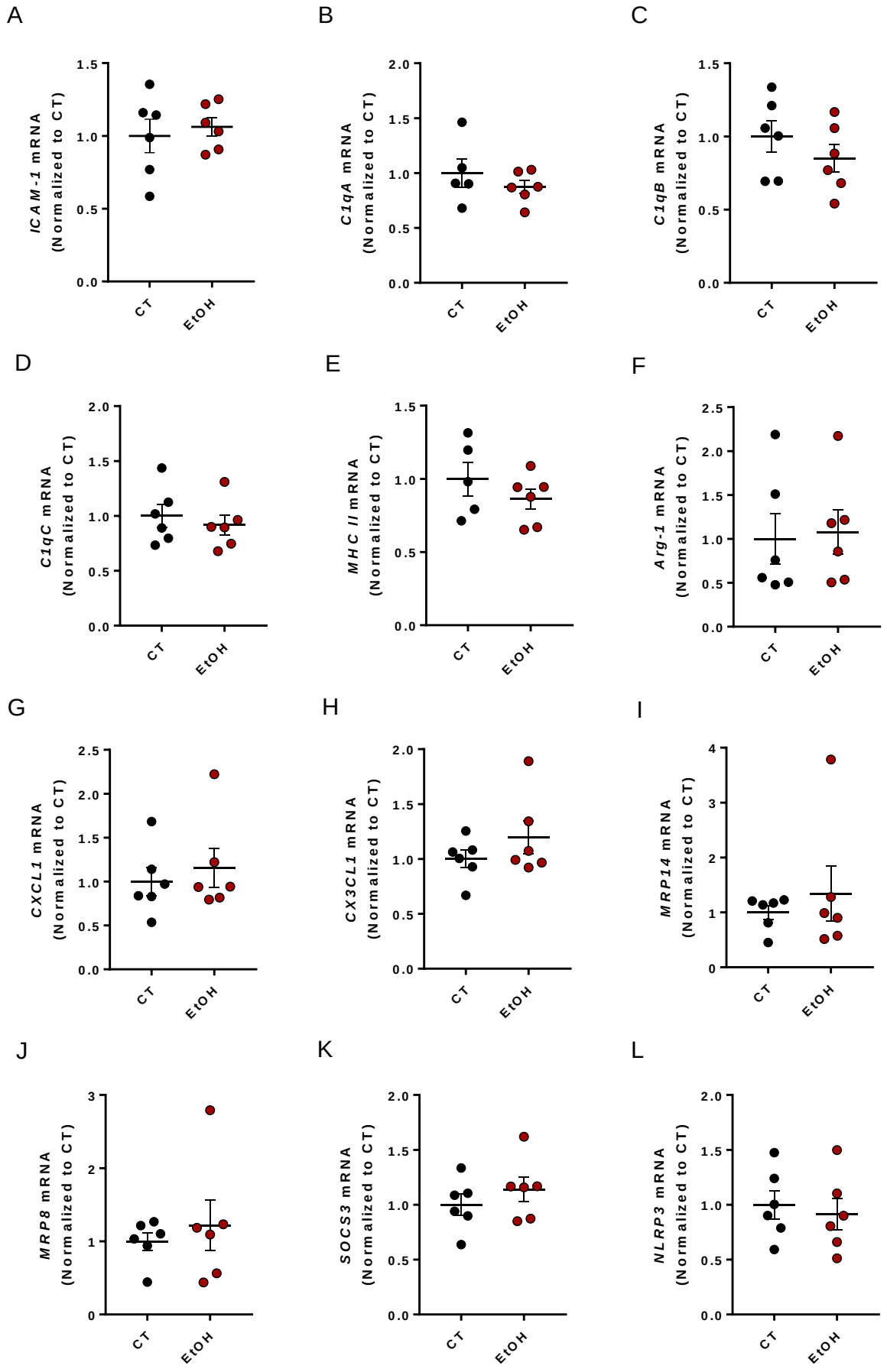


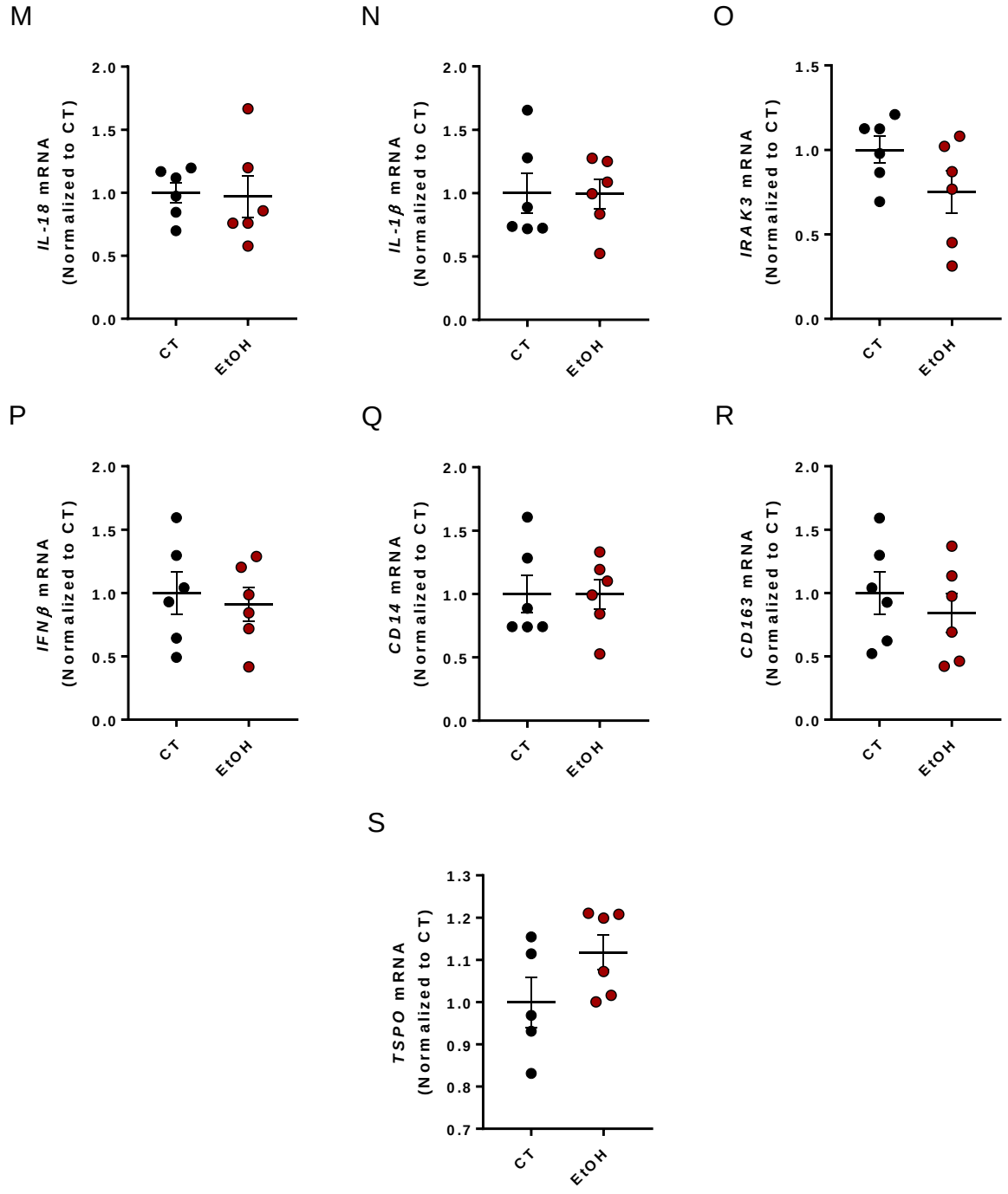
Annex 2. Ethanol and downstream c-Src activation regulate TNF production to induce glutamate release from microglia.

(A) Supernatant of N9 microglia expressing c-Src Y527F or the empty vector (pMSCV) was collected and ELISA determined TNF content. ***P < 0.001, Mann-Whitney test, N=3. (B) Primary cortical microglial cells expressing FLIPE probe were challenged with 25 ng/ml TNF. CFP/FRET emission ratios of the chimera were normalized at 0 min. Line in B.1 represents the mean of 5 cells. Box whiskers in B.2 display the median and min to max amplitude variation in vehicle (HBSS) vs. TNF. ***P < 0.001, N=5, t test. The panels show time-lapse CFP/FRET images coded according to the indicated pseudocolor scale. Calibration bar = 10 µm. (C) Cortical microglia from wild type (WT; black) or TNF knockout ($TNF^{-/-}$; red) mice were transfected with the FLIPE probe. Cells were treated with vehicle (MiliQwater 10 µl; black) and EtOH (70 mM). The CFP/FRET emission ratios of the chimera were normalized at 0 min as in B. Lines in C.1 represent the mean of 4 cells (WT) or 5 cells ($TNF^{-/-}$). Box whiskers in C.2 display the median and min to max amplitude variation in EtOH in WT vs. EtOH in $TNF^{-/-}$. ***P < 0.001, N=4, Mann-Whitney test. The panels in C show time-lapse CFP/FRET images color-coded according to the pseudocolor ramp. Calibration bars = 10 µm. (D) WT or $TNF^{-/-}$ microglia were co-transfected with the FLIPE probe and the RapRSrc construct. CFP/FRET emission ratios were recorded in the presence of rapamycin (50 nM). Lines in D.1 represent the mean of 4 (WT) or 5 (TNF-/-) cells. Box whiskers in D.2 display the median and min to max amplitude variation in vehicle RapRSrc: WT + rapamycin vs. RapR-Src:$TNF^{-/-}$ + rapamycin. ***P < 0.001, N=5, t test. Calibration bar = 10 µm.

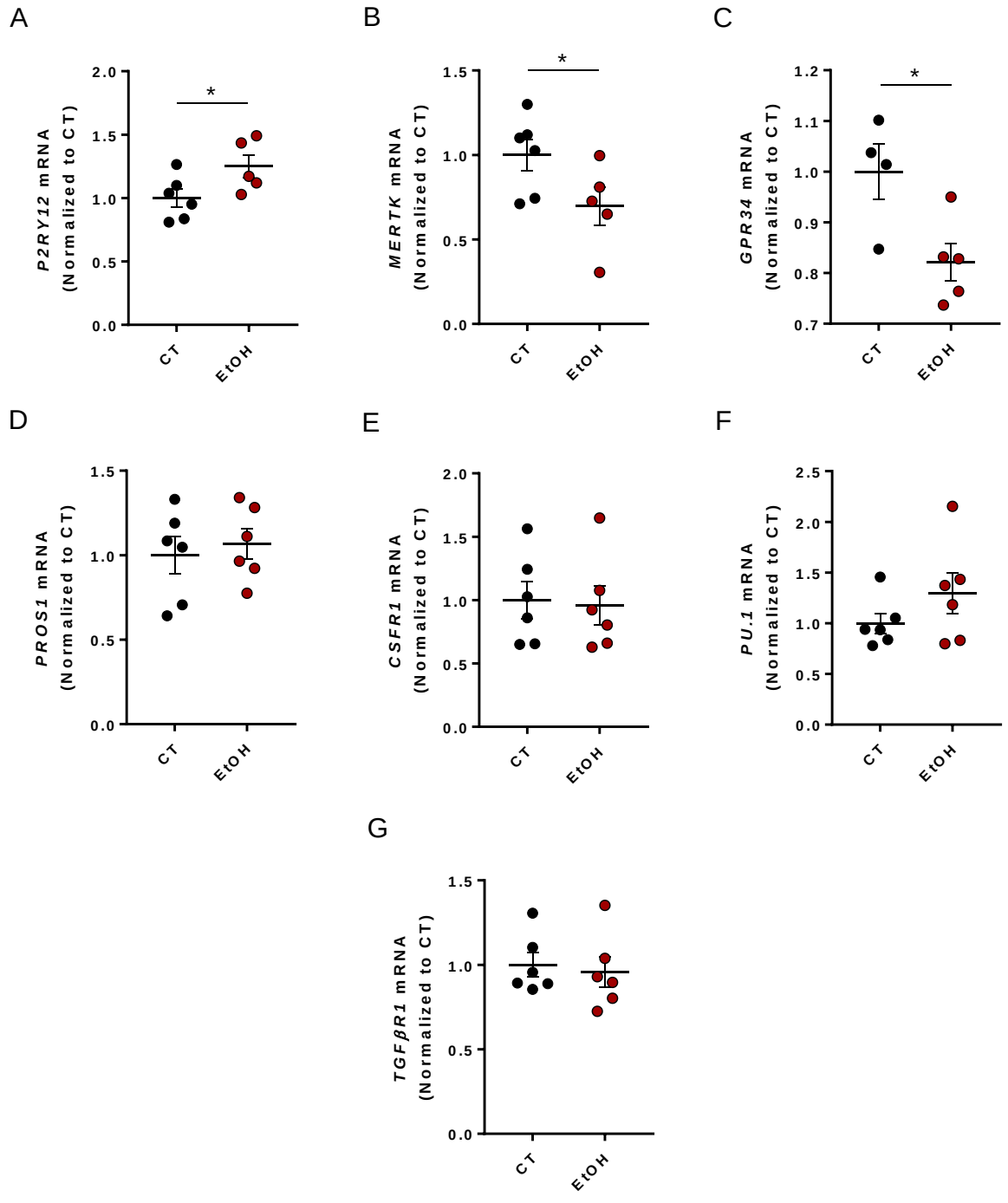


Annex 3. mRNA expression levels of inflammation-related genes in the cerebral cortex of wild-type mice. mRNA was harvested from the cerebral cortex of ethanol-treated and untreated WT mice and the mRNA expression levels of the inflammation-related genes were determined by RT-qPCR. RT-qPCR was executed using *YWHAZ* reference gene. Results are expressed as mean ± SEM; N = 4-6 CT and EtOH WT mice. *P < 0.05. **P < 0.01. Unpaired t-test. Values displayed were normalized to the control group.


A
ICAM-1 mRNA (Normalized to CT)
B
C1qA mRNA (Normalized to CT)
C
C1qB mRNA (Normalized to CT)
D
C1qC mRNA (Normalized to CT)
E
MHC II mRNA (Normalized to CT)
F
Arg-1 mRNA (Normalized to CT)
G
CXCL1 mRNA (Normalized to CT)
H
CX3CL1 mRNA (Normalized to CT)
I
MRP14 mRNA (Normalized to CT)
J
MRP8 mRNA (Normalized to CT)
K
SOCS3 mRNA (Normalized to CT)
L
NLRP3 mRNA (Normalized to CT)
CT
EtOH

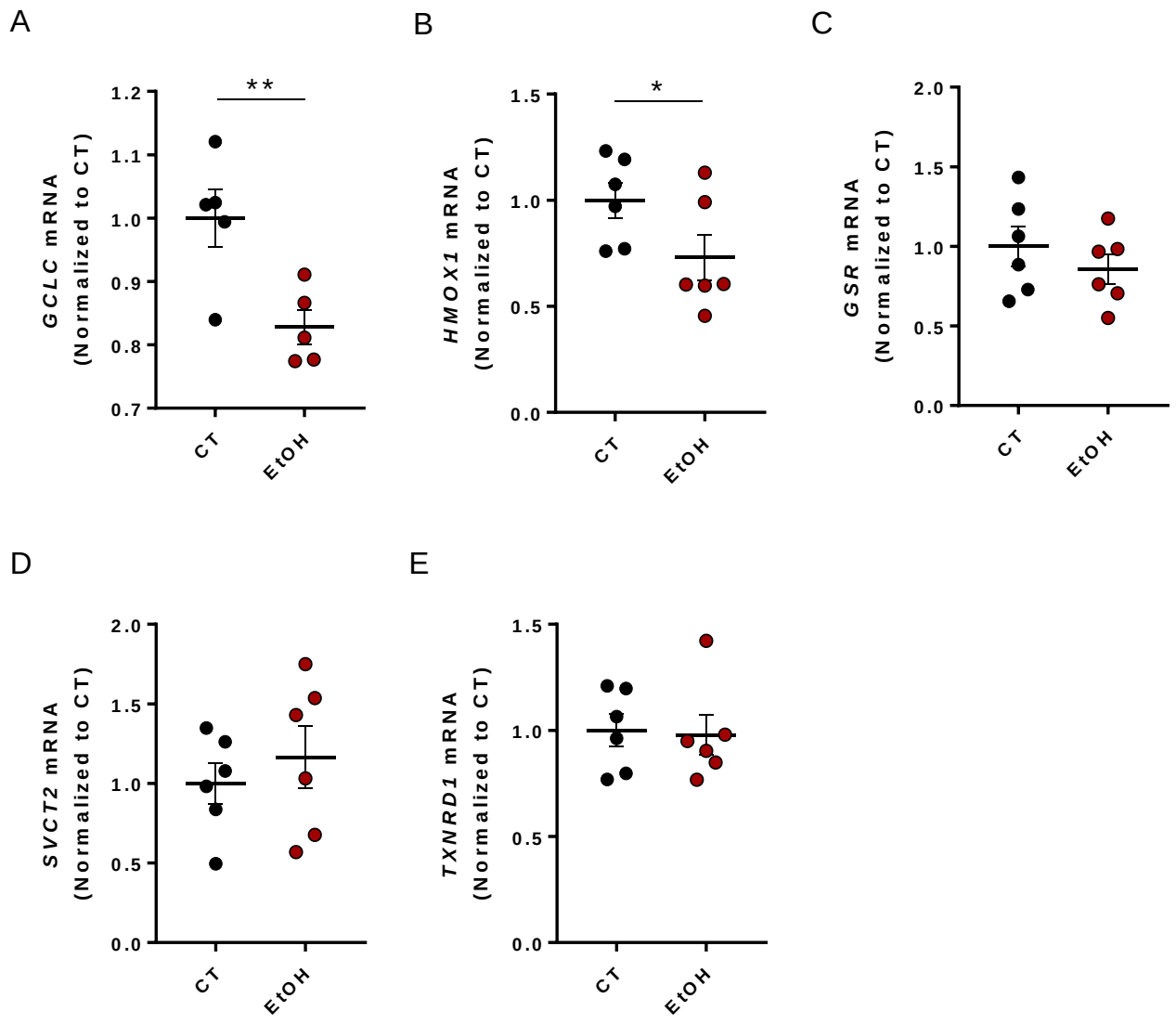


Annex 4. mRNA expression levels of inflammation-related genes in the cerebral cortex of wild-type mice. mRNA was harvested from the cerebral cortex of ethanol-treated and untreated WT mice and the mRNA expression levels of the inflammation-related genes were determined by RT-qPCR. RT-qPCR was executed using *YWHAZ* reference gene. Results are expressed as mean ± SEM; N = 4-6 CT and EtOH WT mice. *P < 0.05. Unpaired t test. Values displayed were normalized to the control group.



Annex 5. mRNA expression levels of microglia signature and homeostasis-related genes in the cerebral cortex of wild-type mice.

mRNA was harvested from the cerebral cortex of ethanol-treated and untreated WT mice and the mRNA expression levels of the microglia signature and homeostasis-related genes were determined by RT-qPCR. RT-qPCR was executed using *YWHAZ* reference gene. Results are expressed as mean ± SEM; N = 4-6 CT and EtOH WT mice. *P < 0.05. Unpaired t test. Values displayed were normalized to the control group.



Annex 6. mRNA expression levels of antioxidant-related genes in the cerebral cortex of wild-type mice. mRNA was harvested from the cerebral cortex of ethanol-treated and untreated WT mice and the mRNA expression levels of the antioxidant-related genes were determined by RT-qPCR. RT-qPCR was executed using *YWHAZ* reference gene. Results are expressed as mean ± SEM; N = 4-6 CT and EtOH WT mice. *P < 0.05. **P < 0.01. Unpaired t test. Values displayed were normalized to the control group.