# The role of the cannabinoid receptor 2 in alcohol-induced neuroinflammation and alcohol addiction

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**Affirmation**

I hereby declare that I prepared this thesis entitled: “The role of the cannabinoid receptor 2 in alcohol-induced neuroinflammation and alcohol addiction” entirely by myself except where otherwise stated. All text passages that are literally or indirectly taken from published or unpublished papers are indicated as such. All materials or services provided by other people are equally indicated.

Bonn, August, 20th, 2014

Bruno Pradier

So eine Arbeit wird eigentlich nie fertig, man muss
sie für fertig erklären, wenn man nach Zeit
und Umständen das Mögliche getan hat.

Johann Wolfgang von Goethe

## Abbreviations

| | |
|---|---|
| 2-AG | 2-arachidonylglycerol |
| 5-$HT_3$ | 5-hydroxytryptophan / serotonin (receptor 3) |
| ACTH | adrenocorticotropic hormone |
| AEA | anandamide |
| ANOVA | analysis of variance |
| BAL | blood alcohol level |
| BCA | bicinchoninic acid |
| BCP | beta-caryophyllene |
| $Ca^{2+}$ | calcium |
| $CB_1$ | cannabinoid receptor 1 |
| $CB_2$ / CNR2 | cannabinoid receptor 2 |
| CCL-2 | chemokine (C-C motif) ligand 2 |
| CD11b | cluster of differentiation molecule 11B |
| cDNA | complementary DNA |
| CNS | central nervous system |
| COX-2 | cyclooxygenase-2 |
| CPP | conditioned place preference |
| CRH | corticotropin-releasing hormone |
| DA | dopamine |
| DAGL | diacylglycerol lipase |

| | |
|---|---|
| DAPI | 4',6-diamidino-2-phenylindole |
| DEPC | diethylpyrocarbonate |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| EtOH | ethanol |
| FAAH | fatty acid amide hydrolase |
| FD | forced drinking |
| G x E | gene – environment interactions |
| GABA | γ-Aminobutyric acid |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GFAP | glial fibrillary acidic protein |
| GIRKs | G protein-coupled inwardly-rectifying potassium channels |
| GluT5 | glucose transporter 5 |
| GPCR | G protein-coupled receptors |
| $H_2O$ | water |
| HIC | handling-induced convulsions |
| HPA | hypothalamic-pituitary-adrenal axis |
| i.p. | intraperitoneal |
| Iba1 | ionized calcium-binding adapter molecule 1 |

| | |
|---|---|
| IFD | intermittent forced drinking |
| IFNγ | interferon gamma |
| IL-X | interleukin-X |
| IR | immuno-reactivity |
| $K^+$ | potassium |
| LPS | lipopolysaccharide |
| MAGL | monoacylglycerol lipase |
| $Na^+$ | sodium |
| NAc | nucleus accumbens |
| nACh | nicotinic acetylcholine receptor |
| NAD | nicotinamide adenine dinucleotide |
| NFκB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NMDA | N-methyl-D-aspartate |
| NO | nitric oxide |
| NR1-3 | NMDA receptor 1 - 3 |
| OD | optical density |
| OEA | oleoylethanolamine |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PEA | palmitoylethanolamide |
| PFA | paraformaldehyde |

| | |
|---|---|
| PVN | paraventricular nucleus of the hypothalamus |
| RNA | ribonucleic acid |
| ROI | region of interest |
| SDS | sodium dodecyl sulfate |
| SEM | standard error of the mean |
| SNP | single nucleotide polymorphism |
| TBS | tris-buffered saline |
| TLR4 | toll-like receptor 4 |
| TNF-α | tumor necrosis factor alpha |
| VTA | ventral tegmental area |
| WHO | world health organization |
| WT | wildtype |

## Abstract

Chronic alcohol abuse leads to severe brain damage, which has been associated with alcohol-induced neuroinflammation. Recently, the cannabinoid receptor 2 ($CB_2$), which is predominantly expressed on immune cells, has been shown to be involved in alcohol addiction. Therefore, this study aimed at investigating the role of the $CB_2$ in alcohol-induced neuroinflammation and at characterising alcohol-related behaviour in $CB_2$ knockout animals. First, the potency of different chronic alcohol models to induce neuroinflammation was analysed. To achieve this, levels of pro- and anti-inflammatory cytokines and glial activation markers were quantified in the cortex of the animals using ELISA and immuno-histochemical approaches. Next, we characterised the modulatory role of the $CB_2$ receptor in alcohol-induced neuroinflammation. We hypothesised that lack of $CB_2$ should be beneficial in alcohol-induced neuroinflammation. Therefore, the neuroinflammatory burden after chronic alcohol consumption was analysed in $CB_2$ deficient animals compared to WT controls. We can conclude that long-term models applied in this study led to neuroinflammation, as revealed by increased expression of pro-inflammatory cytokines. These changes were more pronounced when animals were continuously exposed to alcohol and additionally, we found a strong correlation between the duration of alcohol drinking and the severity of neuroinflammation. In line with this, long-term alcohol drinking led to a pro-inflammatory phenotype of microglia in the cortex. Furthermore, $CB_2$ deficiency dampens the inflammatory response in the cortex. However, this effect was strongly dependent on housing conditions. In a second approach, the alcohol drinking pattern of $CB_2$ deficient animals was analysed in different models that included environmental factors like social isolation, repeated withdrawal of alcohol or foot shock-induced stress. Finally, the development of tolerance, somatic signs of withdrawal and alcohol clearance were characterised in these mice. Interestingly, we detected that the $CB_2$ receptor increased alcohol drinking in a model for social drinking. Additionally, our data suggest that the $CB_2$ receptor modulates alcohol reward. Taken together, these data show that the $CB_2$ receptor is involved in a variety of alcohol-related phenotypes ranging from alcohol-induced neuroinflammation to alcohol reward. In addition, the function of this receptor is strongly modulated by the environment.

# 1 Introduction

Alcohol use disorder is a chronic relapsing disease that is characterised by a "compulsion to seek and to take the drug, loss of control in limiting intake, and the emergence of a negative emotional state when access to the drug is prevented" (Koob & Volkow, 2009). According to the status report of the World Health Organisation (WHO) 19.4 % of adult men and 5.9 % of adult women in Germany drink in a manner posing a risk to health (WHO, 2014a). Moreover, in 2012 5.9 % of all global deaths were attributable to alcohol (WHO, 2014b). Considering the harm alcohol inflicts on users and to their social environment, it is the most harmful drug before heroin in second place (Nutt et al., 2010).

While there is a substantial risk for humans to inherit alcoholism, environmental factors contribute nearly in equal strength to the development of addiction, which underlines the complex nature of this disease (Goldman et al., 2005). Genetic and environmental interaction leads to a large heterogeneity in alcohol-dependent patients in terms of symptom dimensions and severity of disorder. Furthermore, long-term alcohol abuse leads to severe cognitive deficits, which have been – similarly to other neurodegenerative diseases – attributed to neuroinflammation (He & Crews, 2008; Obernier et al., 2002; Pascual, Baliño, et al., 2011).

The endocannabinoid system plays an important role in the modulation of neurological and immunological processes and is therefore a promising candidate in the investigation of different aspects of addiction ranging from initial drug use to cognitive impairments after long-term abuse. Recently, the cannabinoid receptor 2 ($CB_2$) has been associated with alcoholism in humans, and has been implicated in alcohol, nicotine and cocaine addiction in rodents (Al Mansouri et al., 2014; Ignatowska-Jankowska et al., 2013; Ishiguro et al., 2007; Ortega-Álvaro et al., 2013; Xi et al., 2011). These findings were very surprising, as for a long time the $CB_2$ was believed to be absent in the brain and its function was thought to be restricted to immune function. Furthermore, it is involved in stress reactivity (Bahi et al., 2014; García-Gutiérrez & Manzanares, 2011; García-Gutiérrez et al., 2010), thereby possibly modulating alcohol consumption in relation to the environment (Al Mansouri et al., 2014; Ishiguro et al., 2007). These studies indicate an emerging role of the $CB_2$ in alcohol abuse, which might also depend

on gene x environment (G x E) interactions. Moreover, it possibly modulates alcohol-induced neuroinflammation, which can cause cognitive deficits after chronic alcohol use. Altogether, these reports suggest that the $CB_2$ receptor is a valuable target to study alcohol-related behaviour and alcohol-induced neuroinflammation.

## 1.1 Neurobiology of alcohol addiction

Several activities like eating, sex and sport elicit pleasant feelings. They guarantee the survival and reproduction of the individual by acting on the brain's reward system. These so-called 'positive reinforcers' stimulate the mesolimbic dopaminergic system, which is part of the reward pathway. Drugs of abuse activate the same pathway and protracted misuse results in pathologic changes leading to addiction (Koob & Volkow, 2009). Alcohol activates dopaminergic neurons in the ventral tegmental area (VTA), which project to the nucleus accumbens (NAc) and results in the release of dopamine (DA) (Chiara & Imperato, 1988). This effect is a hall-mark of all drugs of abuse and is associated with the pleasant acute effects of the drug (Boileau et al., 2003). As a first feature of the development of addiction, the drug use becomes impulsive and is driven by positive reinforcement (Figure 1). However, as the disease progresses, after long-term alcohol abuse, the drug use is characterised by uncontrolled compulsion to seek and to take the drug. Importantly, at this stage drug intake is driven by negative reinforcement, as a negative emotional state emerges during abstinence (Koob & Volkow, 2009).

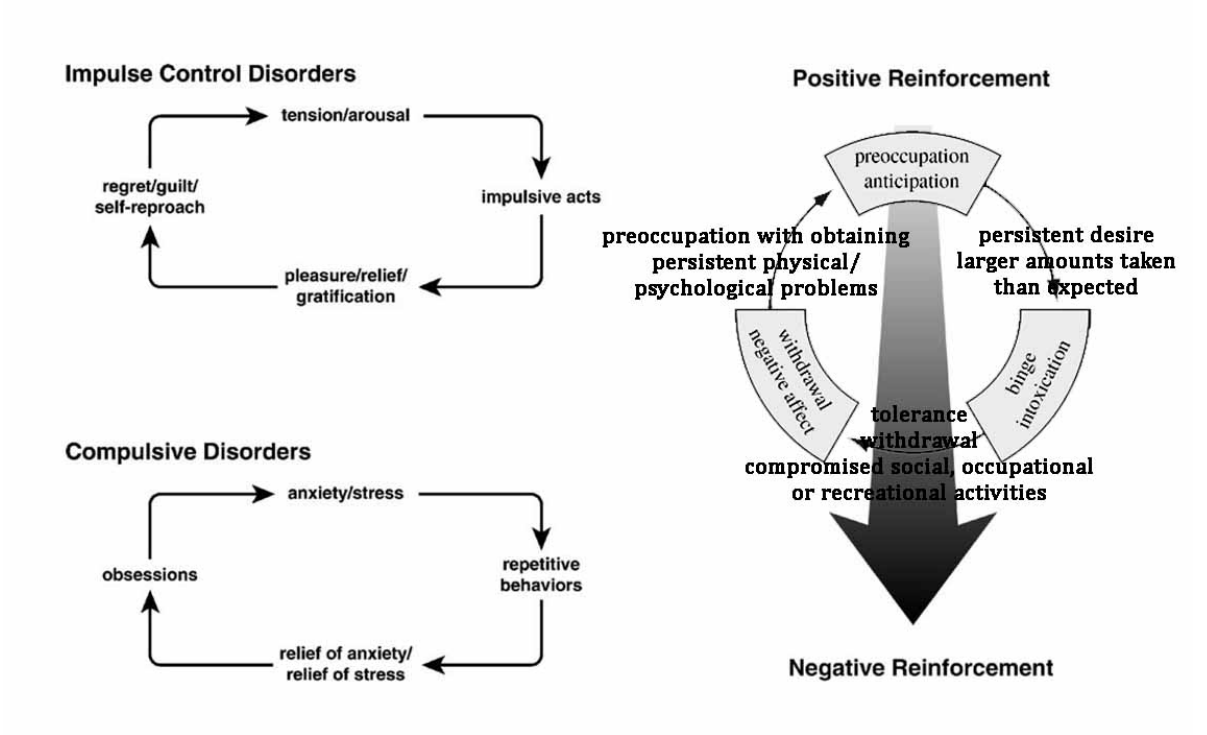



Figure 1. The development of addiction (Modified from Koob et al. 2004; Koob & Le Moal 2008).

From a pharmacologic point of view, alcohol is a 'dirty' drug because it has many primary targets including the γ-aminobutyric acid ($GABA_A$), N-methyl-D-aspartic acid (NMDA), acetyl choline (nACh), glycine and 5-hydroxytryptamine (serotonin, $5\text{-}HT_3$) receptors, as well as G-protein activated inwardly rectifying $K^+$ channels (GIRKs) and L-type $Ca^{2+}$ channels (Spanagel, 2009). However, the effect of alcohol is most thoroughly studied in the case of GABAergic and glutamatergic neurons. The $GABA_A$ receptor is a pentameric ligand-gated chloride channel and the major inhibitory neurotransmitter receptor in the mammalian brain. Acute alcohol intake increases the activity of $GABA_A$ receptors, which results in reduced anxiety, slurred speech, sedation, disinhibition and reduced levels of consciousness (Lingford-Hughes et al., 2010). Chronic alcohol use, however, leads to decreased $GABA_A$ receptor function, which is due to the development of tolerance. This effect is thought to be mediated via a decreased $GABA_A$ receptor density and an altered expression of $GABA_A$ subunits (Spanagel et al., 2008). Alcohol mediates the DA-release indirectly via GABA-ergic neurons. GABA is an important modulator of DA release in the NAc as GABA-interneurons tonically inhibit the activity of VTA DA-neurons that project to the NAc ('GABA-brake') (Shizgal & Hyman, 2013). Alcohol leads to the release of endorphin within the VTA, which acts on μ-opioid

receptors on GABA-interneurons. This results in the inhibition of GABA-interneurons and thereby in disinhibition of DA release in the NAc (Lingford-Hughes et al., 2010). Alcohol also profoundly modulates glutamate signalling by acting on the NMDA receptor. The NMDA receptor is a ligand-gated ion channel and consists of a heteromeric assembly of NR1, $NR2_{(A-D)}$ and NR3 subunits. Acute alcohol intake antagonises NMDA receptor function, which results in reduced excitatory transmission (Spanagel, 2009). Long-term adaptation to alcohol use leads to the enhanced expression of NMDA receptors. Nonetheless, chronic alcohol use leads to a reduced baseline activity in regions of the frontal cortex, which is in part dependent on glutamatergic projections. These regions control executive functions like working memory, attention, decision making and behavioural inhibition. Changes in activity of these regions are profoundly implicated in the development of addiction and compulsive drug use (Nestler, 2005). However, alcohol withdrawal results in excess glutamate activity, which is associated with increased cytotoxicity and is thought to contribute to cognitive impairments (Barron et al., 2008; Tsai & Coyle, 1998).

### 1.2 Gene x environment interactions in addiction

Twin studies revealed that the heritability of alcoholism resides between 50 and 60 %, which indicates that genetic and environmental risk factors equally contribute to the development of addiction (Goldman et al., 2005). Many genes are associated with alcoholism, including genes encoding for alcohol metabolising enzymes or genes that are associated with other psychiatric diseases (Crabbe et al., 2006). Environmental risk factors that favour the development of addiction are manifold and include maternal stress, substance abuse during pregnancy, low birth weight, lack of normal parental care, stressful life events, childhood physical abuse and, toxic exposures (Clarke et al., 2008). Stress is the major environmental risk factor in the development and maintenance of addiction, as any form of negative life events or emotionally disruptive condition may promote relapse (Sinha, 2008). In order to appropriately respond to environmental stimuli the body releases neurotransmitters and stress hormones, thereby activating the hypothalamus-pituitary-adrenal (HPA) axis, which is an important mediator of the homeostatic response (Lightman & Conway-Campbell, 2010). Briefly, during stress response, corticotropin-releasing hormone (CRH) is secreted from the paraventricular nucleus (PVN) of the hypothalamus to the pituitary gland. Here, CRH

induces the release of adrenocorticotropic hormone (ACTH) into the blood stream and stimulates the adrenal cortex to produce glucocorticoid hormones (mainly corticosterone in rodents and cortisol in humans). Corticosterone/cortisol provides negative feedback to the pituitary gland and the PVN in order to stop the stress response. During chronic stress, the negative feedback of the HPA axis is disrupted and leads to prolonged and exacerbated stress responses. During alcohol exposure and detoxification, the HPA axis activity is increased and remains altered for weeks after cessation of alcohol intake. Polymorphisms in the CRH system are associated with heavy drinking, often in interaction with a history of stress experience (Clarke et al., 2008; Zorrilla et al., 2014). Consistently, rodents also show alcohol-dependent increases in CRH and ACTH levels following alcohol exposure. Pharmacologic blockade of the CRH receptor 1 results in reduced alcohol seeking and stress-induced alcohol intake (Sillaber et al., 2002; Sommer et al., 2008). Importantly, glucocorticoids also modulate the reward system leading to enhanced DA levels in the NAc, whereas chronic stress leads to a reduced DA synthesis and turnover (Rodrigues et al., 2011). In summary, the HPA axis is an important system that orchestrates stress responses and is implicated in the development of addiction.

### 1.3 Neuropathomechanism of chronic alcohol consumption:

### A role for neuroinflammation?

Cycles of chronic excessive alcohol consumption and abstinence have long-lasting neurological and behavioural consequences, resulting in cognitive impairment and enhanced compulsivity. Impairments have been observed to include deficits in abstract problem solving, learning and memory, and executive motor functions (Fama et al., 2004). Furthermore, chronic alcohol consumption can lead to alcohol-associated dementia and Wernicke-Korsakoff syndrome, the latter of which is due to thiamine deficiency. Brain imaging techniques have demonstrated that chronic alcohol abuse leads to atrophy of the cerebellum, corpus callosum and frontal cortex (Pfefferbaum & Sullivan, 2005; Sullivan & Pfefferbaum, 2005). Moreover, alcohol abuse leads to severe brain volume loss, which is comparable to that in patients with Alzheimer's disease. This includes shrinkage in cortical and subcortical regions, hippocampus, striatum and brainstem, as well as ventricle enlargement (Sullivan & Pfefferbaum, 2005). The diminished gray and white

matter density suggests a reduced connectivity in the brain. However, the underlying pathomechanisms are not fully understood, although oxidative stress, glutamate excitotoxicity and nutritional deficiency contribute in part to neurological impairments (Crews & Nixon, 2009; Haorah et al., 2008). Recently, another mechanism has been discovered, which could underlie the neuropathologic processes. Signs of alcohol-induced neuroinflammation were demonstrated in human post mortem tissue as revealed by increased expression of CCL-2, microglial (Iba1) and astrocytic (GluT5) markers in various brain regions (He & Crews, 2008). Since then, many studies have provided evidence of alcohol-induced neuroinflammation also in preclinical models (Figure 2) (Collins & Neafsey, 2012; Crews & Vetreno, 2011; Qin et al., 2008). The use of genetically modified mice established the importance of the innate immune system, specifically of the toll-like receptor 4 (TLR4) in alcoholism (Alfonso-Loeches et al., 2010; Fernandez-Lizarbe et al., 2009). Alcohol has been shown to activate the TLR4 pathway in microglia and astrocytes, which leads to the activation of nuclear factor kappa B (NFκB). This in turn, leads to the production of a wide range of pro-inflammatory mediators, including chemokines (CCL-2), cytokines (TNF-α, IL-1β, IL-6) and enzymes like inducible NO-synthase (iNOS) and cyclooxygenase 2 (COX-2) (Alfonso-Loeches et al., 2010; Pascual et al., 2009), which lead to the enhanced production of NO and prostaglandins. Furthermore, cognitive impairments and demyelination were shown to be associated with neuroinflammation, and TLR4-deficient mice were protected against alcohol-induced brain damage (Alfonso-Loeches et al., 2012; Obernier et al., 2002; Pascual, Baliño, et al., 2011).

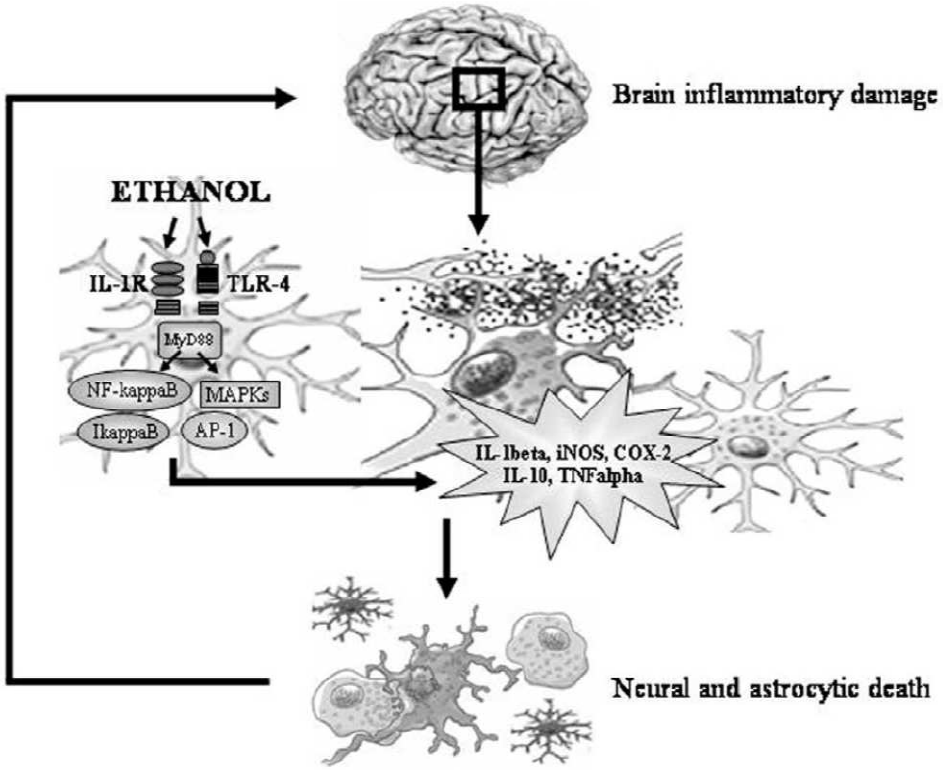



Figure 2. Potential mechanism of alcohol-induced brain damage involving neuroinflammation (Blanco & Guerri, 2007)

## 1.4 The endocannabinoid system

The endocannabinoid system is a modulatory system that alters neural transmission, as well as immune function. It consists of at least two well-described cannabinoid receptors ($CB_1$ and $CB_2$), their endogenous ligands (endocannabinoids) and their synthesis and degradation enzymes. $CB_1$ and $CB_2$ are (mostly) $G_{i/o}$-protein coupled receptors (GPCR) that both act via inhibition of the adenylate cyclase, activation of MAP kinases and modulation of intracellular calcium ($Ca^{2+}$) flux (McAllister & Abood, 2006). Furthermore, activation of $CB_1R$ inhibits voltage-dependent $Ca^{2+}$-channels and activates inwardly rectifying potassium ($K^+$) channels ($K_{ir}$), which leads to reduced neurotransmitter release through a retrograde signalling pathway (Figure 3A) (Piomelli, 2003). Being the most abundant GPCR in the brain, $CB_1R$ is involved in many physiological and pathological conditions (Katona & Freund, 2008).

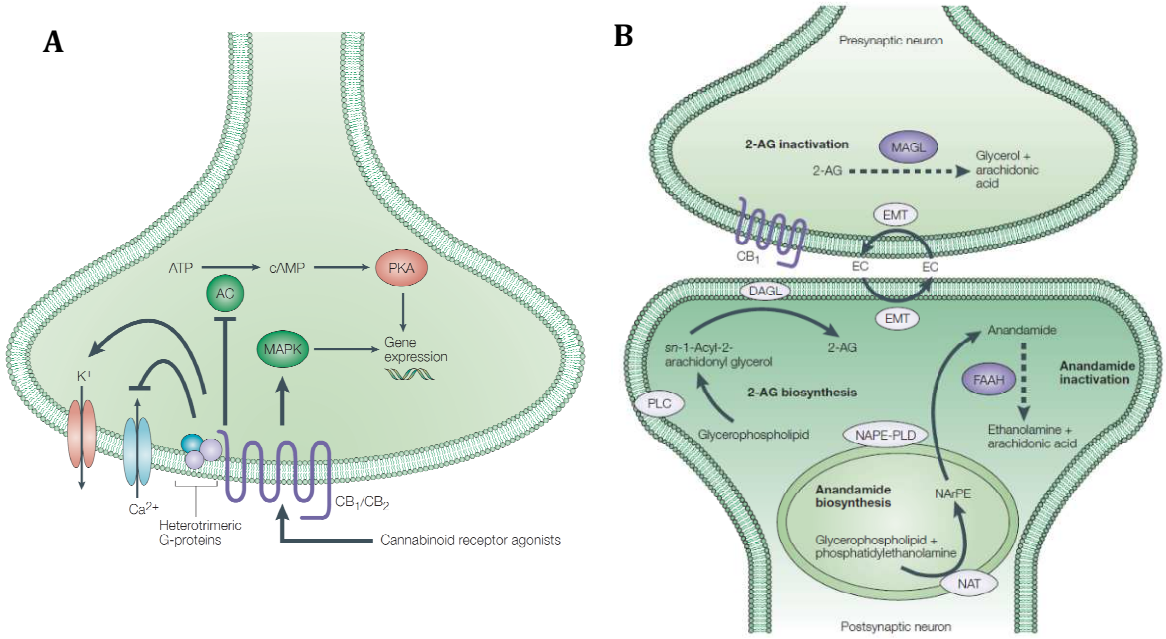



Figure 3. (A) $CB_{1/2}R$ signalling transduction pathways in the pre-synapse. (B) Synthesis and degradation of endocannabinoids. For more details, please see text (taken from Di Marzo, 2004).

On the other hand, expression of the $CB_2$ in the central nervous system (CNS) has long been neglected, and due to its high expression on leukocytes its function has been predominantly restricted to immune modulation (Atwood & Mackie, 2010). However, recent studies detected $CB_2$ mRNA expression in various brain regions, including brain stem, cerebellum, midbrain, cingulate cortex, entorhinal cortex, hippocampus, striatum, nucleus accumbens, amygdala and hypothalamus (Atwood & Mackie, 2010; García-Gutiérrez et al., 2010; Gong et al., 2006; Navarrete et al., 2012; Onaivi et al., 2008; Van Sickle et al., 2005). Furthermore, two reports provide electro-physiologic evidence suggesting a neuromodulatory function of the $CB_2$ in the entorhinal and the prefrontal cortex (Boon et al., 2012; Morgan et al., 2009). However, the cellular site of expression of $CB_2$ receptors in the brain is still highly arguable (Atwood & Mackie, 2010) and its neuro-physiologic relevance still remains elusive.

2-arachidonyl glycerol (2-AG) and anandamide (AEA) are the two most defined endocannabinoids (Figure 3B). Both bind with comparable affinity to the $CB_1R$, whereas 2-AG is a high efficacy agonist at $CB_2$ and AEA shows only low affinity to this receptor (Atwood & Mackie, 2010; Pertwee et al., 2010). Endocannabinoids are produced on

demand, and their synthesis can be triggered by $Ca^{2+}$ influx (into the postsynaptic site) and neuronal activation. Four alternative pathways lead to the biosynthesis of AEA, whereas its major degrading enzyme is the fatty acid amide hydrolase (FAAH) (Di Marzo et al., 2004). Most of the 2-AG pool is synthesized by the diacylglycerol lipase (DAGL) (Di Marzo, 2011), which has two isoforms: DAGLα expression is most prevalent in the brain, whereas DAGLβ is the prominent enzyme for 2-AG synthesis in the liver. 2-AG is mainly degraded by monoacylglycerol lipase (MAGL), yielding arachidonic acid and glycerol (Lichtman et al., 2010). Beside 2-AG and AEA, several other endocannabinoids that do not bind to $CB_1R$ or $CB_2$ have been recently identified in the brain (called non-classical or orphan endocannabinoids), like oleoylethanolamide (OEA) and palmithoylethanolamide (PEA). OEA has been shown to bind to GPR 55, whereas PEA is a ligand of both GPR55 and GPR119 (Godlewski et al., 2009), which have been suggested as novel receptors of the endocannabinoid system (Brown, 2007).

## 1.5 The role of the $CB_2$ receptor in neuroinflammation

The $CB_2$ receptor has been connected with many pathologic conditions including cardiovascular disease, allergic dermatitis, inflammatory pain, obesity as well as liver and bone disorders (Buckley, 2008; Cluny et al., 2012; Karsak et al., 2007; Klauke et al., 2014; Pacher & Mechoulam, 2011). The expression of $CB_2$ is predominantly found on immune cells and is also present in brain resident microglia cells (Stella, 2010). The $CB_2$ has been implicated in neuroinflammatory conditions like Alzheimer's disease (AD), Huntington's disease, multiple sclerosis and neuropathic pain (Ashton & Glass, 2007; Cabral & Griffin-Thomas, 2010; Racz, Nadal, et al., 2008). Analysis of post mortem tissues of AD patients showed a strong increase in $CB_2$ expression, as well as $CB_2$ and FAAH in microglia clusters at β-amyloid plaques (Benito et al., 2003; Solas et al., 2013). Furthermore, administration of $CB_2$ agonists (MDA7, JWH-133 and WIN 55,212) has been shown to reduce microglial activation and reduced the cognitive impairment in rodent models of AD (Aso et al., 2013; Ramírez et al., 2005; Wu et al., 2013). Moreover, in a rat chronic lesion model for Huntigton's disease the $CB_2$ expression was increased in a subpopulation of microglia in the lesioned striatum (Fernández-Ruiz et al., 2007). Recently it has been demonstrated in a model for multiple sclerosis that $CB_2$ expression increases with progression of disease, specifically in activated microglia and macrophages (Maresz et al., 2005). Finally, activation of the $CB_2$ by JWH-133 or beta-

caryophyllene (BCP) reduced thermal hyperalgesia, mechanical allodynia and microglial activation after partial nerve ligation, whereas the inflammatory response was exacerbated in $CB_2$ deficient animals (Klauke et al., 2014; Racz, Nadal, et al., 2008).

Evidence from *in vitro* studies show that $CB_2$ activation modulates B- and T-cell differentiation, reduces proliferation and phagocytosis of macrophages, as well as production of inflammatory mediators like NO, IL-12 and TNF-α. Moreover, it is implicated in migration of leukocytes (Ashton & Glass, 2007; Cabral & Griffin-Thomas, 2010). Pro-inflammatory stimulation of microglia with lipopolysaccharid (LPS) activates the TLR4 pathway, which leads to a massive cellular response including phosphorylation of MAP kinases, the production of TNF-α, IL-1β, IL-6, and induction of iNOS expression as well as secretion of NO. Many studies demonstrate that activation of the $CB_2$ signalling blocked the LPS-induced pro-inflammatory response (Ashton & Glass, 2007; Cabral & Griffin-Thomas, 2010; Gertsch et al., 2008). On the other hand, application of $CB_2$ inverse agonists showed the same effects (Gertsch, 2008). In accordance with that, primary microglia isolated from $CB_2$ deficient mice showed an impaired LPS response as revealed by decreased ICAM, CD40, IL-6, CCL-2 and TNF-α production (Schmöle et al., submitted.). These pleiotropic effects are probably due to ligand bias or altered receptor coupling. To sum up, activation of the $CB_2$ signalling has been shown to be anti-inflammatory in many conditions, although this effect is not always consistent and may depend on the ligand and the cell type.

## 1.6 The emerging role of the $CB_2$ receptor in drug abuse

Recently, a single nucleotide polymorphism (SNP) in the CNR2 gene locus, R63Q, has been associated with psychiatric disorders including schizophrenia, depression (Ishiguro et al., 2010; Onaivi et al., 2008), and alcoholism (Ishiguro et al., 2007). This SNP leads to a missense mutation in the first intracellular domain, which results in a decreased cellular response to $CB_2$ ligands (Ishiguro et al., 2010). Based on these reports, preclinical studies also implied the $CB_2$ in animal models of psychiatric disorders, including depression, impulsivity, anxiety and schizophrenia (García-Gutiérrez et al., 2010; Navarrete et al., 2012; Ortega-Alvaro et al., 2011). Furthermore, there is emerging evidence to support the role of the receptor in drug addiction. Xi and co-workers discovered that high dose application of JWH-133 led to a decreased cocaine

self administration. This effect was independent of the $CB_1R$ and seemed to be specific for the $CB_2$ (Xi et al., 2011). In the same study, application of the $CB_2$ agonist led to a dose-dependent decrease in baseline dopamine levels in the NAc. This finding has been validated in mice overexpressing the $CB_2$. Enhanced $CB_2$ activity resulted in reduced cocaine conditioned place preference (CPP) and decreased cocaine self administration (Aracil-Fernández et al., 2012). On the other hand $CB_2$ activity appears to have opposing effects in nicotine addiction. Inhibition of $CB_2$ with SR144528 at low doses decreased nicotine CPP, whereas stimulation with O-1966 enhanced nicotine CPP. Moreover, $CB_2$ deficient animals did not develop nicotine CPP (Ignatowska-Jankowska et al., 2013). This finding has been replicated in another study reporting that antagonist treatment (AM630) and $CB_2$ deficiency reduced nicotine CPP and reduced nicotine self administration (Navarrete et al., 2013). Contrary to this, Gamaleddin and colleagues reported no effect of either $CB_2$ agonist (AM1241) or antagonist (AM630) on nicotine self administration, reinstatement and nicotine seeking in rats (Gamaleddin et al., 2012). However, the reason for these conflicting reports may be species-related differences. Furthermore, $CB_2$ deficient mice on a CD1 background showed enhanced alcohol CPP, preference and consumption whereas alcohol self administration was not altered (Ortega-Álvaro et al., 2013). Moreover, the $CB_2$ expression seems to decrease, when animals develop alcohol preference compared to those that did not develop (Onaivi et al., 2008). On the other hand, neither chronic pharmacologic treatment with JWH-015 nor AM630 altered alcohol consumption (Ishiguro et al., 2007). Interestingly, in the same study chronic $CB_2$ stimulation resulted in enhanced alcohol consumption after chronic mild stress compared to vehicle-treated stressed control animals. This finding indicates that $CB_2$ receptor activity is modulated by stress, which points to putative G x E interaction of the $CB_2$ receptor.

## 1.7 Aims of this study

The first part of this study was aimed at **(1)** establishing a model for the study of alcohol-induced neuroinflammation. In order to do so, the potency of different models for alcohol consumption to induce neuroinflammation was analysed (including chronic, forced and intermittent forced drinking). For this, levels of neuroinflammatory markers (pro- and anti-inflammatory cytokines and glial activation marker) were quantified in the cortex of the animals with different techniques (ELISA, Western blot and immunohistochemistry). **(2)** We characterised the modulatory role of the $CB_2$ in alcohol-induced neuroinflammation. We hypothesised that lack of $CB_2$ should be beneficial in alcohol-induced neuroinflammation ultimately leading to a reduced activation of the TLR4 pathway. Therefore, the cognitive performance and the neuroinflammatory burden after chronic alcohol consumption were analysed in $CB_2$ deficient animals and compared to WT controls. Moreover, the study aimed at **(3)** the phenotypic analysis of alcohol drinking patterns in $CB_2$ deficient animals in different models that included environmental risk factors like social isolation, frequent withdrawal of alcohol or foot shock-induced stress. Finally, the development of tolerance, somatic signs of withdrawal and alcohol clearance were characterised in these mice.

# 2 Material

## 2.1 Equipment

| | |
|---|---|
| Analytical balance | BP 121 S, Sartorius |
| Bioanalyzer | Agilent 2100 bioanalyzer, Agilent Technologies |
| CCD camera | AxioCam MRm, Zeiss |
| Centrifuges | Biofuge fresco, Heraeus Instruments |
| | Biofuge pico, Heraeus Instruments |
| Cryostate | CM 3050 S, Leica |
| Homogenisers | Precellys® 24, Bertin Technologies |
| | Ultra-Turrex®, IKA Werke, Staufen, Germany |
| | Ultrasound homogenizer, Bandelin Sonoplus |
| | 1 ml glass homogeniser, Wheaton, USA |
| Incubator | CB210, Binder |
| Laser Scanning Microscope | SP8, DMI 6000 CS, Leica |
| Lux meter HI 97500 | Hanna Instruments, Hamburg, Germany |
| Magnetic stirrer | MR 3001 K, Heidolph, Fisher |
| Microscopes | Axioplan 2, Zeiss Axioscope 40, Zeiss |
| PCR iCycler | iCycler, Bio-Rad Laboratories |
| pH meter | inoLab, WTW |
| Polypropylene vials (15, 50 ml) | BD Bioscience, Pharmingen, San Diego, CA, USA |
| Polystyrene vials (5 ml) | BD Bioscience, Pharmingen, San Diego, CA, USA |
| Real-Time Cycler | 7500 Real-Time PCR Detection System, |
| | Applied Biosystems |
| Rectal thermometer BAT-12 | Physiotemp, New Jersey, USA |
| Rota-Rod | UgoBasile, Italy |
| Safe-lock vials (0.5, 1.5, 2 ml) | Eppendorf, Hamburg, Germany |
| Scanner | Epson Perfection 4990, Epson |
| Spectrophotometers | MRX TCII, Dynex |
| | NanoDrop ND-1000, Thermo Scientific |
| | Ultrospec 2100 pro, GE Healthcare |
| Superfrost Plus® slides | Menzel-Gläser, Braunschweig, Germany |
| Startle response System | TSE Systems, Germany |

| | |
|---|---|
| Vortexer | Vortex-Genie 2, Scientific Industries |
| 96-well-plate | Greiner, Bio-one, Frickenhausen, Germany |

## 2.2 Chemicals

If not mentioned otherwise, all reagents were purchased from Carl Roth (Karlsruhe, Germany), Invitrogen (Darmstadt, Germany), Merck (Darmstadt, Germany), or SIGMA-Aldrich (Steinheim, Germany).

## 2.3 Antibodies

| 1st Antibody | Host | Dilution | Company |
|---|---|---|---|
| GFAP | Rabbit | 1/1500 | Abcam |
| Iba1 | Rabbit | 1/200 | WAKO |
| IL-1β | Goat | 1/50 | R&D |
| NeuN | Rabbit | 1/500 | Sigma |

Table 1. List of primary antibodies used in this study

| 2nd Antibody | Host | Dilution | Company |
|---|---|---|---|
| Anti-goat Alexa Fluor 488 | Donkey | 1/500 | Invitrogen |
| Anti-rabbit Alexa Fluor 488 | Donkey | 1/500 | Invitrogen |
| Anti-rabbit Alexa Fluor 488 | Goat | 1/500 | Invitrogen |
| Anti-rabbit Cy3 | Goat | 1/500 | Invitrogen |

Table 2. List of secondary antibodies used in this study

## 2.4 ELISA and assay kits

| Kit | Company |
|---|---|
| BCA assay | ThermoFisher (23227) |
| Corticosterone Enzyme Immunoassay Kit | Arbor Assays (K014) |
| Mouse CCL-2 ELISA | eBioscience (88-7391) |
| Mouse GM-CSF ELISA | eBioscience (88-7334) |
| Mouse IFN-γ ELISA | eBioscience (88-7314) |
| Mouse IL-10 ELISA | eBioscience (88-7804) |
| Mouse IL-1β ELISA | eBioscience (88-7013) |
| Mouse IL-6 ELISA | eBioscience (88-7064) |
| Mouse IL4 ELISA | eBioscience (88-7044) |
| Mouse TNF-α ELISA | eBioscience (88-7324) |

| | |
|---|---|
| Mouse TNF-α ELISA | R&D Systems (DY410) |
| NAD-NADH Reagent Multiple Test Vial | Sigma-Aldrich (n7160) |

Table 3. List of ELISA and assay kits used in this study.

## 2.5 Buffers and solutions

| | |
|---|---|
| Blocking buffer (immuno staining) | 1 x PBS (pH = 7.2) |
| | 1 % BSA |
| | 10 % NS |
| Glycine buffer | 0.5 M glycine per 100 ml $H_2O$ |
| | adjust pH with $Na_2CO_3$ to pH 9.0 |
| Narcotic solution | 5 ml Xylariem |
| | 2.5 ml Ketamin |
| | 52.5 ml 0.9 % (w/v) NaCl |
| Permeabilisation buffer | 1x PBS (pH = 7.2) |
| (Immuno staining) | 0.5 % Triton-X 100 |
| Protein lysis buffer | 10 mM Tris (pH = 8) |
| (RIPA buffer) | 150 mM NaCl |
| | 0.5 mM EDTA |
| | 1 % IGEPAL (Nonidet P40) |
| | 0.1 % (w/v) SDS |
| | 0.5 % deoxycholic acid |
| | (prior to use: 1 tablet Complete Mini |
| | Protease Inhibitor per 10 ml buffer) |
| | 0.1 % Tween-20 |
| Stop solution (ELISA) | 1 M $H_3PO_4$ |
| 10 x TBS | 0.2 mM Tris-HCl (pH 7.6) |
| | 1.37 mM NaCl |
| Washing buffer (ELISA) | 1x PBS |
| | 0.05 % (v/v) Tween-20 |
| Washing buffer (Immuno staining) | 1 x PBS |
| 4% PFA | 40 g paraformaldehyde per 1 l 1x PBS (pH 7.2) |
| 10 % SDS | 100 g SDS per 1 l 1 x PBS (pH 7.2) |

| | |
|---|---|
| 10 % sucrose solution | 100 g sucrose per 1 l 1 x PBS (pH 7.2) |
| 20 % sucrose solution | 200 g sucrose per 1 l 1x PBS (pH 7.2) |

## 2.6 Software

| | |
|---|---|
| ActiMot | TSE Systems, Germany |
| Adobe Photoshop | CS3, Version 10.0.1, 2007 Adobe Systems |
| AxioVision LE | Carl Zeiss, Germany |
| Ethovision | Noldus, Version XT 8.15 |
| ImageJ | Wayne Rasband, NIH, USA ,Version 1.41o |
| Leica Application Suite | Leica, Germany |
| Microsoft Office 2011 | Microsoft, Germany |
| Mouse-E-Motion | Infra-E-Motion, Germany |
| Mendeley | Mendeley Ltd., USA |
| NanoDrop | NanoDrop 1000, V 3.7 |
| Prism | GraphPad Software, Inc., Version 5 (2010) |
| Revelation | Dynex Technologies, Inc |
| Sequence Detection Software | Applied Biosystems, Version 2.2.2 |
| Sarto Connect | Sartorius, Germany |
| Statistica | StatSoft, Inc. Version 7.1 (2005) |
| Startle response system | TSE Systems, Germany |
| VideoMot | TSE Systems, Germany |

# 3 Methods

## 3.1 Animals

Male mice (2-8 months old) carrying two truncated alleles of *CNR2* ($CB_2$) and wildtype (WT) littermates on a C57BL/6J background (backcrossed for > 8 generations) were used in this study (Buckley et al., 2000). Animals were kept in a reversed light/dark cycle (light off between 7:00 AM and 7:00 PM) and received food and water *ad libitum*. The housing conditions were maintained at 21 ± 1°C and 55 ± 10 % relative humidity. Experimental procedures complied with all regulations for animal experimentation in Germany and were approved by *Landesamt für Natur, Umwelt und Verbraucherschutz in Nordrhein-Westfalen*, Germany.

## 3.2 Alcohol models

### 3.2.1 Chronic alcohol administration

Two models were applied to study chronic alcohol consumption: the forced drinking (FD) and the intermitted forced drinking paradigms (IFD). In the FD procedure animals were supplied with a 16 % ethanol solution as the only source of liquid (Racz et al., 2012; Trebicka et al., 2011). To familiarise animals with alcohol a 4 % alcohol solution was given for three days before starting the procedure. Then the alcohol concentration was raised to 8 % for a further four days. After one week, animals received a 16 % alcohol solution for the following six months. If animals refused to drink in the beginning of the protocol and lost more than 10 % of their initial body weight they got an intra-peritoneal (i.p.) injection of 1 ml saline to counteract dehydration. During the whole experiment, alcohol intake (g / kg body weight and day), body weight (g) and food consumption (g) were measured weekly. In the IFD protocol, the alcohol administration was non-continuous: it was interrupted for three days per week to model social drinking. The ethanol consumption was determined at the end of every drinking session; the food consumption and body weight were measured weekly.

### 3.2.2 Two-bottle choice paradigm

Ethanol preference was determined using the two-bottle choice paradigm as previously described (Racz et al., 2003; Racz, Schürmann, et al., 2008). In this paradigm,

two drinking bottles with 8 % v/v alcohol (EtOH) or drinking water were available for the animals *ad libitum*. In order to avoid the development of a side preference, the positions of the bottles were changed daily. The consumption of liquid and food, as well as the body weight were measured twice per week and the intake of alcohol was quantified as g (EtOH) / kg (body weight) per day (g/kg*day). The preference was calculated in percent as the ratio of consumed alcohol to total fluid (alcohol + water) consumption.

### 3.2.3 Stress-induced drinking

To assess stress induced-drinking of animals in the two-bottle choice paradigm, mice were exposed to mild foot shocks (0.5 mA, 100 ms) after 12 weeks of alcohol access (Racz et al., 2012; Racz, Schürmann, et al., 2008). For this, they were placed in an isolated, dark chamber (Startle response, TSE) with a continuous white noise (65 dB) for 5 minutes. Warning signals (sound and light) were presented a few seconds before the electric foot shocks, which were delivered five times through a grid floor. The interval between the foot shocks was 55–60 seconds. We recorded the behavioural responses (jumping reactions in g) of the animals during the stress procedure and then calculated the mean of the five startle reactions. 45 minutes after the stress exposure we sampled blood from the orbital sinus to determine the level of corcicosterone expression. The mice were then returned to their home cages, and alcohol as well water intake was determined 24 and 96 hours after the shock and calculated as average daily consumption. These values were compared to the ethanol preference of the last four weeks before the stress exposure.

### 3.2.4 Acute alcohol effects and development of tolerance

To assess acute alcohol effects in WT and $CB_2$ knockout mice, animals were injected i.p. with 2 or 3.5 g / kg ethanol or saline as published previously (Racz et al., 2003; Racz, Schürmann, et al., 2008). The animals' body temperature was measured with a rectal thermometer immediately before and 45 min after alcohol injection to determine alcohol-induced hypothermia. Blood was sampled 15 and 45 minutes after injection to assess blood alcohol levels (BAL) in plasma. To study the development of tolerance, animals underwent the forced drinking procedure for 5 weeks. Then acute alcohol injections were carried out as described above. Subsequently body temperature and

BALs were determined again. If the animals developed tolerance for alcohol they would show a reduced decrease in body temperature and lower BALs after chronic alcohol treatment.

### 3.2.5 Somatic signs of withdrawal

To study physical signs of withdrawal after chronic alcohol consumption we used the handling-induced convulsion protocol (Watson & Little, 1999). The scoring procedures were performed as described previously (Racz, Schürmann, et al., 2008). Briefly, animals were lifted gently by the tail and rotated for 5 s close to a light source (2200 lux). The elicited behavioural reaction was rated on a score from 0 – 3 as follows: 0 = no tremor or convulsion, 1 = mild tremor on lifting and turning, 2 = continuous severe tremor on lifting and turning, 3 = clonic forelimb extensor spasm on lifting. Animals were scored twice: the first time during the ethanol drinking procedure and the second time 3 hours after the animals had been withdrawn from alcohol. The experimenter, who performed the scoring, was blind to the treatment and genotype.

## 3.3 Behavioural tests

### 3.3.1 Dark / Light box test

An open field arena (45 x 45 x 22 cm) was weakly illuminated with 15 lux. A dark compartment (45 x 18 x 22 cm) was placed at one side of the arena, with an opening facing the centre, which permitted the transition of animals. In the beginning of the test individual animals were placed in the dark compartment facing the opening, and 10 min testing-trials were automatically recorded by the ActiMot system (TSE Systems GmbH). Time spent in the dark compartment (%) was documented as a level of anxiety, whereas distance travelled (%) was used to analyze locomotion (Bilkei-Gorzó et al., 2004).

### 3.3.2 Elevated O-Maze test

To study if alcohol withdrawal induces anxiety, the animals were analyzed in the elevated O-maze test (Racz et al., 2003). The maze was 40 cm elevated above the floor and consisted of a ring-shaped white platform with an inner diameter of 47 cm. The O-maze was divided into four compartments of the same size with two opposed quadrants closed by a non-transparent wall. The open part was illuminated with a desk lamp by

light sources with varying intensities (40, 120 and 700 lux) to increase aversion, whereas the closed quadrants remained dark (20-40 lux). For the test, animals were placed in the middle of an open arm facing a closed compartment and were allowed to freely explore the maze for 5 min. During the test, mice were automatically traced with a video-tracking system (Ethovision, Noldus) to assess time spent and distance travelled in the open areas in percent (Bilkei-Gorzó, Otto, et al., 2008; Bilkei-Gorzó, Racz, et al., 2008).

### 3.3.3 Home cage activity

Home cage activity was monitored in single housed animals by an infrared sensor connected to a recording and storing system (Mouse-E-Motion, Infra-e-motion) placed in the lid of each cage (A. Becker et al., 2010). Mouse movements were sampled every second and accumulated for every 30 min. The animals were observed throughout three weeks to investigate withdrawal-induced changes in the active and the inactive phases.

### 3.3.4 Morris water maze test

The Morris water maze test was performed to assess the spatial memory performance of mice after chronic alcohol treatment (Albayram et al., 2011). Each mouse was tested in four consecutive sessions daily over ten days (Figure 4). The platform was hidden just under the surface of murky water and remained at a fixed spatial location (N) for the seven-day acquisition period. For each trial session the mice were released from a different escape sector facing the wall of the maze. During the first two days, animals were placed at the same starting point (S) for each session. From days three to seven, animals were placed to one of the four positions (N, E, S, W), respectively, for each session. A trial ended when the mouse reached the hidden platform and managed to remain there for 5 s. If a mouse did not manage to escape from the water to the platform within 70 s, it was gently guided to the platform and the trial was recorded as an escape failure with a latency of 70 s. The mouse was dried and left in the home cage for a brief 15 s inter-trial interval. After the seven-day acquisition phase learning flexibility was assessed with the reversal phase. In this part of the test the platform was moved to the opposing quadrant (S), and each mouse was analysed for three consecutive days. To assess retention of spatial memory, the platform was removed from the maze at the end of the test. The animals were tested 24 hrs after the final trial.

In this probe trial, lasting for 70 s, each mouse was placed into the water as described for the training trials. The time (s) to reach the target quadrant and the time spent in the target quadrant was recorded.

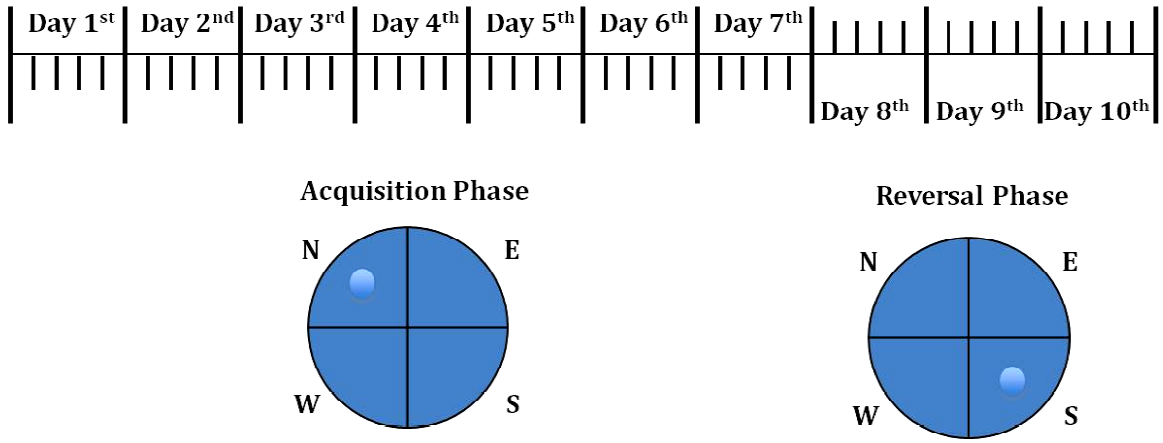



Figure 4. Schedule of the Morris-water maze. During the acquisition phase the hidden platform remained always in the same quadrant (N). The platform was moved to opposite quadrant (S) during the reversal phase (modified from Albayram 2012).

### 3.3.5 Open field test

Exploratory locomotor activity and anxiety of mice was investigated in an open field arena (45 x 45 x 22 cm) under red light in a sound-attenuated compartment (Bilkei-Gorzó et al., 2002). The animals were tested during the drinking period or three days after withdrawal. The animals were placed in the centre of the arena. During an observation period of 10 min, vertical and horizontal activity of the animals was monitored with an automated system (Actimot, TSE Systems). The time and the distance that animals spent in the centre, as well as the overall distance travelled, were analysed.

### 3.3.6 Object recognition test

The object recognition test was used to study the declarative memory of the animals (Pascual, Baliño, et al., 2011). In this test, animals were first habituated for 5 min during a three-day period to a sawdust-covered arena (45 x 45 x 30 cm), which contained two marbles with a diameter of 2 cm. On the fourth day, mice explored two identical Lego® objects. Three trials each lasting 6 min with an inter-trial period of 10 min were performed. In the test trial, one familiar object was replaced by a novel Lego® object.

The test trial was performed after different intervals (10, 30 or 60 min), and the exploration time (s) was assessed with an automated tracking system (Ethovision, Noldus). Animals with intact declarative memory spent more time exploring the novel object and thus showed increased novel preference. To increase the object interaction time, objects were paired with different neutral odours (cinnamon – familiar object; lemon – novel object). Animals spending less than total 10 s with the objects were excluded from analysis.

### 3.3.7 Social preference test

This test was used to determine the preference for a social partner compared with an empty cage (Bilkei-Gorzó et al., 2005). Animals were habituated to a sawdust-covered arena (45 x 45 x 30 cm) on three consecutive days for 5 min containing two empty grid cages with a diameter of 10 cm. During the test session on the following day, an age matched mouse was placed in one of the empty cages. The exploration time spent at both cages was recorded with an automated system for 6 min (Ethovision, Noldus). The preference ratio for the social partner was calculated in percent.

### 3.3.8 Social recognition test

This test was used to determine the declarative memory with respect to social, emotional elements (Bilkei-Gorzó et al., 2005). Similar to the object recognition test animals were habituated on three consecutive days for 5 min to a sawdust-covered arena (45 x 45 x 30 cm) containing two empty grid cages with a diameter of 10 cm. On the fourth day, the mice explored two cages containing one mouse each. The test consisted of three trials each lasting 6 min with an inter-trial period of 10 min. In the test trial, one of the familiar animals was replaced by a novel interaction partner. The test trial was performed after different intervals (10, 30 or 60 min) and the exploration time (s) was assessed with an automated tracking system (Ethovision, Noldus). Animals with an intact declarative memory spent more time exploring the novel partner. Animals spending less than total 10 s with their partners were excluded from the analysis. Furthermore, only animals that did not show preference (50 ± 10 % ) for one partner in the pre-trial were included in the analysis.

### 3.3.9 Y-Maze test

This task is specifically prefrontal cortex-dependent and investigates the working memory of mice (Darvas et al., 2009). The maze consisted of three 60 cm long arms oriented in a Y-shaped maze. Non-transparent 10 cm high walls surrounded the arms. The maze was illuminated with desk lamps to 20 lux. Mice were placed in the centre of the maze and were allowed to freely explore the arms during 10 min. The activity and the sequence of arm entries of the animals were recorded with an automated tracking system (Videomot, TSE-Systems). The working memory was assessed as the percentage of return (i.e. entering into the same arm) and spontaneous alternations (i.e. three consecutive enterings into new arms) from the total of transitions.

## 3.4 Organ dissection and isolation protocols

### 3.4.1 Brain removal for immunohistochemistry

Mice were anesthetized by i.p. injection of 500-1,000 µl narcotic solution, and afterwards fixed on a grid plate lying on the back. Abdomen and thorax were opened, and the mice were transcardially perfused with first 24 ml of ice-cold PBS followed by 24 ml of ice-cold 4 % PFA with a flow rate of 4 ml per minute. The skull was opened, the brain was removed and post-fixed overnight in 4 % PFA at 4°C followed by an incubation in 10 % (w/v) sucrose solution for 24 h, and then in 20 % (w/v) sucrose solution for 24 h. Brains were then snap-frozen in isopentane on dry ice and stored at -80°C until use. Alternatively, animals were killed by cervical dislocation and fresh brains were directly removed, snap-frozen in isopentane on dry ice and stored at -80°C until use as previously published (Pradier et al., 2013).

### 3.4.2 Isolation of organs for protein analysis

After cervical dislocation, brains were isolated and snap-frozen in isopentane on dry ice and stored at -80°C until use. Brain regions of interest were then isolated at the cryostat using the punching technique. For this the brains were cut into 1 mm thick coronal sections using a brain matrix. The regions of interest were then identified with the help of a mouse brain atlas and sampled using needles with an inner diameter of 400 to 2500 µm or cut out with a scalpel. Alternatively, fresh brains were dissected using the punching technique in ice cold PBS. To assess the potency of the applied alcohol model

on the peripheral organs, the liver (a part of the larger lobe) was also collected, snap-frozen and stored at -80°C until use.

### 3.4.3 Blood sampling and plasma preparation

Blood was collected from the orbital sinus under short isoflurane anaesthesia. 10 µl of 0.21 mmol K-EDTA was added to prevent clotting. The blood samples were centrifuged at 4000 rpm for 20 minutes at 4°C. Supernatant was collected and frozen immediately and stored at -20°C until use. To assess BALs blood was collected either after an acute injection of alcohol or during chronic alcohol consumption (at least 5 weeks of alcohol consumption), subsequently BALs were determined. During the chronic alcohol consumption the blood was consequently taken at 11 o´clock (2 hours after the start of active phase).

## 3.5 Biochemical methods

### 3.5.1 Protein isolation

Frozen brain tissues were weighed and 10 µl/mg brain tissue of ice-cold RIPA buffer was added. Brain tissues were homogenized with the Ultra-Turrax homogeniser for 30 s on ice (5 cycles, 60 % power) and cooled on ice for 20 min. Samples were then centrifuged at 13,300 rpm for 20 min at 4°C. The supernatant was collected, aliquoted in 50 µl and stored at -80°C.

Proteins from liver samples were isolated using the Precellys™ homogeniser. For this liver tissue and 1 ml of lysis buffer was added to Precellys tubes, which contain ceramic bead. The tissue was homogenised at 5000 rpm for 20 s and then centrifuged at 13,000 rpm for 20 min at 4°C. The supernatants were collected and stored at -80°C.

### 3.5.2 Protein quantification

To determine total protein concentration a commercial available kit was used (Pierce). The Bicinchoninic acid (BCA) assay is based on the biuret reaction. BCA forms a color-intense stable complex in the presence of proteins and copper ions with a maximal absorbance at 562 nm. Samples were diluted 1:10 and the total protein concentration was measured following manufacturer's instructions. In brief, 25 µl of the prediluted

samples and the standard were loaded in duplicates on a 96 well plate and 200 µl of the working reagent was added to each well. After 15 min the OD was measured with the plate reader and the protein concentration was calculated.

### 3.5.3 Enzyme-linked immunosorbent assays (ELISA)

The principle of ELISA is based on the antibody-mediated detection of a specific protein in a complex matrix such as tissue homogenates, plasma, or cell culture supernatants. The concentration of the target protein is directly correlated to a light-sensitive detection signal given by enzymatically oxidized 3,3',5,5'-tetramethylbenzidine (TMB). The detection signal is measured at 450 nm. Mouse cytokines and chemokines were detected in tissue homogenates and serum following manufacturer's instructions. Serum samples were assayed undiluted, whereas 100 µg/well of brain homogenates and 50 µg/well of liver lysates were loaded on a 96 well plate. The determination of the concentration of cytokines or chemokines was carried out in accordance with the manufacturer's instructions.

### 3.5.4 Determination of blood alcohol levels

Plasma alcohol levels were determined using a NAD-NADH Reagent. In this colorimetric assay the enzyme alcohol dehydrogenase oxidizes ethanol to acetaldehyde with the simultaneous reduction of NAD to NADH. The consequent increase in absorbance at 340 nm is directly proportional to the concentration of alcohol in the sample. To calculate the absolute concentration of alcohol in the sample, a reference standard with a range of 0.8 % to 0.008 % was applied in parallel to the samples. The assay was performed in accordance with manufacturers' description. BALs were routinely determined for all models applied.

### 3.5.5 Corticosterone Enzyme Immunoassay (EIA)

To measure the stress response of WT and $CB_2$ deficient animals to the foot shock in the stress-induced drinking paradigm, blood was taken from the orbital sinus one week prior to and 45 min after the stress. Due to circadian fluctuations in the blood corticosterone level, it is important to note that the blood sampling has always to be carried out at the same time of day. Next, plasma was prepared and stored at -20°C until use. Plasma samples were then assayed at a dilution of 1:50. The determination of

corticosterone concentration was carried out in accordance with the manufacturer's instructions.

## 3.6 Immuno-histochemical methods

### 3.6.1 Iba1, GFAP, NeuN and IL-1β immunostaining

Brains from wild type and knockout animals were prepared as described above (3.4.1). Brains were then embedded in Tissue-Tek® and consecutively sectioned at 16 µm using a cryostat. Slices were dried at 37°C for 30 min and subsequently stored at -80°C until use. For the immuno-staining procedures slices were processed as previously published (Pradier et al., 2013). Briefly, slices were thawed at 37°C, washed in PBS and then permeabilised in 0.5 % Triton X-100 (Sigma) for 1 h. After blocking in 3 % bovine serum albumin, the primary antibody was applied directly onto the slices, which were incubated overnight in a moist compartment at 4°C (for dilutions of antibodies see Table 1 and Table 2). The next day, slices were washed three times for 10 min, and the secondary antibody was applied in 0.5 % BSA for 1 h. Next, slices were washed three times before mounting in DAPI Fluoromount-G™. Sealing with water varnish prevented sections from drying-out.

### 3.6.2 Image acquisition and analysis of area fraction

For area fraction analysis, immuno fluorescent images were acquired on a Zeiss Axioplan microscope and recorded with a monochrome Zeiss Axiocam. Image analysis was performed using different macros for each staining in ImageJ. For staining quantification, the image contrast was enhanced and the brain regions of interest were traced in accordance with the mouse brain atlas (Figure 5) (Paxinos & Franklin, 2001). Next, a threshold was set and applied to all images. Depending on the staining, cell counts and/or percentage of the stained area was measured. Six to eight sections per animal were evaluated.

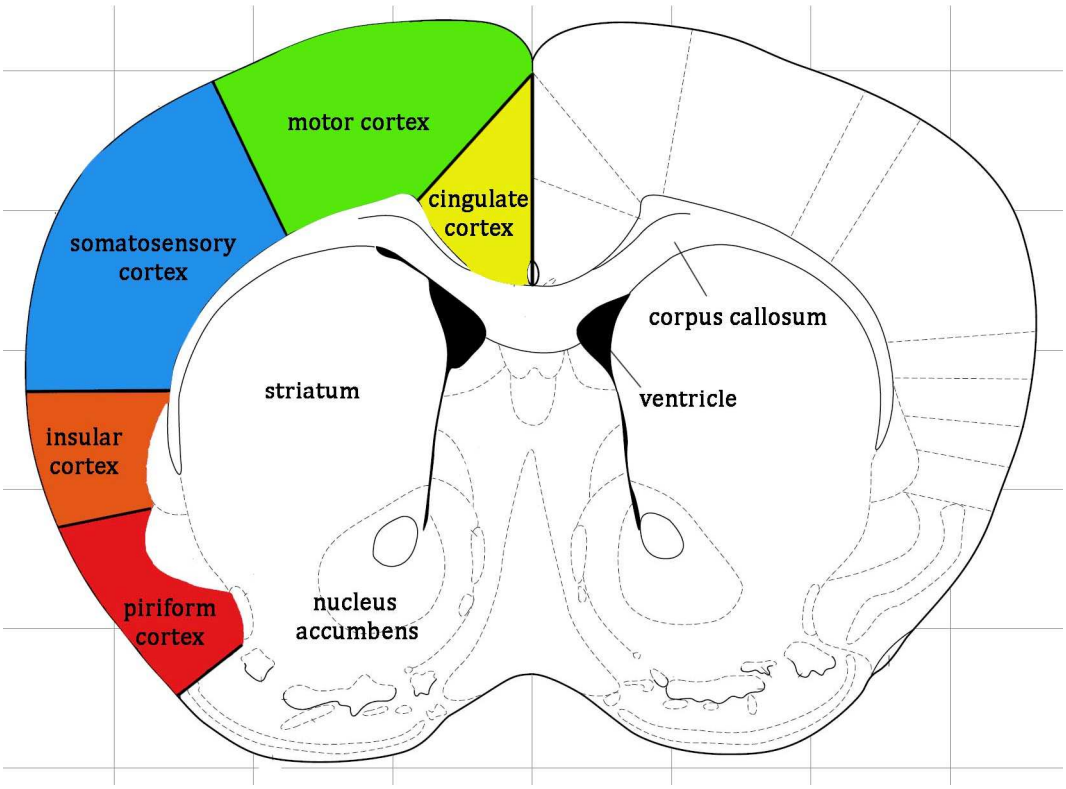



Figure 5. Cortical brain regions of interest (coloured). Cingulate-, motor-, somatosensory-, insular- and piriform cortex were selected in accordance with the mouse brain atlas (modified from Paxinos & Franklin 2001).

### 3.6.3 Quantitative analysis of microglial cells

For stereological quantification of microglia in the cortex every 6th slice was selected and stained. I analyzed in total 6 - 8 sections per region of interest from Bregma 1.18 to 0.02 in both hemispheres per animal. The numbers of stained microglia were counted with ImageJ; next, the total number ($n$) of Iba1-immunoreactive microglia was estimated using the optical fractionator technique as described previously (Bondolfi et al., 2002; Grathwohl et al., 2009; Gundersen, 1986):

$$n = \sum(number\ of\ cells\ counted) \times \frac{1}{ssf} \times \frac{1}{asf} \times \frac{1}{tsf} \times 2$$

Where *ssf* is the section sampling fraction (i.e. one-sixth of the total sections used), *asf* is the area sampling fraction (in this case the entire region is the dissector, thus *asf* = 1), and *tsf* is the thickness of sampling fraction (depth of field estimated as *tsf* = 1).

Multiplication by factor 2 corrects for the hemispheres. The volume ($V$) was calculated as follows:

$$V = \sum(investigated\ area) \times \frac{1}{ssf} \times \frac{1}{tsf} \times 2$$

With *tsf* = 16 µm being the total thickness of each section.

### 3.6.4 Quantification of IL-1β expression in Iba1⁺ cells

The standard staining protocol was used for the co-staining of IL-1β (green) and Iba1 (red) on cortical slides. To study the co-localization of IL-1β and Iba1, Z-stacks were acquired on a Leica SP8 confocal microscope. Five stacks of the cingulate cortex were recorded for each mouse with a 20x objective. The distance between the optical sections was 1.5 µm. The stacks were acquired with a sequential frame scan, i.e. first the weaker IL-1β signal was scanned; then, separately from the first scan, the stronger Iba1 staining was recorded.

For the co-localization analysis the section with the strongest Iba1/IL-1β signal was selected from the Z-stacks using ImageJ. Then Iba1-positive cells were outlined and defined as regions of interest (ROI) in the red channel; next, the area fraction of IL-1β expression was determined in the green channel within the respective ROIs.

## 3.7 Molecular biological methods

### 3.7.1 RNA isolation

Total RNA was extracted from brain tissue using the TRIzol® reagent (Life Technologies). The TRIzol® reagent is a monophasic solution of phenol and isothiocyanate and is an improvement to the method of Chomczynski (Chomczynski & Sacchi, 1987). It maintains RNA integrity during lysis and homogenization and allows RNA isolation from small amounts of tissue. Dissected tissue samples were transferred to 1.4 ml Precellys tubes with ceramic beads (peqLab, Erlangen, Germany), then 800 µl TRIzol® was added to the tubes. The tubes were put into the Precellys centrifuge, where the specimens were homogenized in 2 cycles at 5000 rpm for 20 sec. The samples were then centrifuged at 11400 rpm for 10 min at 4°C, and the supernatants were transferred

to a new 1.5 ml Eppendorf tube. Then 160 µl of chloroform was added to separate the solution into an aqueous (containing RNA) and an organic phase. The tubes were vortexed for 30s, allowed to remain 3 minutes at room temperature and centrifuged at 11400 rpm for 10 minutes at 4°C. The upper, RNA containing phase was transferred into a new tube, and 400 µl isopropyl alcohol was added to precipitate RNA. The tubes were vortexed for 30 sec and allowed to incubate for 10 minutes at room temperature, and were then centrifuged at 11400 rpm for 10 min at 4°C. The resulting pellet contained the precipitated RNA. Supernatants were removed, and the pellet was washed 3 times with 1 ml of 75 % ethanol, each washing step was followed by a centrifugation (11400 rpm, 5 minutes, 4°C). The RNA pellet was then dried for 5-10 minutes at 50°C and dissolved in 20 µl of RNAse-free water (Qiagen, Germany) for 10 minutes at room temperature. The RNA concentration (ng/ml) was determined using a spectrophotometer (NanoDrop 1000).

### 3.7.2 cDNA synthesis

RNA was transcribed into cDNA using the SuperScript First-Strand Synthesis System for RT-PCR Kit (Life Technologies, Carlsbad, CA, USA). A given amount of RNA (30 ng in the assays used) was pipetted and adjusted to a volume of 10 µl with RNAse-free water. Then, 1 µl of Oligo-dT and 1 µl of dNTP were added to the samples, incubated for 5 min at 65°C and then for 3 min at 4°C in a PCR cycler (Biorad). 6 µl of master mix, consisting of 4 µl 5x First Strand Buffer and 2 µl 0.1 M DTT was subsequently pipetted into each tube. The samples were incubated for 2 min at 42°C and for 3 min at 4°C. Then 1 µl of Reverse Transcriptase was added to each sample. The probes were incubated for 1 h at 42°C (transcription stage), followed by 15 min at 70°C (inactivation stage) and remained at least for 10 min at 4°C. The resulting cDNA was again diluted with an appropriate amount of DEPC water and stored at -20°C. 30ng (10 µl) of cDNA was used for each TaqMan reaction (per well). Samples containing only DEPC water were used as a control for possible contaminations.

### 3.7.3 Quantitative polymerase chain reaction

Differences in mRNA expression were determined by custom TaqMan® Gene Expression Assays (Applied Biosystems, Darmstadt, Germany) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control to standardize the amount of target

cDNA as described previously (Albayram et al., 2011; A. Becker et al., 2010). Samples were processed in a 7500 Real-Time PCR Detection System (Applied Biosystems, Darmstadt, Germany), and further analysis was performed using the 7500 Sequence Detection Software version 2.2.2 (Applied Biosystems, Darmstadt, Germany). Relative quantitative gene expression was calculated with the $2^{-\Delta\Delta}$Ct method (Livak & Schmittgen, 2001).

### 3.8 Statistical data analysis

All statistical analyses were carried out using STASTICA software package. Datasets containing only one independent variable (e.g. treatment or model) were analyzed by one-way analysis of variance (ANOVA). Datasets containing two independent variables (e.g. treatment and genotype or treatment and age) were evaluated using two-way ANOVA, whereas datasets containing three independent variables (treatment, genotype and housing conditions) were analyzed by three-way ANOVA. Repeated measures ANOVA combined with one-, two- or three-way ANOVA was applied when data from the same animals were collected over a given period (within effect: time). The ANOVA was considered to be significant at a 95 % confidence interval; the analysis was followed by a post-hoc test. In the case of $n \leq 4$ Bonferroni correction was applied, for $n \geq 5$ Fisher-LSD correction was performed. p-values between 0.1 and 0.06 were considered as tendency. For non-parametrical datasets and scores, the Kolmogorov-Smirnov test was used. Data are represented as mean values ± standard error of mean (S.E.M).

# 4 Results

## 4.1 Comparison of chronic alcohol models to induce neuroinflammation

In the following part I investigate the potency of two different chronic alcohol models (FD and IFD) and different treatment durations of alcohol consumption to induce neuroinflammation in our laboratory. Next, I characterise the inflammatory phenotype of microglia after long-term alcohol drinking in the cortex. Furthermore, I analysed the level of systemic inflammation in the liver to monitor the effect of chronic alcohol drinking.

### 4.1.1 Two months of forced and intermittent forced drinking

In previous publications from our laboratory we analysed behavioural changes of animals after two months of chronic alcohol treatment (Racz et al., 2003). For this purpose we started to investigate alcohol-induced neuroinflammation after eight weeks of chronic alcohol consumption. Figure 6A compares the alcohol consumption of WT animals in both chronic alcohol models: after two months of treatment mice drank the same amount of alcohol [$F_{(1, 25)} = 0.22$; $p = 0.64$]. The food consumption was significantly reduced by the model [$F_{(1, 37)} = 42.94$; $p = 0.000$] (Figure 6B). In the forced drinking procedure animals showed a decreased food intake compared to their water-treated peers. Due to the intermittent alcohol access mice showed an intermediate food consumption compared to the water and forced drinking groups. During the treatment period the body weight did not change in any of the models [$F_{(1, 37)} = 1.44$; $p = 0.25$] (Figure 6C).

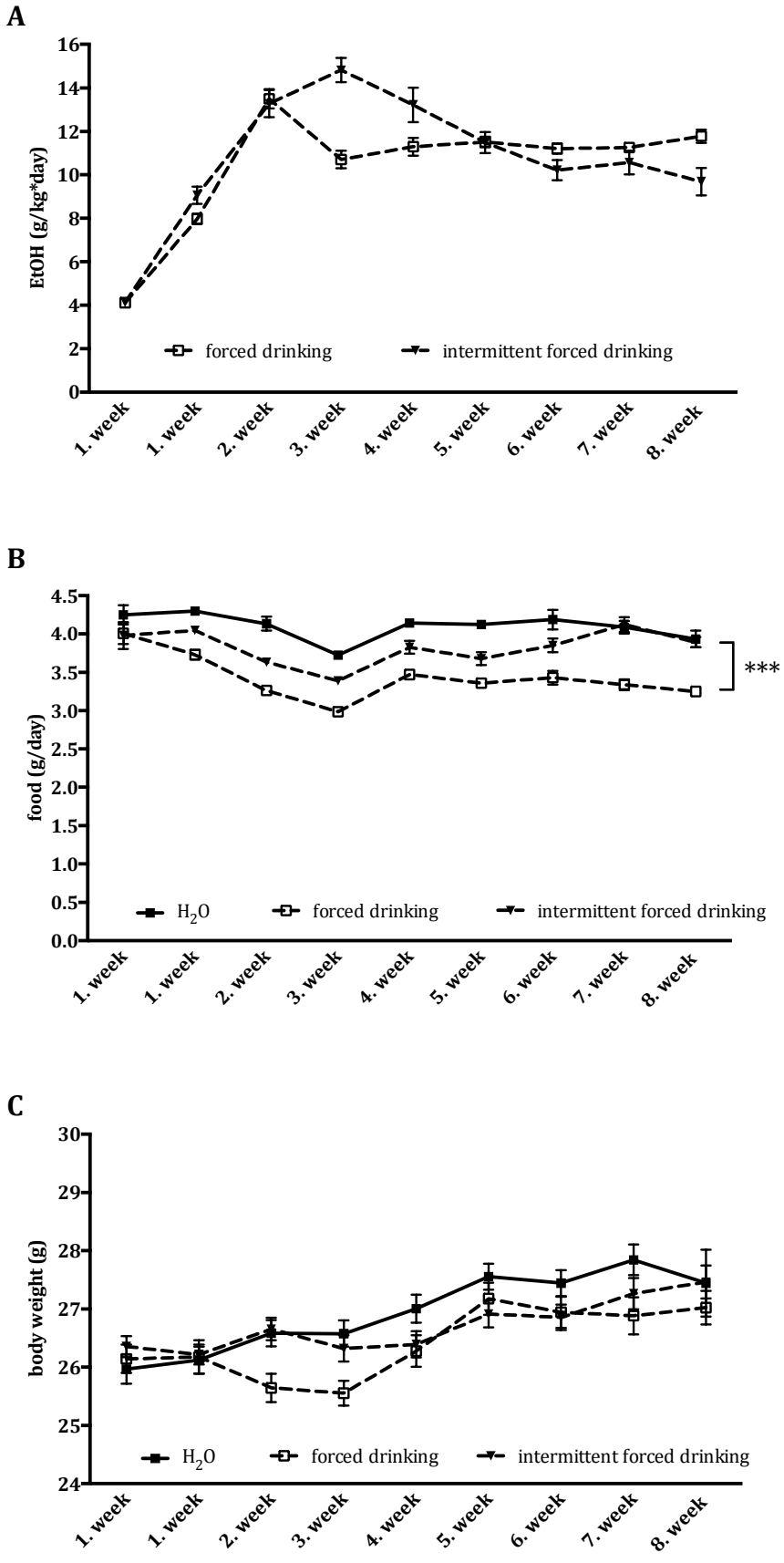

A
EtOH (g/kg*day)
16
14
12
10
8
6
4
2
0
forced drinking
intermittent forced drinking
1. week
1. week
2. week
3. week
4. week
5. week
6. week
7. week
8. week
B
food (g/day)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
H2O
forced drinking
intermittent forced drinking
1. week
1. week
2. week
3. week
4. week
5. week
6. week
7. week
8. week
C
body weight (g)
30
29
28
27
26
25
24
H2O
forced drinking
intermittent forced drinking
1. week
1. week
2. week
3. week
4. week
5. week
6. week
7. week
8. week

Figure 6. Ethanol and food consumption and body weight of WT animals after two months of forced and intermittent forced drinking. (A) WT animals drank similar amounts of alcohol independent of the model. (B) The forced drinking procedure led to strongly decreased food consumption, whereas animals with an intermittent alcohol exposure showed intermediate food consumption. (C) The body weight was not significantly changed after eight weeks of alcohol treatment (n = 13 - 14 per group). Data were analysed by repeated measures one-way ANOVA (main factor: model, within effect: time) and represented as mean value ± SEM. ***$p < 0.001$.

Two months of alcohol treatment did not induce an inflammatory response in the cortex as revealed by ELISA analysis (Figure 7). The expression of neither pro- nor anti-inflammatory cytokines was changed by the two alcohol treatments compared to water control animals [TNF-α: $F_{(1,12)} = 1.03$, $p = 0.38$; IL-1β: $F_{(1,12)} = 0.20$, $p = 0.81$; IL-6: $F_{(1,12)} = 2.34$, $p = 0.13$; IFN-γ: $F_{(1,12)} = 1.76$, $p = 0.21$; CCL-2: $F_{(1,12)} = 3.64$, $p = 0.057$; IL-10: $F_{(1,12)} = 0.97$, $p = 0.40$].

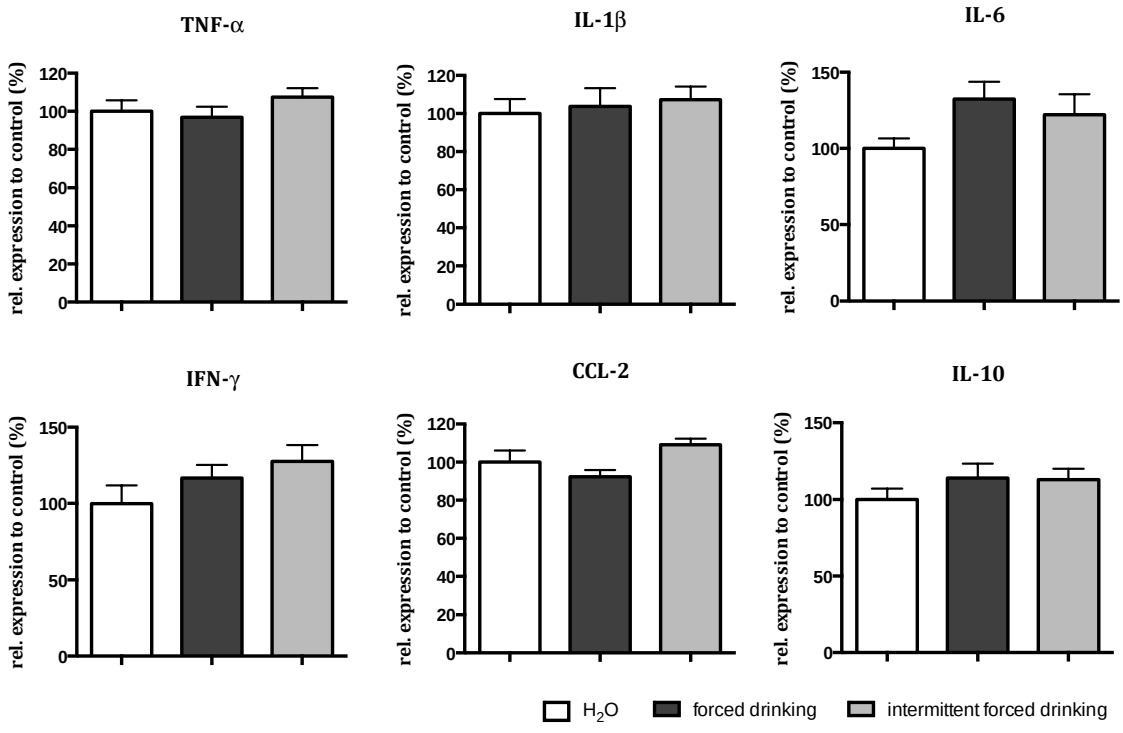
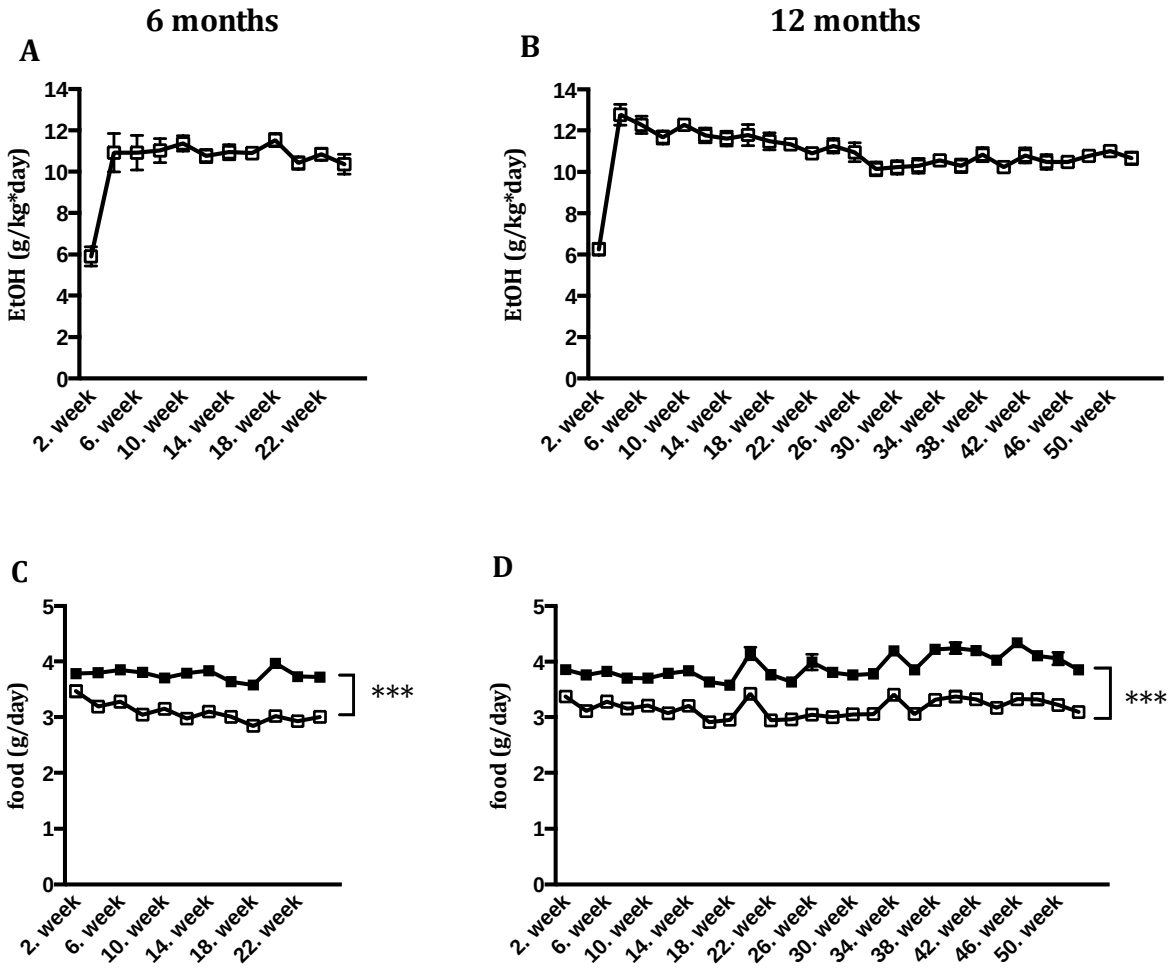



Figure 7. Expression of inflammatory markers in the frontal cortex after two months of alcohol administration. The expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IFN-γ), chemokine CCL-2 and anti-inflammatory cytokine IL-10 were not changed after the different alcohol treatments (n = 5 per group). Data were analysed by one-way ANOVA (main factor: model) and represented as mean value ± SEM.

### 4.1.2 Six and twelve months of forced drinking

The previously introduced alcohol models suggest that the duration of the treatment is a crucial factor in inducing a neuroinflammatory response. Therefore we analysed different durations of alcohol access in the forced drinking procedure. The panel in Figure 8 shows two different cohorts of WT animals that had free and continuous alcohol access for six and twelve months, respectively. Both cohorts drank similar amounts of alcohol (Figure 8A and B) and alcohol-treated mice presented a reduced food consumption [FD 6M: $F_{(1,12)} = 76.45$, $p = 0.000$; FD 12M: $F_{(1,14)} = 97.01$, $p = 0.000$] (Figure 8C and D) compared with water controls. The body weight was also reduced by the treatment, but only in the cohort that underwent the six-month treatment [$F_{(1,12)} = 5.12$, $p = 0.04$]. (Figure 8E). Surprisingly the animals that drank alcohol for twelve months showed an unchanged body weight compared to the water controls (Figure 8F) [$F_{(1,14)} = 0.02$, $p = 0.87$].



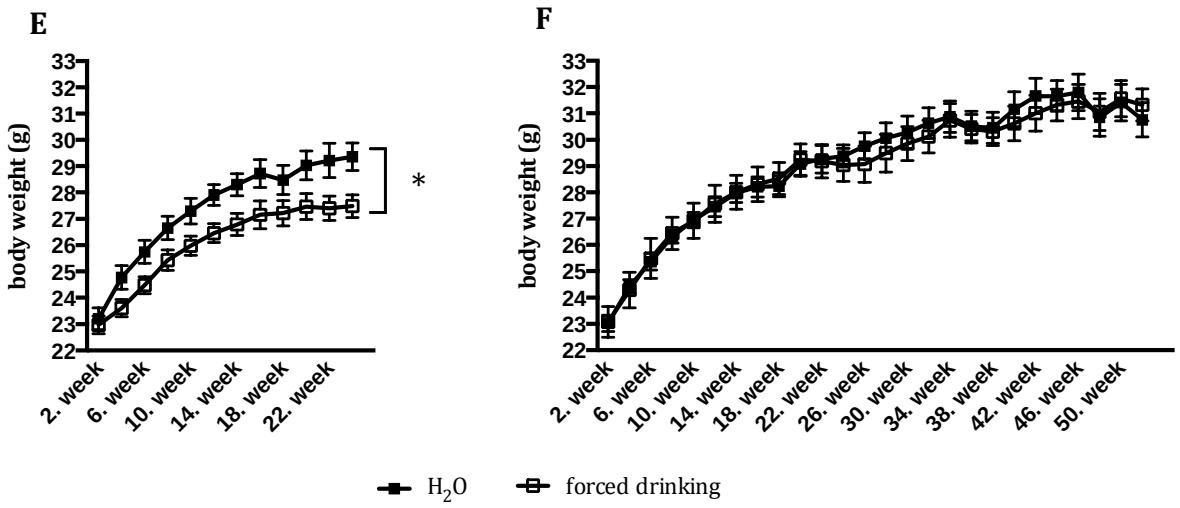



Figure 8. Ethanol and food consumption and body weight during six and twelve months of chronic alcohol treatment. (A and B) show the alcohol consumption of WT animals during six and twelve months. Both cohorts drank similar amounts of alcohol. (C and D) The forced alcohol consumption resulted in significantly decreased food consumption in both cohorts. (E and F) The body weight was only decreased by chronic alcohol treatment in the cohort of six months of forced drinking. Surprisingly, the body weight of the second cohort (twelve months forced drinking) was not changed by the treatment (n = 7 – 8 per group). Both cohorts were subjected to the alcohol treatment at the same time. Data were analysed by repeated measures one-way ANOVA (main factor: treatment, within effect: time) and represented as mean value ± SEM. *p > 0.05, ***p > 0.001.

In the literature it is established that five months of alcohol treatment are enough to induce neuroinflammation in the cortex, which is shown by increased expression of CD11b, GFAP and pro-inflammatory cytokines (Alfonso-Loeches et al., 2010; Pascual, Fernández-Lizarbe, et al., 2011). I analysed Iba1 expression (Figure 9), which is another microglial activation marker, in the frontal cortex of animals treated for six- and twelve-month with alcohol. In order to more clearly show the onset of development of neuroinflammation, I further added an earlier time point, two months, for the following immuno-histological analysis (taken from the previous study). The area fraction of Iba1 immuno-reactivity (IR) was analysed in different cortical areas and showed a significant increase over time (Figure 10). This effect was enhanced in alcohol-treated animals. Post-hoc analysis revealed that this finding was most prominent in the cingulate [$F_{(1,12)}$ = 8.92, p = 0.004], motor [$F_{(1,12)}$ = 10.73, p = 0.002] and piriform cortex [$F_{(1,12)}$ = 10.85, p =

0.002], where Iba1-IR was tripled after twelve months of alcohol treatment compared to two months of treatment. However, the changes in Iba1-IR were also significant for the somatosensory [$F_{(1,12)} = 5.32$, $p = 0.02$] and insular cortex [$F_{(1,12)} = 5.89$, $p = 0.01$].

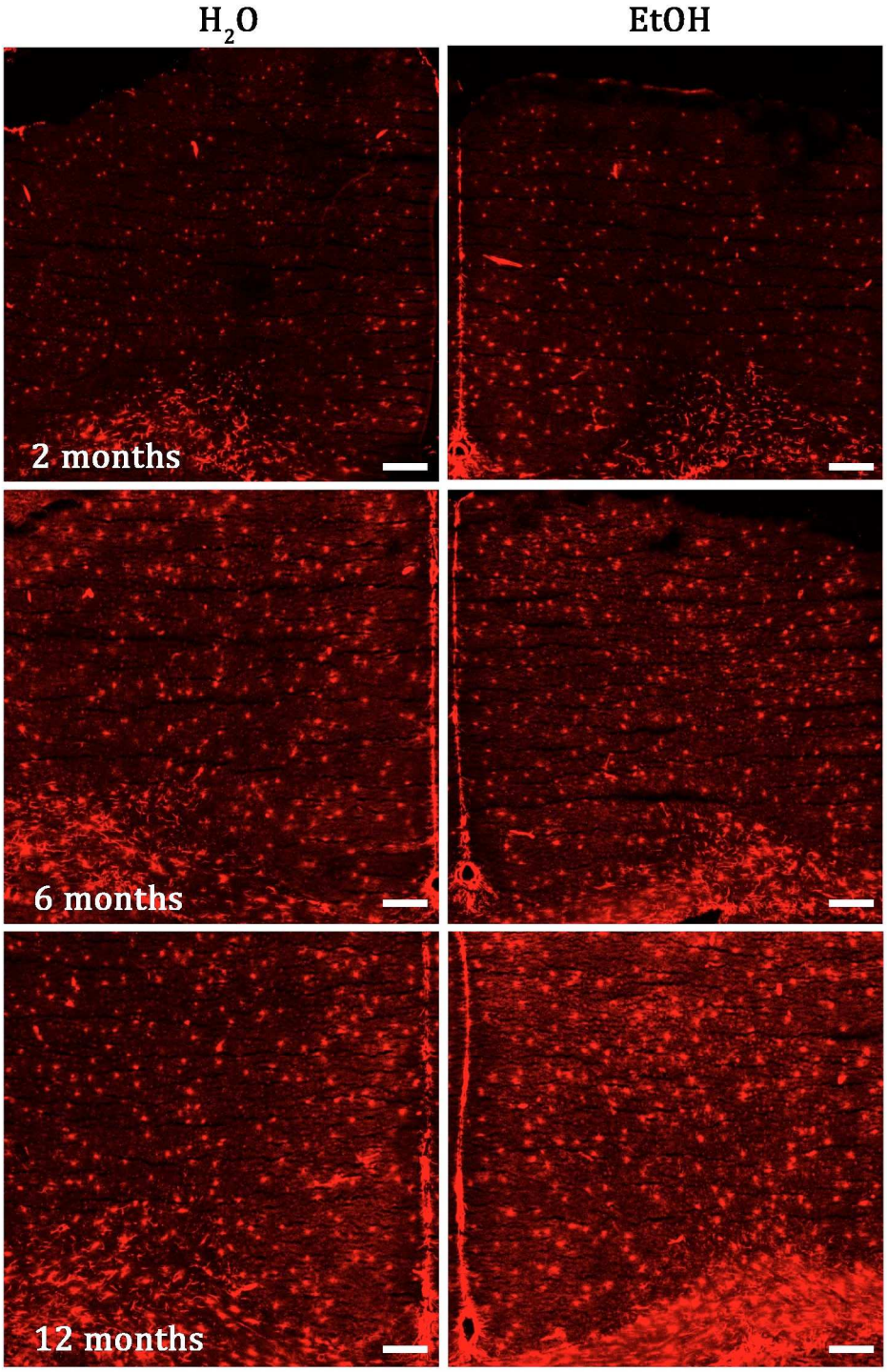



Figure 9. Representative images of Iba1-IR in the cingulate cortex of WT animals after 2, 6 and 12 months of alcohol consumption. Scale bar represents 100 µm

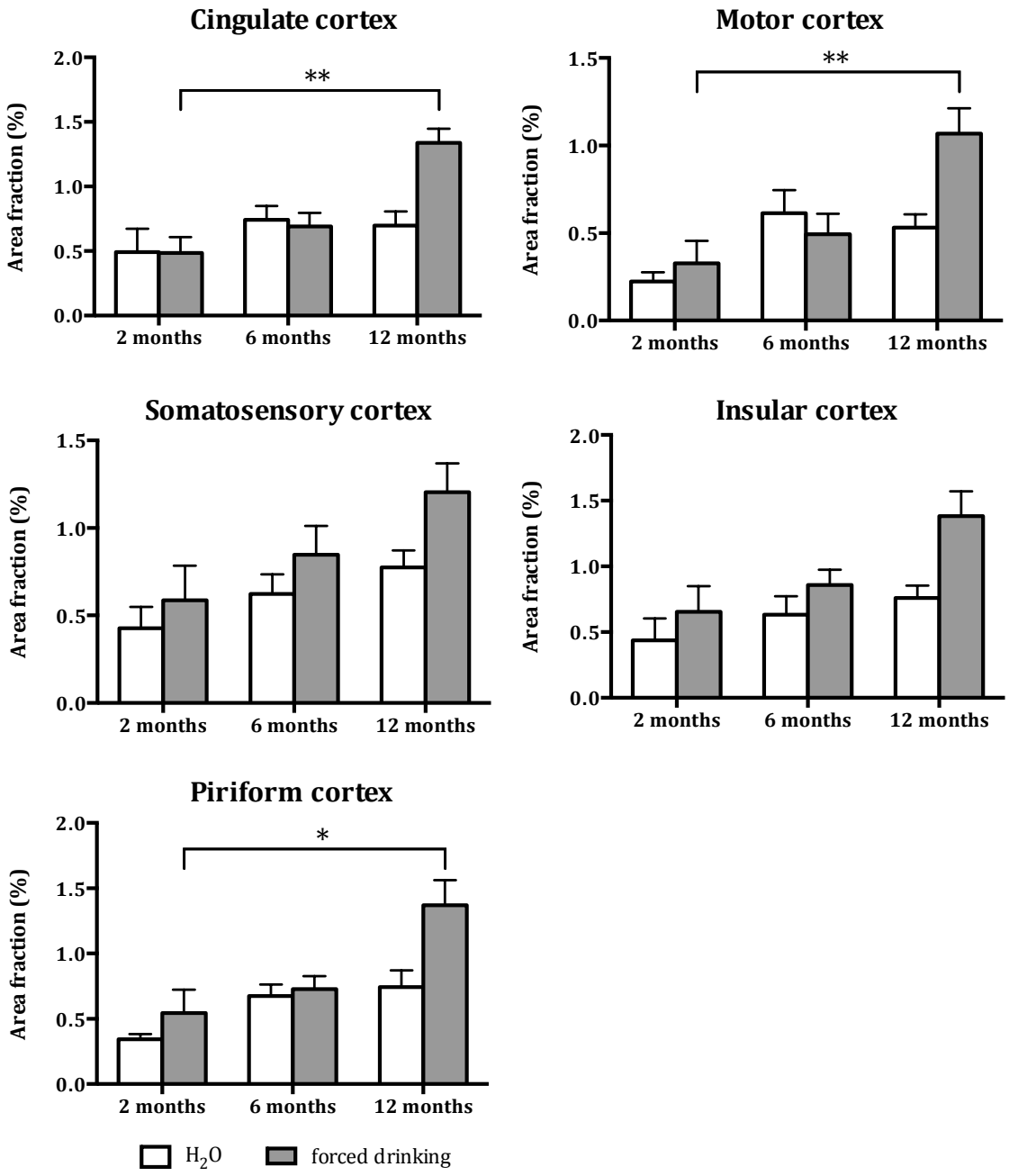
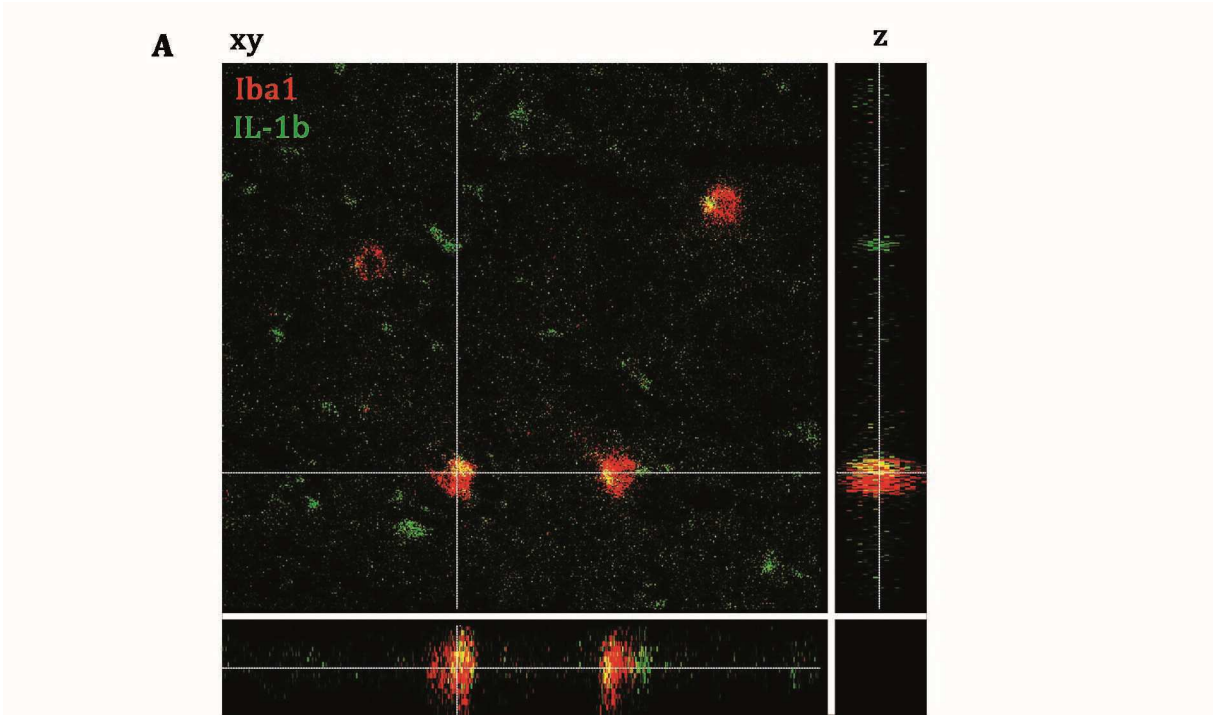



Figure 10. Area fraction of Iba1-IR in various cortical regions after chronic alcohol consumption. There is a significant increase in the area fraction of Iba1-IR after twelve months of alcohol treatment (n = 3 per group). Data were analysed by two-way ANOVA (main factors: treatment and age) and represented as mean value ± SEM. *$p < 0.05$; **$p < 0.01$.

Increased microglial activation after long-term alcohol consumption is associated with an increased production of pro-inflammatory cytokines. However, clear *in vivo*

evidence for the cellular source of pro-inflammatory cytokines and the assumed pro-inflammatory phenotypic shift of microglial cells is missing. Therefore I co-stained IL-1β with Iba1 to monitor its cellular source in microglia and to prove that microglial cells shift to a pro-inflammatory phenotype after long-term alcohol treatment. The orthogonal section clearly shows the localisation of IL-1β (green) within the microglia (red) in xy-, but also in z-direction (Figure 11A). The panel in Figure 11B displays the different alcohol treatment durations and the respective age-matched water control animals. Iba1 is depicted in red, IL-1β in green. In the channel overlay DAPI is added in blue (Figure 11B).



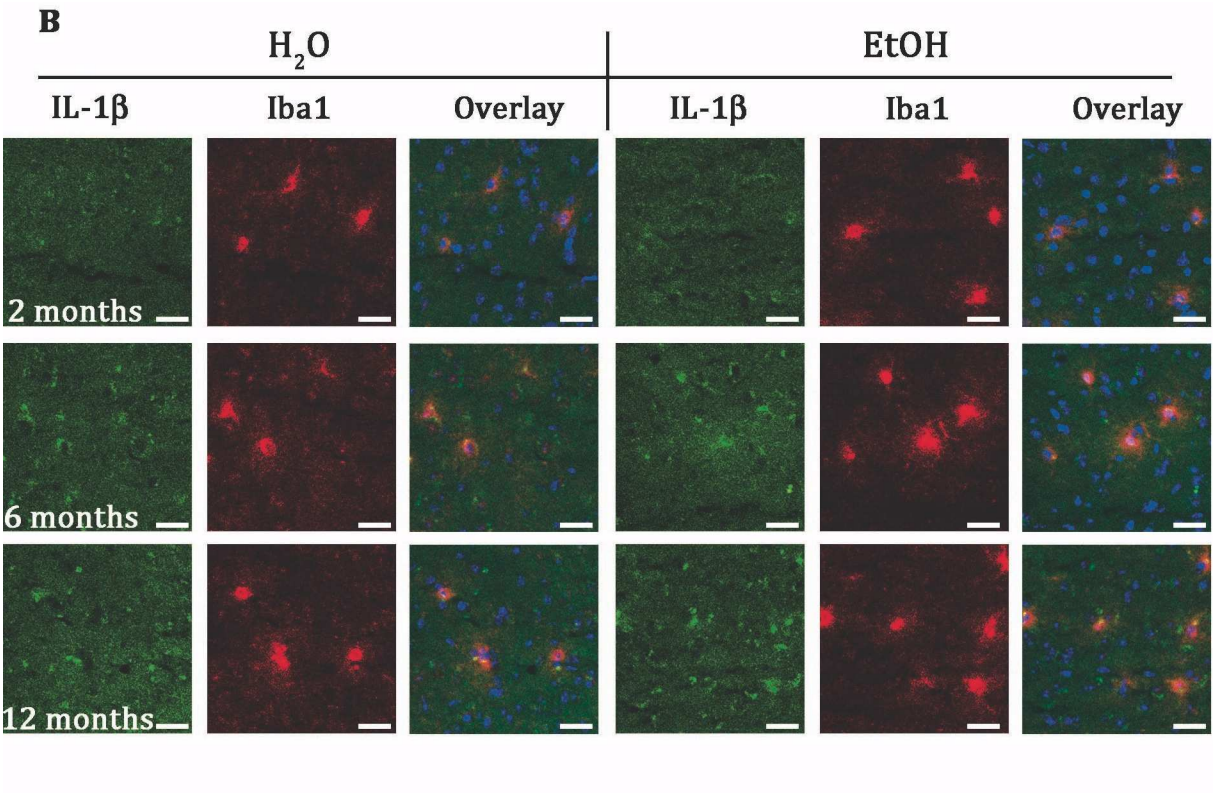



Figure 11. Representative confocal images of Iba1 and IL-1β co-localisation in the cingulate cortex. (A) Orthogonal confocal image of Iba1$^{+}$ cells (red) clearly showing IL-1β expression (green) within the cell body of microglia. (B) Confocal images of water and alcohol-treated animals of IL-1β-IR (green), Iba1-IR (red) and the overlay with DAPI (blue) in the cingulate cortex. Microglia show clearly enhanced expression of IL-1β over time. Scale bar represents 25 µm.

Stereological analysis of Iba1-IR cells was carried out as described in the material and method section (see 3.6.3). In accordance to the Iba1 stained area fraction the total number of microglial cells also reveals an increased number of microglia in the cingulate cortex [$F_{(1,12)}$ = 10.00, p = 0.002] (Figure 12A). The investigated cortical volumes of the six different experimental groups were equal in size [$F_{(1,12)}$ = 0.24, p = 0.78] (Figure 12B). Next, I quantified the area fraction of IL-1β expression exclusively in microglia. An arbitrary threshold defining low and high levels of IL-1β expression gives a number of IL-1β$^{low}$ and IL-1β$^{high}$ expressing microglia, which is best represented as a ratio in percent (Figure 12C and D). The analysis clearly shows age-dependent changes in the ratio of IL-1β expression revealing a decrease in IL-1β$^{low}$ and an increase in IL-1β$^{high}$ microglia over time [$F_{(1,12)}$ = 11.11, p = 0.001]. With knowledge of the total number of

microglia in the cingulate cortex, it is possible to calculate the total number of IL-1β$^{low}$ and IL-1β$^{high}$ microglia from the ratio (Figure 12E and F). There is no significant change in the total number of IL-1β$^{low}$ expressing microglia [$F_{(1,12)}$ = 2.29, p = 0.14], but there is a substantial increase in IL-1β$^{high}$ expressing microglia over time [$F_{(1,12)}$ = 23.95, p = 0.000], which was enhanced by alcohol treatment.

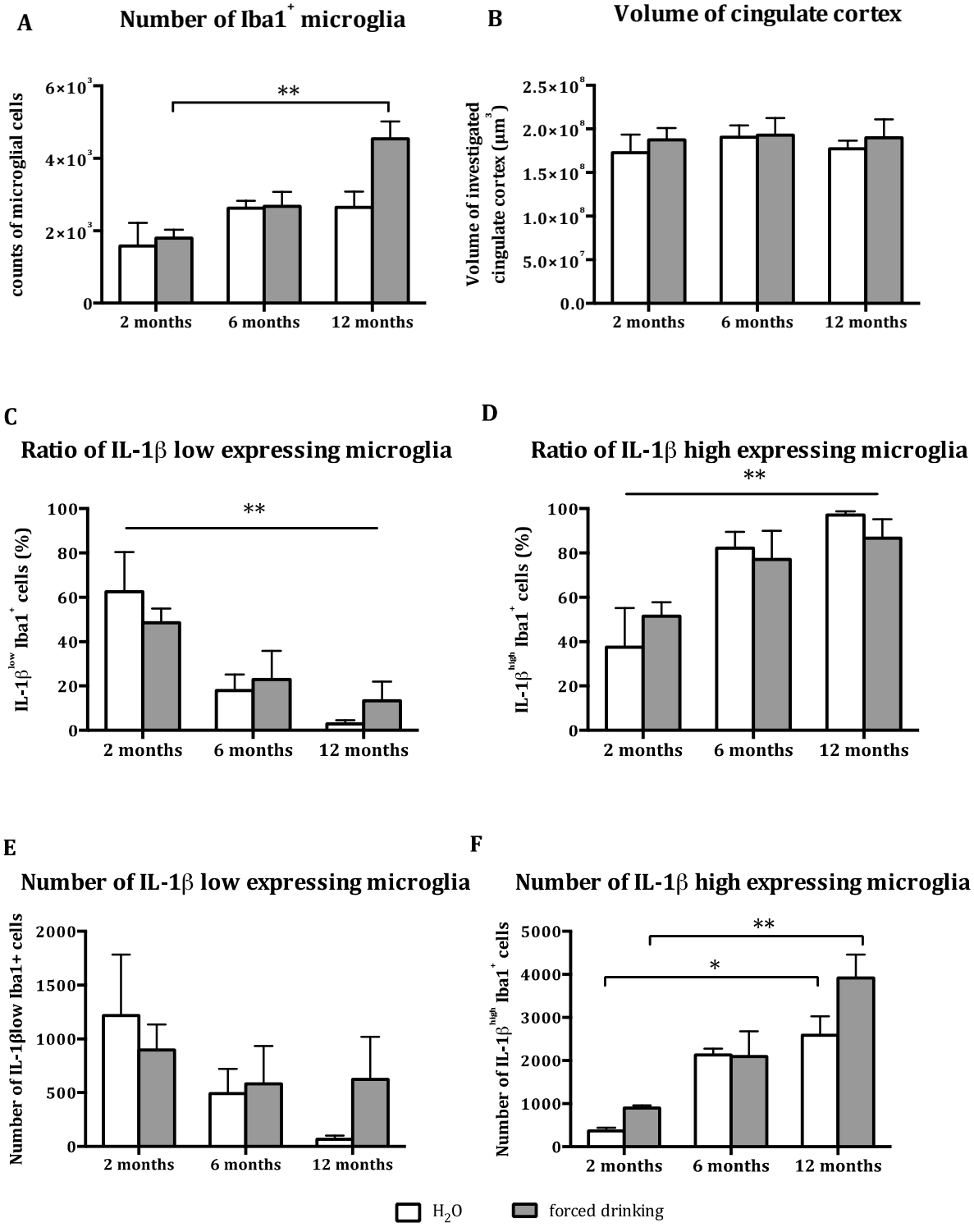


Figure 12. Counts of Iba1-IR cells and quantification of co-localized IL-1β-IR in the cingulate cortex. (A) There is a significant increase in the number of microglia after twelve months of alcohol treatment compared to water controls. (B) The investigated volume was similar in each the group. (C) The ratio of IL-$1\beta^{low}$ expressing microglia decreases over time (D) whereas the ratio of IL-$1\beta^{high}$ expressing microglia increases significantly over age. (E) The total number of IL-1β low expressing microglia was not significantly altered. (F) There is a significant increase in IL-$1\beta^{high}$ expressing cells in age. This effect is stronger after chronic alcohol treatment (n = 3 per group). Data were analysed by two-way ANOVA (main factors: treatment and age) and represented as mean value ± SEM. *$p < 0.05$; **$p < 0.01$.

Having established that long-term alcohol treatment leads to a pro-inflammatory phenotypic shift of microglia, I investigated possible consequences on neurons, as *in vitro* studies suggest a neurotoxic potential for microglial derived IL-1β (Block et al., 2007). I therefore examined the neuronal numbers using the common neuronal marker NeuN, which labels the vast majority of neurons (Figure 13).

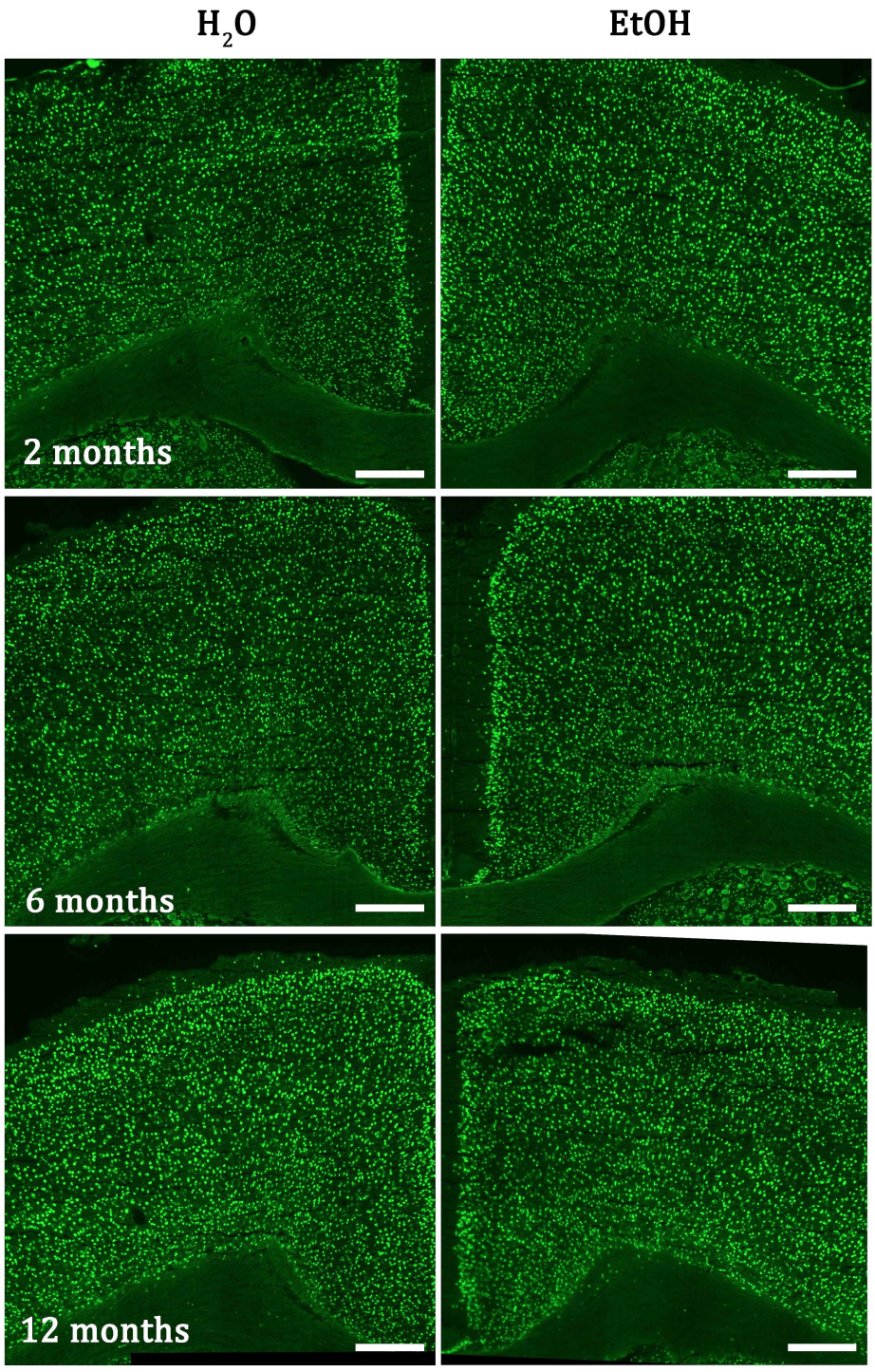



Figure 13. Representative images of NeuN-IR in the cingulate cortex of WT animals after 2, 6 and 12 months of alcohol consumption. Scale bar represents 100 μm

Surprisingly, quantification of the NeuN area fraction revealed neither alcohol- nor age-specific changes in any of the investigated cortical regions [CC: $F_{(1,12)} = 0.46$, $p = 0.63$; MC: $F_{(1,12)} = 0.05$, $p = 0.94$; SSC: $F_{(1,12)} = 0.73$, $p = 0.49$; IC: $F_{(1,12)} = 0.06$, $p = 0.94$; PC: $F_{(1,12)} = 0.18$, $p = 0.83$] (Figure 14).

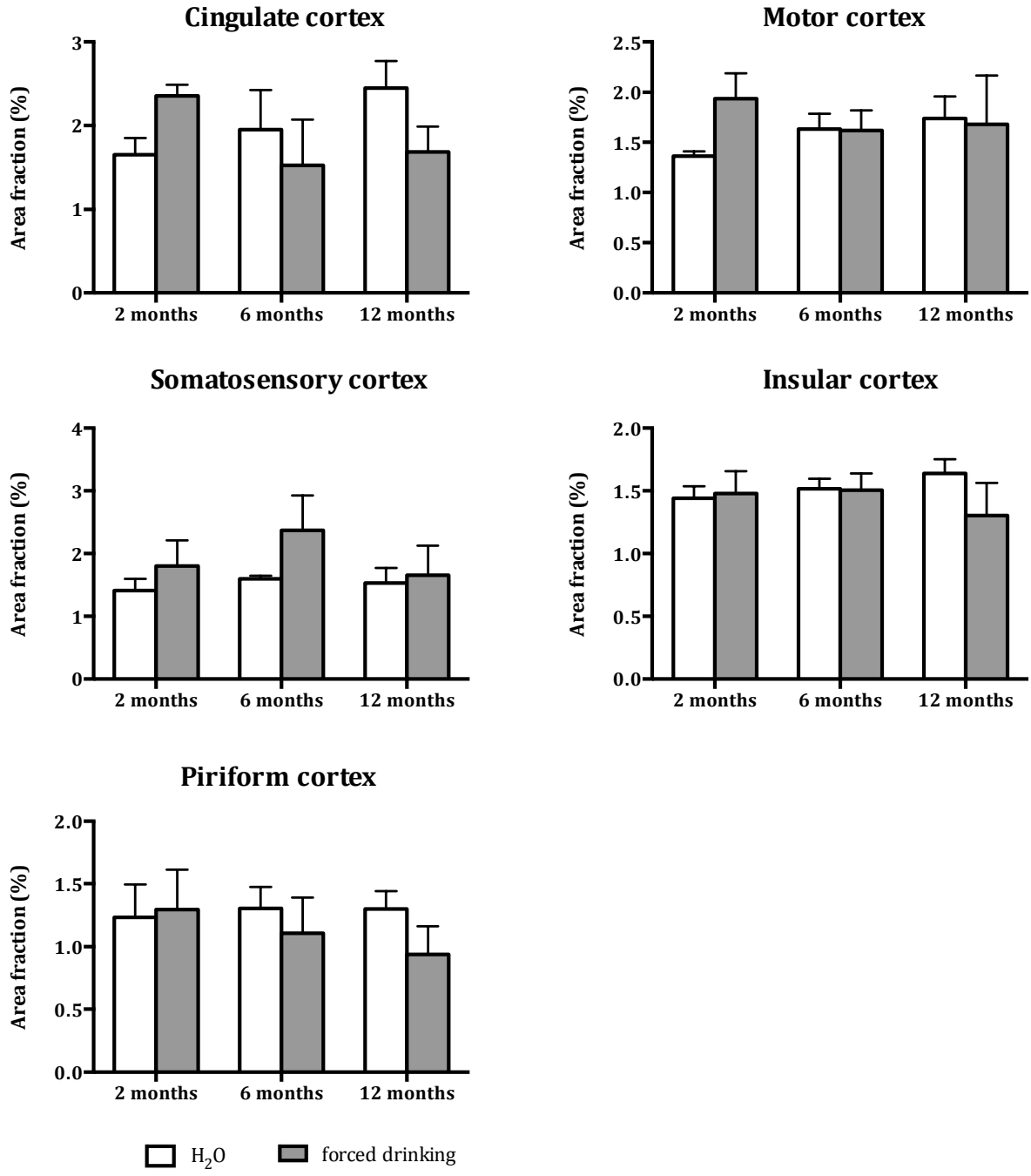



Figure 14. Area fraction of NeuN-IR neurons in various cortical regions after chronic alcohol consumption. There were no changes in the area fraction of NeuN-IR neurons induced by the chronic alcohol treatment (n = 3 per group). Data were analysed by two-way ANOVA (main

factors: treatment and age) and represented as mean value ± SEM. Greta Krusch (technical assistant trainee) performed these experiments under my supervision.

To monitor the systemic effect of alcohol on the animals, I quantified the cytokine burden in the liver as internal reference (Figure 15). Six months of alcohol treatment did not enhance cytokine production; however, the twelve-month alcohol treatment dramatically increased the expression of pro-inflammatory cytokines TNF-α [$F_{(1,20)}$ = 12.85, p = 0.001], IL-1β [$F_{(1,20)}$ = 9.98, p = 0.004] and IL-6 [$F_{(1,20)}$ = 17.39, p = 0.000]. Furthermore, the anti-inflammatory cytokines IL-4 [$F_{(1,20)}$ = 17.73, p = 0.000] and IL-10 [$F_{(1,20)}$ = 22.36, p = 0.000] were also strongly increased by long-term alcohol treatment. The chemokine CCL-2 only showed a moderate increase in expression after twelve months of alcohol drinking [$F_{(1,20)}$ = 24.67, p = 0.000].

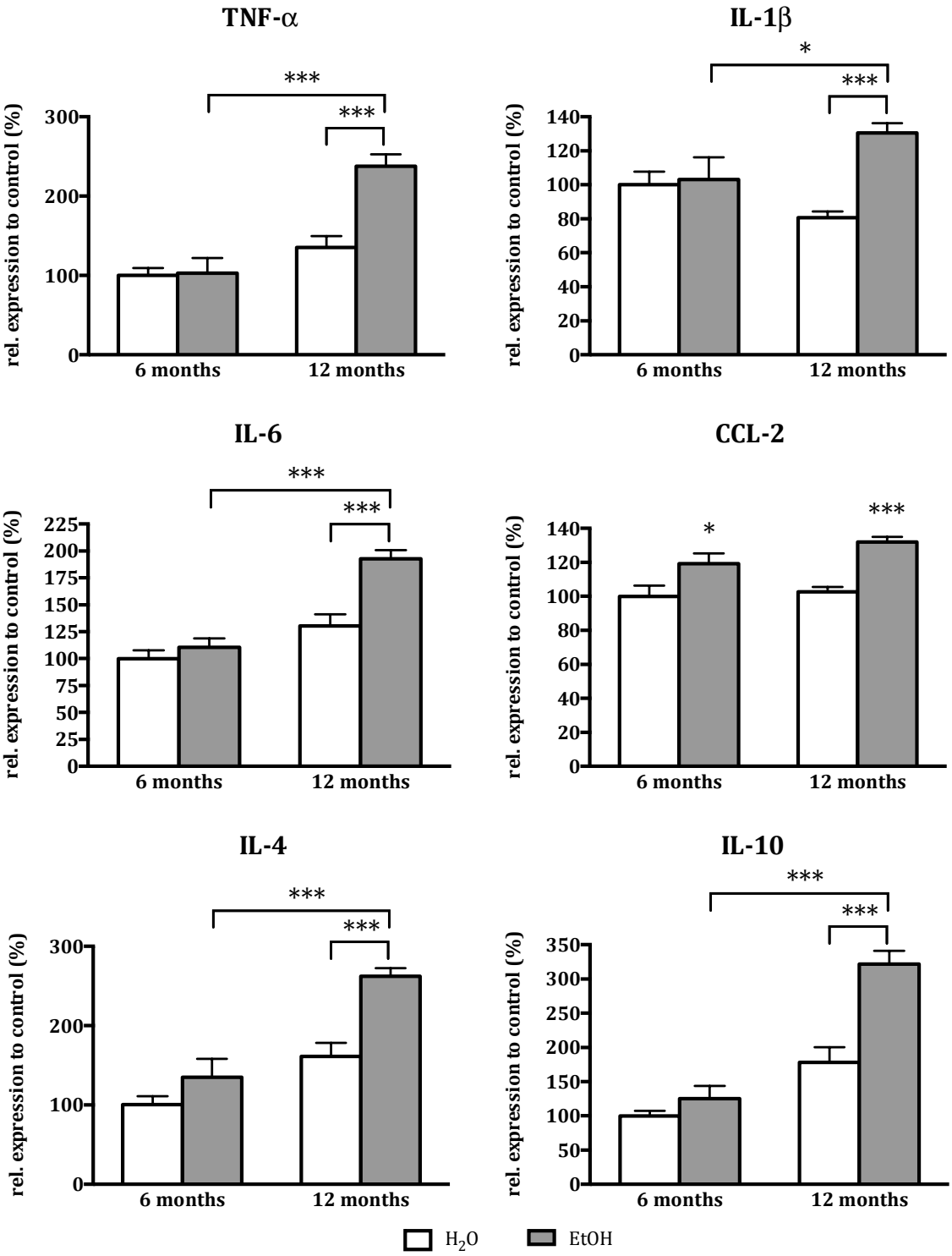



Figure 15. Cytokine expression in the liver of WT animals after six and twelve months of forced drinking. Six months of chronic alcohol treatment elevated the levels of the chemokine CCL-2. After twelve months of alcohol drinking there was a strong induction in pro-inflammatory cytokines TNF-α, IL-1β and IL-6 detectable. The levels of anti-inflammatory cytokines IL-4 and IL-10 were also strongly increased (n = 5 per group). Data were analysed by two-way ANOVA

(main factors: treatment and age) and represented as mean value ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

## 4.2 Effects of the $CB_2$ on alcohol-induced neuroinflammation

In this study we investigated the role of $CB_2$ receptors in alcohol-induced neuroinflammation by histological and molecular analysis. We employed and compared two different models for chronic alcohol administration: the continuous forced drinking (FD) and the discontinuous intermittent forced drinking (IFD) during six months. We used to routinely keep mice singly housed to more precisely monitor their individual alcohol and food consumption. However, as social isolation strongly affects behaviour (Koike et al., 2009; Kwak et al., 2009; Martin & Brown, 2010) and cytokine expression (Krügel et al., 2014) we also kept animals in groups of two to three per cage thus introducing the novel factor 'social environment' to the models.

### 4.2.1 Single-housed animals in the FD model

The analysis of cortical cytokine expression after six months of FD in single-housed animals revealed increased levels of IL-1β [$F_{(1,20)}$ = 5.79, p = 0.02] and IL-10 [$F_{(1,20)}$ = 4.92, p = 0.03] in WT animals compared to water controls (Figure 16). $CB_2$ deficient animals were resistant to the alcohol-induced effects as the expression levels did not change compared to the water controls. Moreover, the alcohol treatment did not alter the protein level of the pro-inflammatory cytokine TNF-α [$F_{(1,20)}$ = 0.23, p = 0.63] or IL-6 [$F_{(1,20)}$ = 0.64, p = 0.43]. The lack of $CB_2$ receptors led to a reduced expression of IL-6 [$F_{(1,20)}$ = 4.83, p = 0.03] and the anti-inflammatory cytokine IL-4 [$F_{(1,20)}$ = 45.78, p = 0.000]. The levels of the chemokine CCL-2 were increased in the $CB_2$ deficient animals [$F_{(1,20)}$ = 14.45, p = 0.001]. In this setup the results suggest that lack of $CB_2$ receptors inhibit the alcohol-induced changes in cytokine expression.

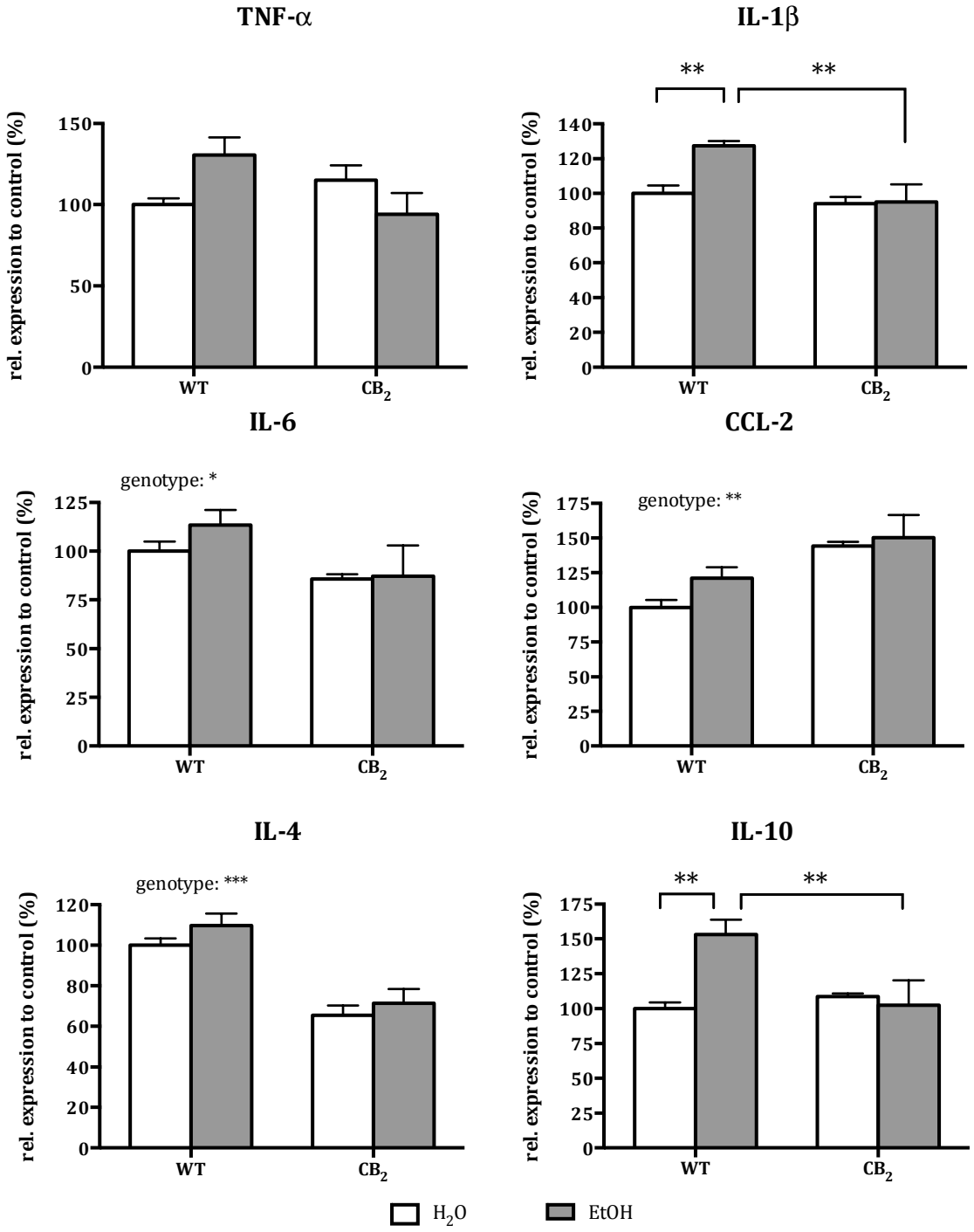



Figure 16. Cytokine expression in the frontal cortex of single-housed WT and $CB_2$ deficient animals in the FD model. WT animals showed an increase in the pro-inflammatory cytokine IL-1β and the anti-inflammatory cytokine IL-10. We could not detect any elevations in the $CB_2$ deficient animals. Furthermore, there was a genotype-specific elevation of the chemokine CCL-2 and a decrease in IL-6 in $CB_2$ deficient mice (n = 6 per group). Data were analysed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Histological analysis of microglia staining in the cortex (Figure 17) revealed that six-month alcohol drinking did not increase the area fraction of Iba1-IR in WT animals in any of the regions (Figure 18). However, there was a genotype-dependent increase in Iba1-IR in $CB_2$ deficient mice pointing to a generally more activated state of microglia in those animals [CC: $F_{(1,12)} = 15.22$, $p = 0.002$; MC: $F_{(1,12)} = 16.62$, $p = 0.001$; SSC: $F_{(1,12)} = 11.77$, $p = 0.004$; IC: $F_{(1,12)} = 5.88$, $p = 0.03$, PC: $F_{(1,12)} = 9.28$, $p = 0.01$].

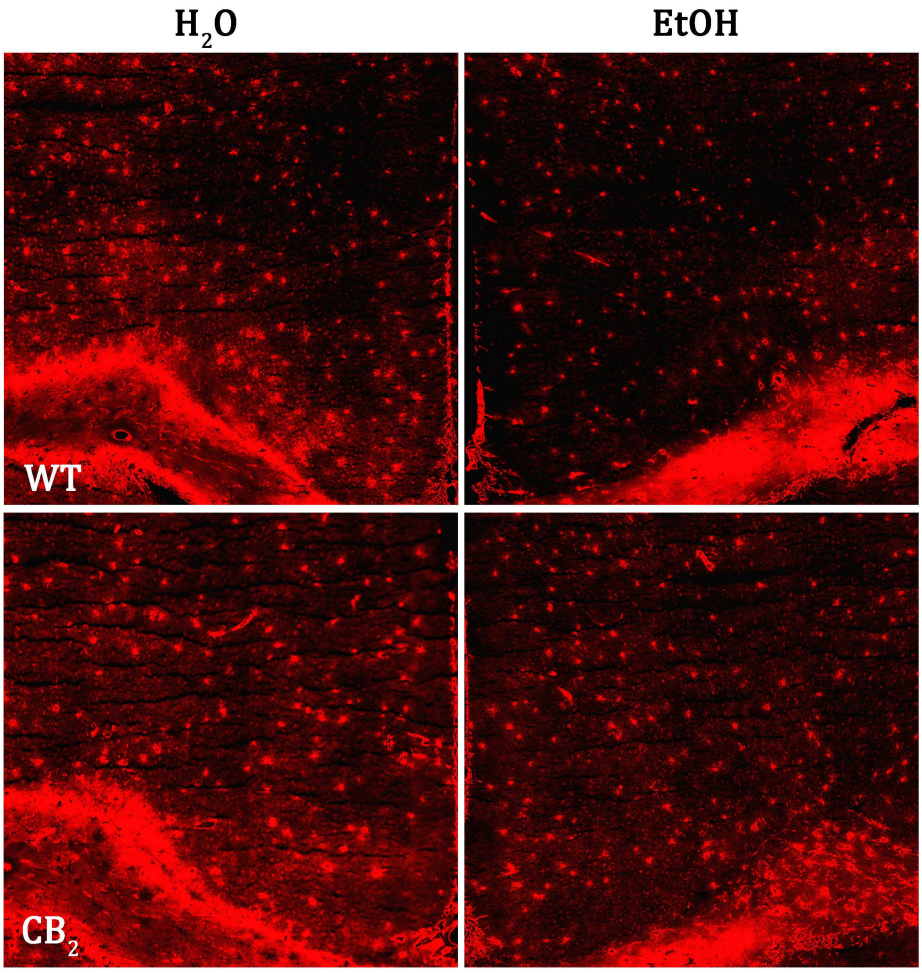



Figure 17. Representative images of Iba1-IR in the cingulate cortex of single-housed WT and $CB_2$ deficient animals in the FD model.

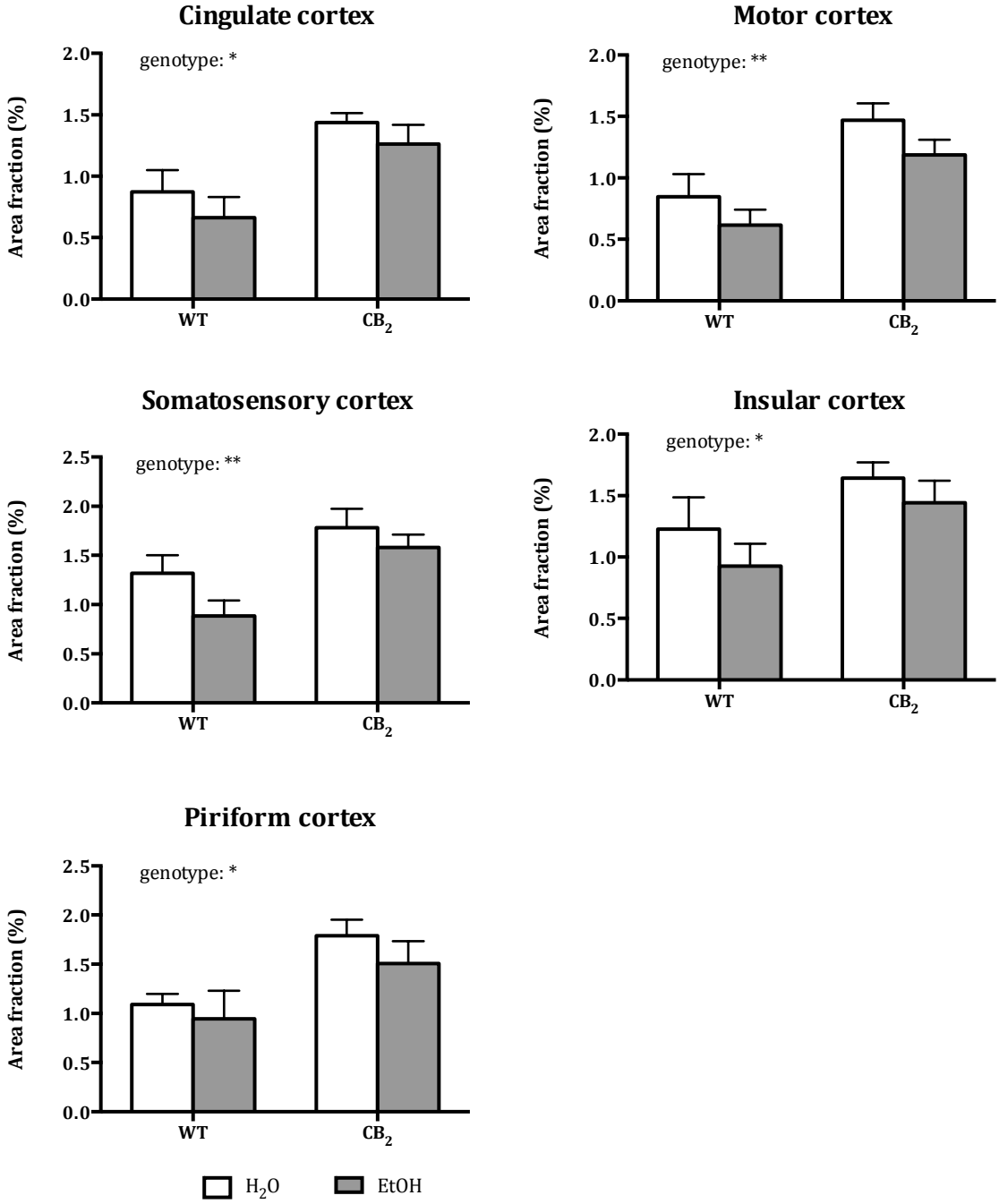



Figure 18. Area fraction of Iba1-IR in various cortical regions of single-housed WT and $CB_2$ deficient animals in the FD model. There is a significant increase in the area fraction of Iba1-IR in $CB_2$ deleted animals (n = 4 per group). Data were analysed by two-way ANOVA (main factors: treatment and age) and represented as mean value ± SEM. *p > 0.05; **p < 0.01.

Analysis of astrocytes in the cortex (Figure 19) revealed an alcohol-dependent increase in the area fraction of GFAP-IR specifically in the cingulate cortex [$F_{(1,12)}$ = 47.50, p = 0.000] (Figure 20). The ectorhinal [$F_{(1,12)}$ = 0.71, p = 0.41] or piriform [$F_{(1,12)}$ =

0.001, p = 0.97] cortex did not display alcohol-dependent changes. These effects were not affected by the genotype.

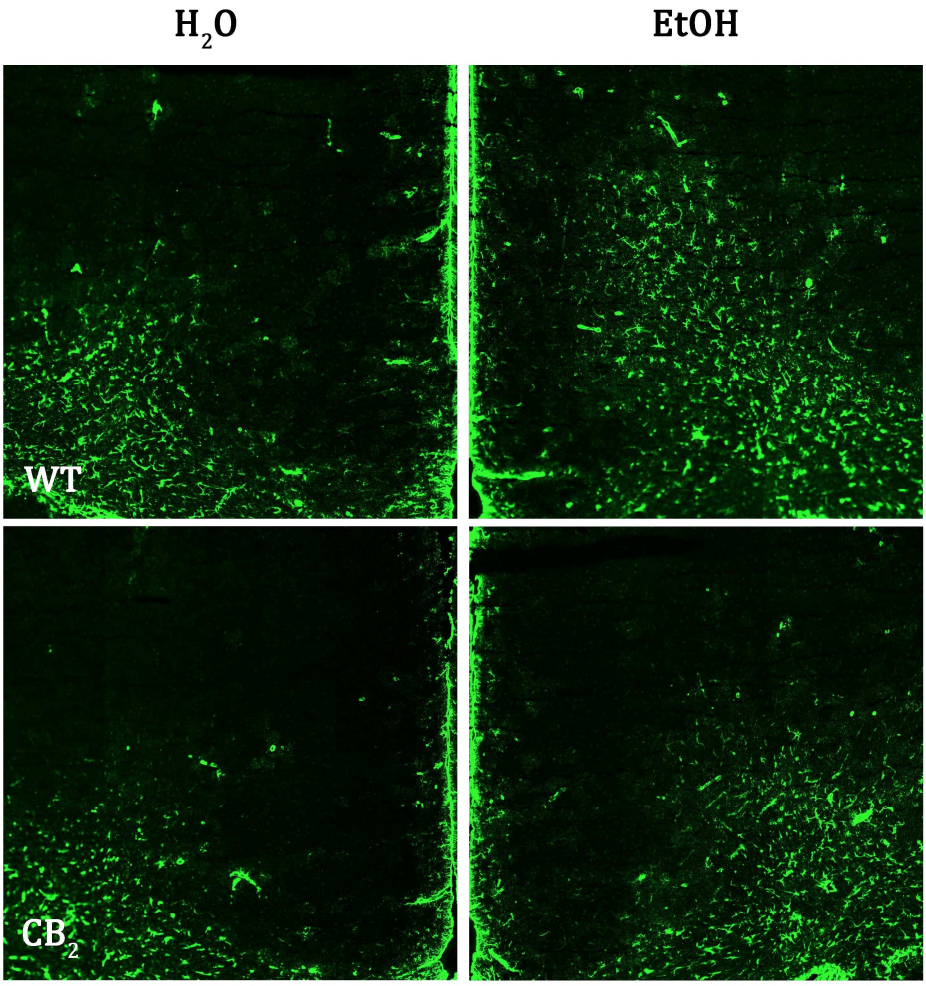



Figure 19. Representative image of GFAP-IR in the cingulate cortex of single-housed WT and $CB_2$ deficient animals in the FD model.

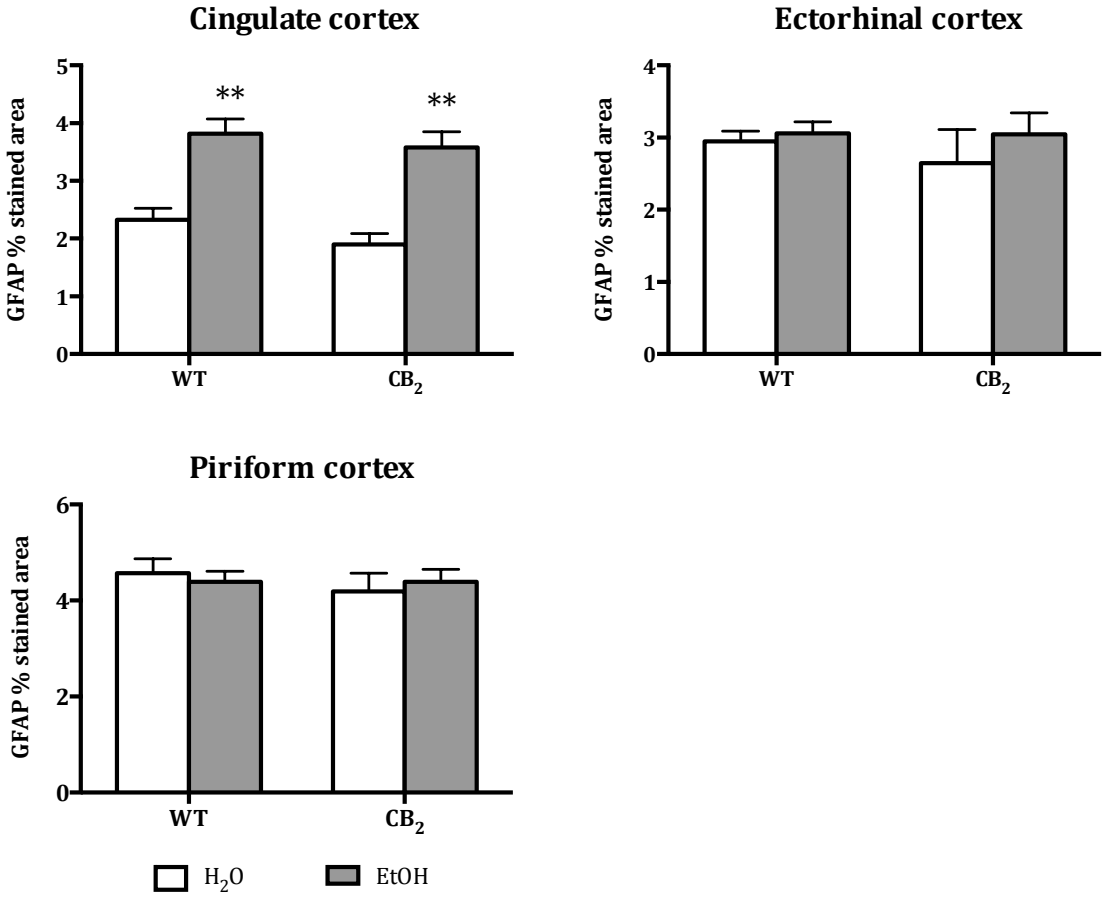



Figure 20. Area fraction of GFAP-IR in various cortical regions of single-housed WT and $CB_2$ deficient animals in the FD model. There is a significant increase in the area fraction of GFAP in the cingulate cortex after chronic alcohol treatment. Data were analysed by two-way ANOVA and represented as mean value ± SEM. (n = 4 per group). **p < 0.01.

As the $CB_2$ receptor seems to modulate the inflammatory response to alcohol the expression of CNR2 transcripts was analysed in different brain regions (Figure 21). The mRNA expression analysis revealed only a strong tendency towards increased levels of CNR2 expression in the investigated brain regions after six months of alcohol treatment [$F_{(1,12)}$ = 5.44, p = 0.05]. The expression in the respective brain regions was similar [$F_{(1,12)}$ =0.33, p = 0.72]. Spleen was used as control tissue and displayed strongest CNR2 expression.

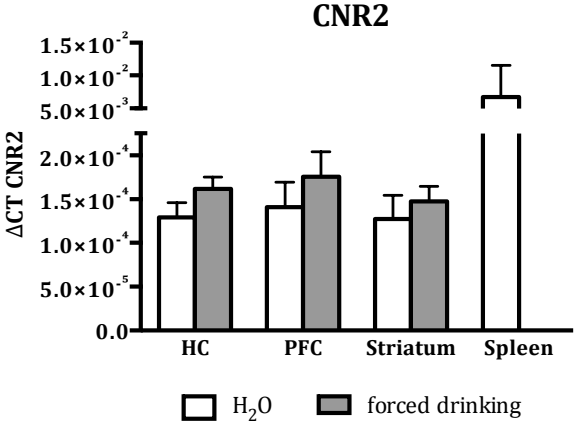



Figure 21. mRNA expression of CNR2 after six months of forced drinking. Expression of CNR2 showed a trend to increased expression in brain regions ($p = 0.05$) ($n = 4$ per group). Spleen tissue served as control. Data were analysed by repeated measures one-way ANOVA (main factor: treatment; within effect: region) and represented as mean value ± SEM.

Quantification of the cytokine expression in the liver of WT and $CB_2$ knockout animals revealed similarly to Figure 15 no alcohol-dependent changes in WT animals after six months of forced drinking (Figure 22). Lack of $CB_2$ expression accelerated the alcohol-induced liver damage represented by the increased expression of the pro-inflammatory cytokines TNF-α [$F_{(1,20)} = 6.50$, $p = 0.01$], IL-1β [$F_{(1,20)} = 13.62$, $p = 0.001$] and IL-6 [$F_{(1,20)} = 4.47$, $p = 0.04$] the anti-inflammatory cytokines IL-4 [$F_{(1,20)} = 17.73$, $p = 0.000$] and IL-10 [$F_{(1,20)} = 16.39$, $p = 0.000$] and the chemokine CCL-2 [$F_{(1,20)} = 18.90$, $p = 0.000$]. These findings are consistent with previously published reports (Louvet et al., 2011). Together, these data suggest that the $CB_2$ receptor might have a pro-inflammatory role in the brain and an anti-inflammatory effect in the liver.

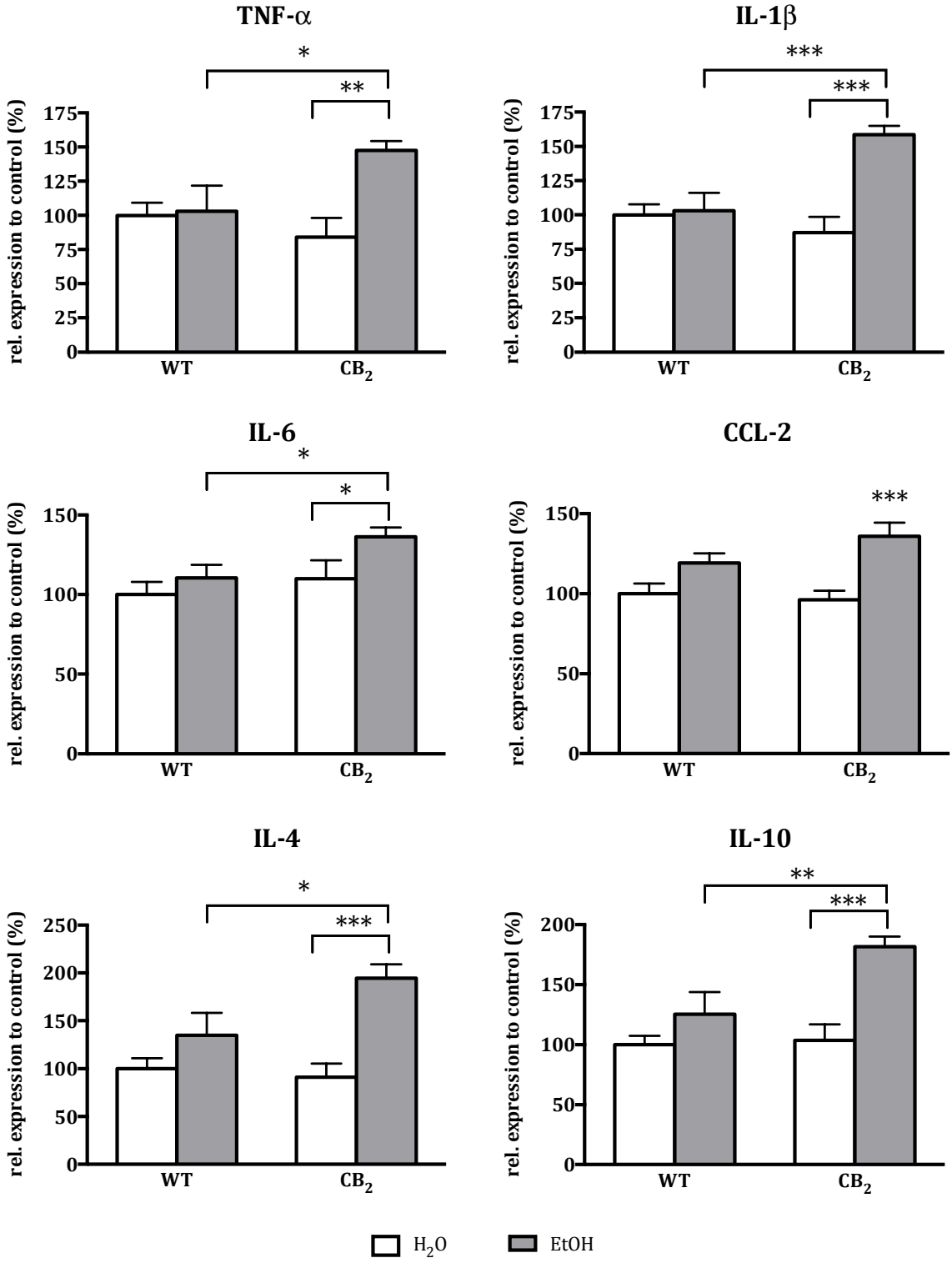



Figure 22. Cytokine expression in the liver of single-housed WT and $CB_2$ deficient animals in the FD model. WT animals did not show a significant increase in any of the investigated cytokines and chemokines. However, $CB_2$ deficient animals showed elevated cytokine levels of TNF-α, IL-1b and IL-10 and the chemokine CCL-2 (n = 6 per group). Data were analysed by two-way

ANOVA (main factors: treatement and genotype) and represented as mean value ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

### 4.2.2 Single-housed animals in the IFD model

Animals in this study were analysed in cognitive performance. They were abstinent for the duration of the testing during the last month of the IFD procedure. The quantification of cytokine expression followed the behavioural analysis and revealed elevated levels of IL-1β [$F_{(1,16)}$ = 4.95, p = 0.04] in the prefrontal cortex (Figure 23). The levels of TNF-α were not affected by the treatment [$F_{(1,16)}$ = 0.22, p = 0.63] . Deletion of the $CB_2$ receptor did not influence the cytokine expression.

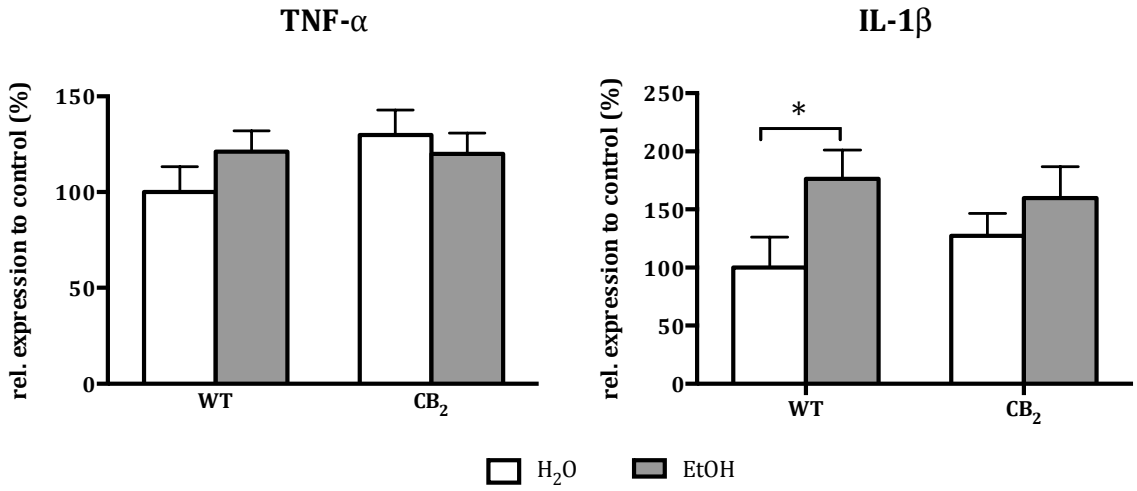



Figure 23. Cytokine expression in the prefrontal cortex after five months of alcohol consumption and one month withdrawal of WT and $CB_2$ animals in the IFD model. WT and $CB_2$ deficient mice showed increased expression of IL-1β after alcohol treatment. The expression of TNF-α was not changed (n = 5 per group). Data were analysed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM. *p < 0.05.

The cytokine expression in the liver showed moderate treatment effects and did not reach the level of significance in the post-hoc analysis (Figure 24). Treatment effects could be observed for the expression levels of TNF-α [$F_{(1,15)}$ = 6.08, p = 0.02], CCL-2 [$F_{(1,15)}$ = 4.81, p = 0.04] and IL-10 [$F_{(1,15)}$ = 6.84, p = 0.01]. The expression of IL-1β, IL-6 and IFN-γ was not significantly changed.

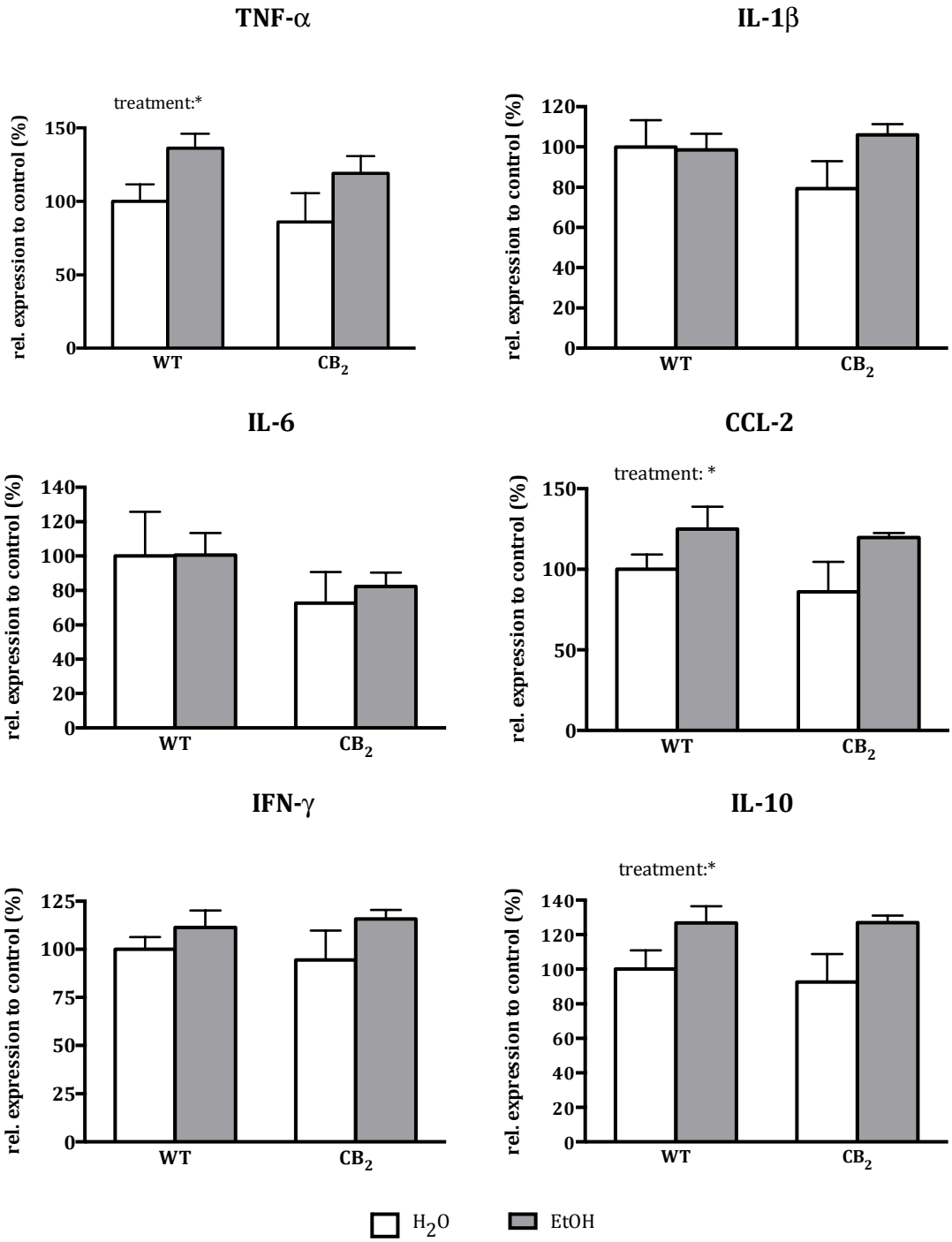
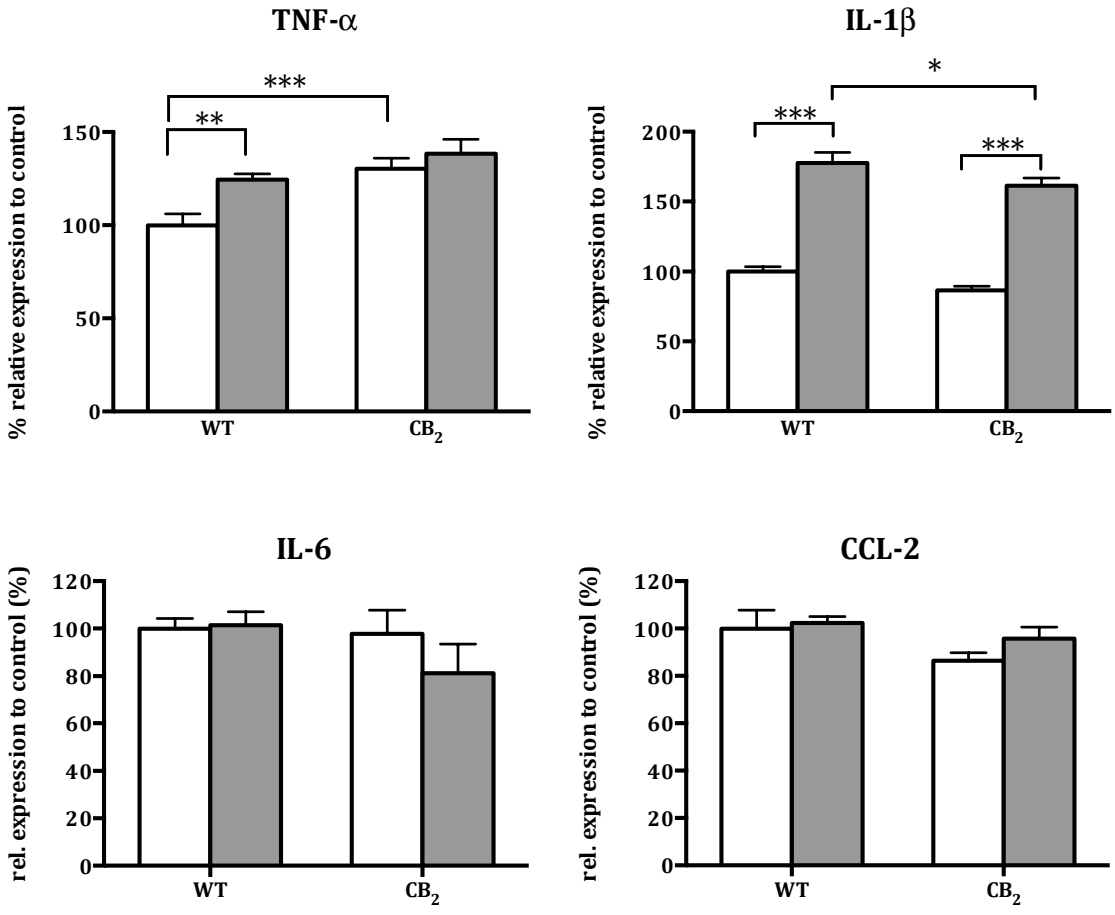



Figure 24. Cytokine expression in the liver after five months of alcohol consumption and one month withdrawal of WT and $CB_2$ deficient animals in the IFD model. WT and $CB_2$ deficient mice showed significant alcohol effects in the expression of TNF-α, CCL-2 and IL-10 (n = 5 per group). Data were analysed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM. *$p < 0.05$.

### 4.2.3 Group-housed animals in the FD model

Quantification of cytokine expression in the frontal cortex of group-housed animals showed an increase in TNF-α [$F_{(1,29)}$ = 7.71, p = 0.009] and IL-1β [$F_{(1,28)}$ = 221.58, p = 0.000] expression due to the alcohol treatment in WT animals (Figure 25). However, the expression levels of IL-6, IL-10, GM-CSF and CCL-2 were not changed. Deletion of the $CB_2$ receptor increased basal levels of TNF-α [$F_{(1,29)}$ = 14.21, p = 0.000], which were not affected by the alcohol treatment. The expression of IL-1β in alcohol-treated $CB_2$ knockout animals was increased by alcohol treatment [$F_{(1,28)}$ = 8.53, p = 0.006]. In contrast to WT animals IL-10 expression level was significantly increased in $CB_2$ deficient animals [$F_{(1,27)}$ = 4.96, p = 0.03]. Similar to WT animals, expression of IL-6, GM-CSF and CCL-2 was not changed in $CB_2$ deficient mice after treatment.



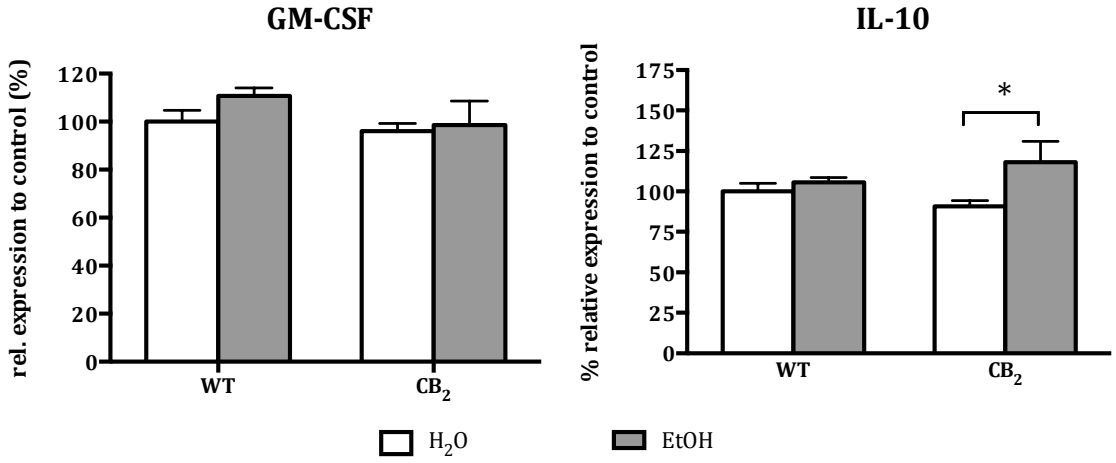



Figure 25. Cytokine expression in the frontal cortex of group-housed WT and $CB_2$ deficient animals in the FD model. WT and $CB_2$ deficient mice showed an increase in IL-1β expression after the alcohol treatment. Furthermore, WT animals showed an increase in TNF-α expression (n = 5 – 8 per group). Data were analysed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

In contrast to single-housed WT animals, the cytokine expression in the liver revealed an increased expression of TNF-α [$F_{(1,14)}$ = 12.56, p = 0.003] and IL-10 [$F_{(1,14)}$ = 11.96, p = 0.003] (Figure 26). Furthermore, there were significant treatment effects for the cytokines IL-1β [$F_{(1,14)}$ = 9.66, p = 0.007], IL-6 [$F_{(1,14)}$ = 8.17, p = 0.01] and GM-CSF [$F_{(1,14)}$ = 7.54, p = 0.01]. The expression of CCL-2 was not changed. Deletion of $CB_2$ did not alter the cytokine expression.

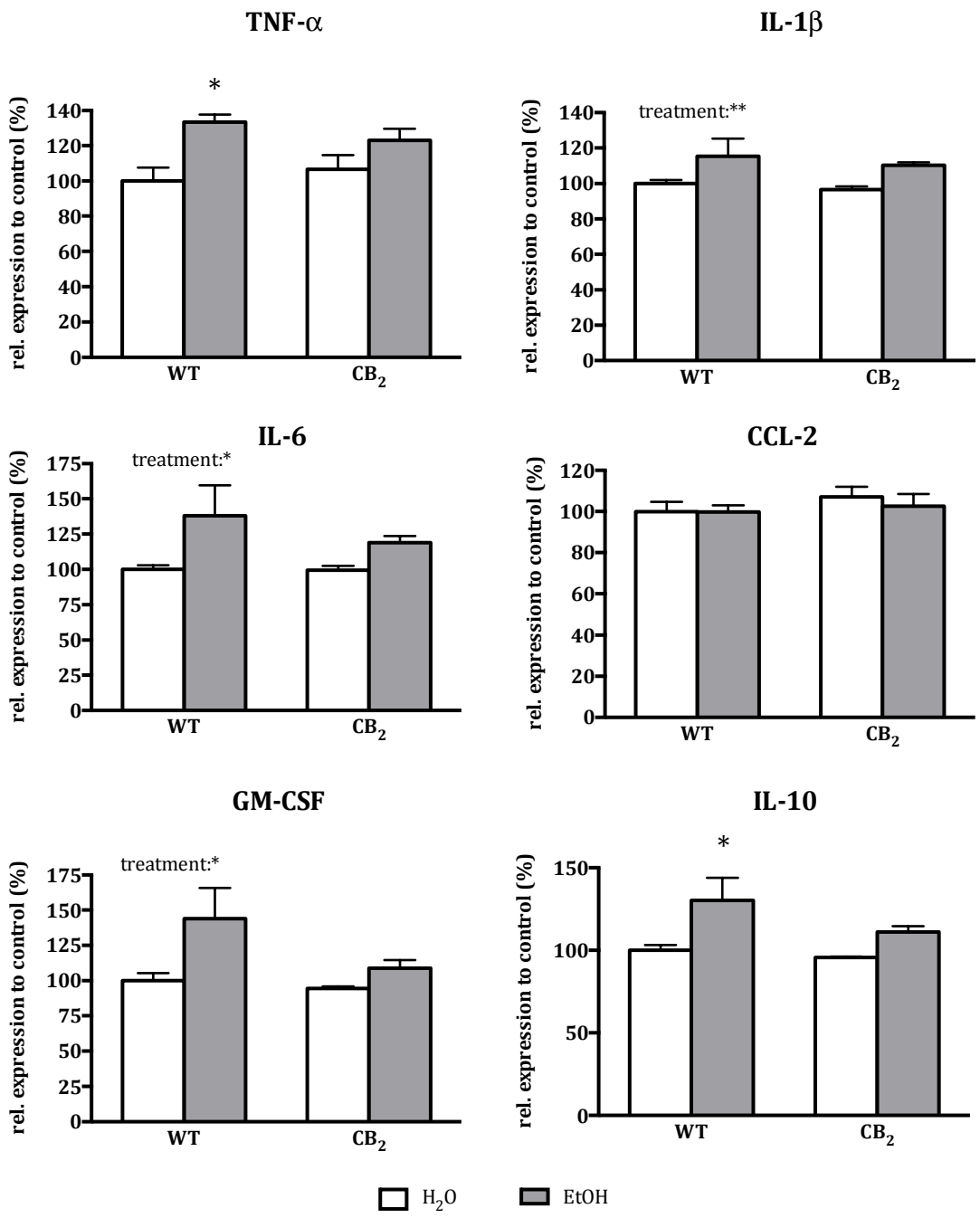



Figure 26. Cytokine expression in the liver of group-housed WT and $CB_2$ deficient animals in the FD model. WT animals show an increase in the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10. We could not detect any elevations in the $CB_2$ deleted animals (n = 5 per group). Data were analysed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

### 4.2.4 Group-housed animals in the IFD model

Quantification of the cytokine expression showed treatment effects in both of the genotypes for IL-10 [$F_{(1,26)}$ = 4.82, p = 0.03], which did not reach the level of significance in the post-hoc analysis (Figure 27). IL-1β showed a strong tendency to increased expression levels due to alcohol treatment [$F_{(1,27)}$ = 4.09, p = 0.05]. The levels of TNF-α were significantly elevated due to $CB_2$ deletion [$F_{(1,28)}$ = 10.49, p = 0.003] and not to treatment [$F_{(1,28)}$ = 0.15, p = 0.69].

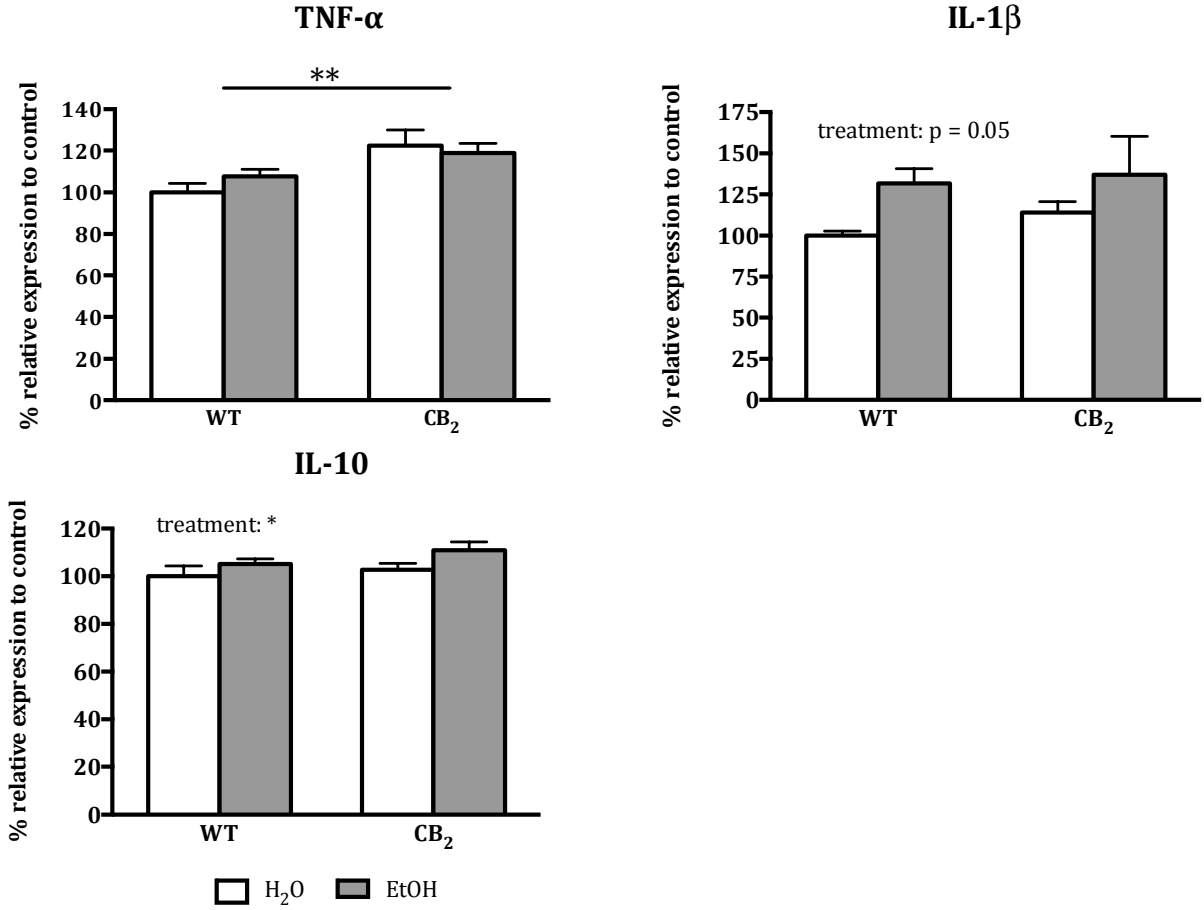



Figure 27. Cytokine expression in the frontal cortex of group-housed WT and $CB_2$ deficient animals in the IFD model. WT and $CB_2$ deficient mice showed increased expression of IL-1β and IL-10 after alcohol treatment. The expression of TNF-α was slightly increased in $CB_2$ deficient animals (n = 8 per group). Data were analysed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM. * indicates genotype effect. **p < 0.01.

The treatment had similar effects on cytokine expression in the liver (Figure 28). The factorial ANOVA showed significant effects for the treatment in TNF-α [$F_{(1,16)}$ = 6.92, p = 0.01] and IL-1β [$F_{(1,16)}$ = 12.19, p = 0.003] in both of the genotypes. The levels of IL-10 were neither altered by the treatment nor by the genotype [$F_{(1,16)}$ = 3.99, p = 0.58].

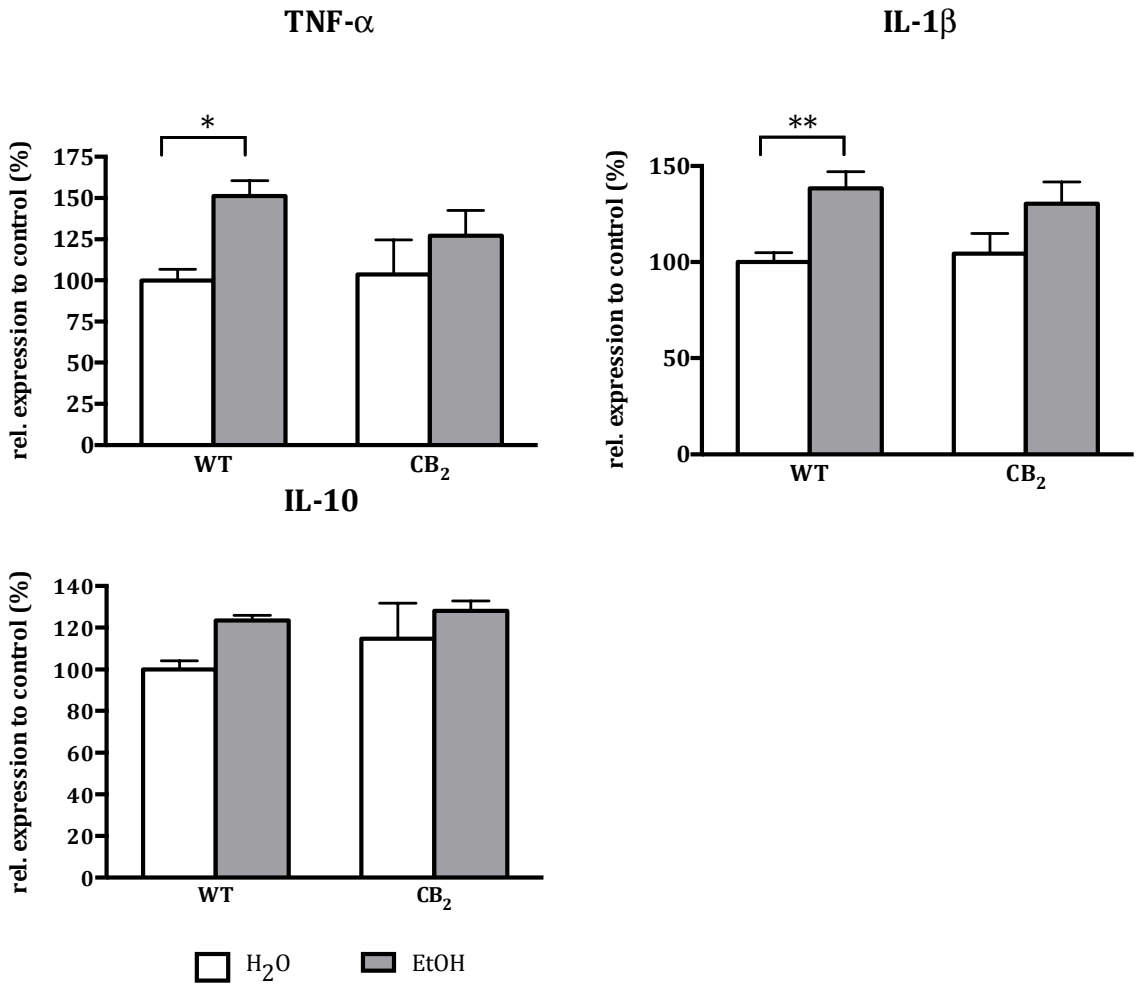



Figure 28. Cytokine expression in the liver of group-housed WT and $CB_2$ deficient animals in the IFD model. Both, WT and $CB_2$ deficient mice showed increased IL-1β and TNF-α expression after chronic alcohol treatment (n = 5 per group). Data were analysed by two-way ANOVA (main factors: treatment and gentype) and represented as mean value ± SEM. *p < 0.05, **p < 0.01.

## 4.3 Effects of alcohol on cognition, locomotion and anxiety

In this part I present the data of the behavioural testing of animals in different chronic alcohol models. It is important to note that, depending on the experiment,

animals were examined during alcohol abstinence or while having free access to alcohol. This is, however, always indicated separately.

### 4.3.1 Two months of chronic alcohol consumption: FD and IFD

To monitor behavioural changes induced by alcohol, I first examined the animals' exploratory activity in the open field after two months of FD and IFD. Animals were at the age of four months at the time of testing. Generally, alcohol treatment enhanced the activity of the animals [$F_{(1,37)}$ = 4.15, p = 0.02] (Figure 29A). Animals that had intermittent alcohol access were tested during a withdrawal phase and appeared to be strongly hyperactive. Animals that had continuous alcohol access were tested without withdrawal and also showed slightly enhanced locomotion in the open field arena. Moreover, the centre field activity, which is a readout for anxiety-like behaviour was not significantly affected by the model [$F_{(1,37)}$ = 2.77, p = 0.07] (Figure 29B).

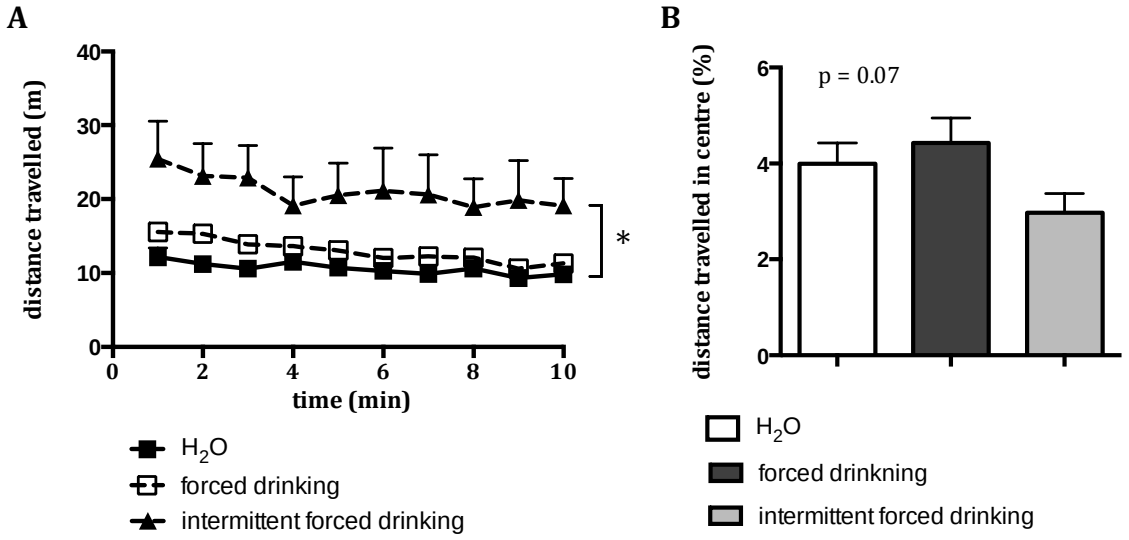



Figure 29. Activity in the open field of WT animals after two months of alcohol treatment in the FD and IFD model. (A) Overall distance travelled. (B) Distance travelled in percent in the centre of the open field (n = 13 - 14 per group). Forced drinking animals were analysed while they had free access to alcohol. Animals from the intermittent forced drinking group were tested during the withdrawal phase. Data were analysed by (A) repeated measures one-way ANOVA (main factor: model) or (B) one-way ANOVA (main factor: model) and represented as mean value ± SEM. * indicates comparison between the models. *p > 0.05.

Next, working memory performance was assessed in the Y-maze (Figure 30). Two parameters are presented: 'returns' and 'alternations'. 'Returns' describe the percentage of performed working memory failures, which are characterised by the return into the same arm. This parameter was not changed by the model [$F_{(1,37)}$ = 1.30, p = 0.28]. 'Alternations' describe the percentage of correct choices, which are revealed by entering a new arm. There was also no treatment effect [$F_{(1,37)}$ = 1.79, p = 0.18].

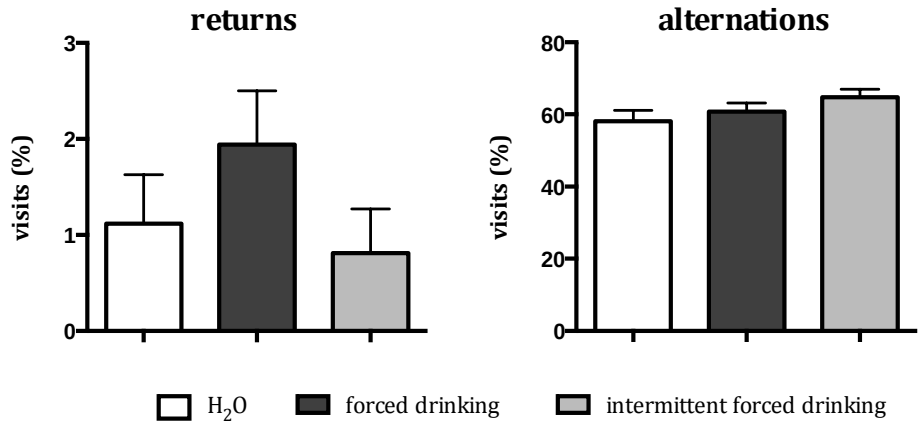



Figure 30. Working memory performance in the Y-maze test of WT animals after two months of alcohol treatment in the FD and IFD models. Different parameters were analysed including correct alternations and returns in percent (n = 13 - 14 per group). Forced drinking animals were analysed while they had free access to alcohol. Animals from the intermittent forced drinking group were tested during the withdrawal phase. Data were analysed by one-way ANOVA (main factor: model) and represented as mean value ± SEM.

Next, the O-maze test was performed to investigate anxiety-related behaviour. Two parameters were analysed: the time and the distance spent in the open arms of the maze in per cent. Neither distance [$F_{(1,37)}$ = 1.22, p = 0.30] nor time spent in the open arms [$F_{(1,37)}$ = 2.01, p = 0.14] were significantly altered by the alcohol treatment (Figure 31).

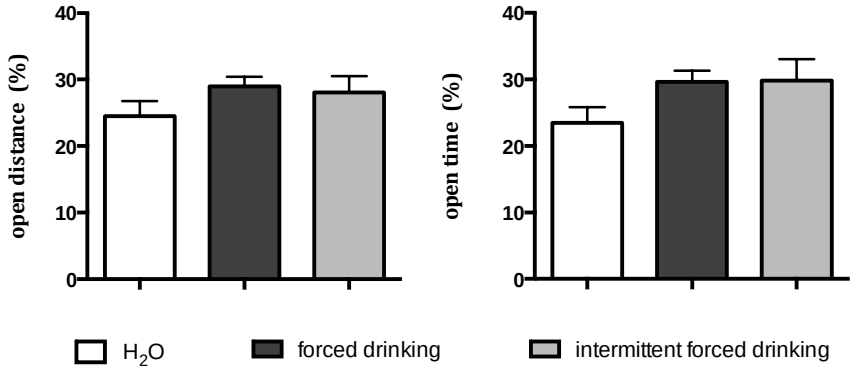



Figure 31. Anxiety-related behaviour in the O-maze test of WT animals after two months of alcohol treatment in the FD and IFD model. The distance and the time spent in the open parts of the O-maze was calculated in percent (n = 13 - 14 per group). Forced drinking animals were analysed while having free access to alcohol. Animals from the intermittent forced drinking group were tested during the withdrawal phase. Data were analysed by one-way ANOVA (main factor: model) and represented as mean value ± SEM.

### 4.3.2 Six months of forced drinking

After six months of FD animals were at the age of eight months and they were examined while they had free access to ethanol. There was no significant change in the working memory performance in the Y-maze test (Figure 32). The percentage of neither working memory failure [$F_{(1,14)} = 2.36$, $p = 0.14$] nor alternations [$F_{(1,14)} = 0.01$ $p = 0.89$] was significantly affected.

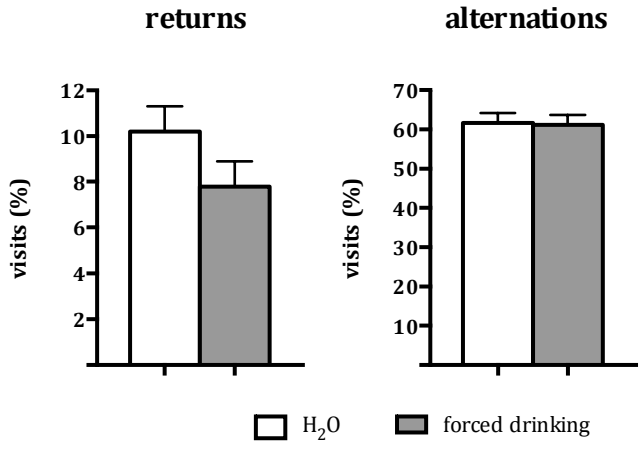



Figure 32. Working memory performance in the Y-maze test of WT animals after six months in the FD model. Different parameters were analysed including alternations and returns in percent.

The treatment did not affect the working memory performance (n = 8 per group). Alcohol-treated animals were analysed without withdrawal. Data were analysed one-way ANOVA (main factor: treatment) and represented as mean value ± SEM.

The anxiety in the O-maze test was also not significantly different between the groups. Both parameters 'distance travelled' [$F_{(1,14)}$ = 1.79, p = 0.20] and 'time spent in the open arms' [$F_{(1,14)}$ = 1.11, p = 0.30] were not significantly changed by alcohol drinking (Figure 33).

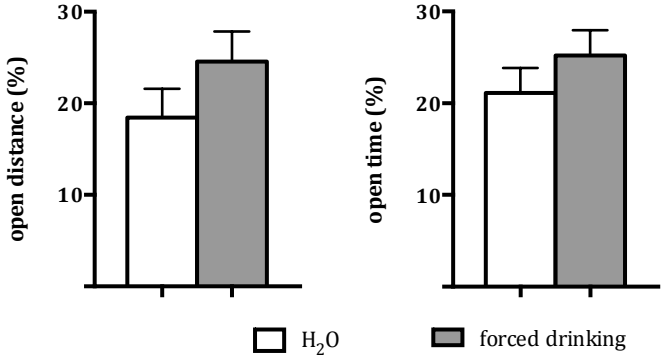



Figure 33. Anxiety in the O-maze test of WT animals after six months in the FD model. The distance and the time spent in the open parts of the O-maze was calculated in percent (n = 8 per group). The test was performed at 700 lux. Alcohol-treated animals were analysed without withdrawal. Data were analysed by one-way ANOVA (main factor: treatment) and represented as mean value ± SEM.

As six months of alcohol treatment did not significantly affect the behaviour of the animals in the Y- and in the O-maze tests, a more complex and challenging test was performed to investigate cognitive functions after chronic alcohol treatment. The Morris-water maze is a test of spatial learning and memory in an aversive environment (Figure 34). During the acquisition phase (Figure 34A), the animals learned to find the platform, which is reflected by changes in latency and path length to reach the platform. As early as the second day of training the latency was reduced by more than 50 % and after seven days the animals managed to find to the platform within twelve seconds. Surprisingly, chronic alcohol treatment did not influence memory acquisition during seven days [latency: $F_{(1,18)}$ = 0.00, p = 0.96; path length: $F_{(1,18)}$ = 0.01, p = 0.90]. The locomotor activity was also not changed by alcohol, as revealed by the swim speed,

which was similar in both groups [$F_{(1,18)}$ = 0.08, p = 0.77] (Figure 34B). To test the flexibility of learning, the position of the platform was changed to the opposite quadrant on the eighth day (Figure 34C). On the second day of the reversal phase, the latency was reduced by 50 %. The learning flexibility was not affected by the alcohol treatment during the three-day reversal phase [latency: $F_{(1,18)}$ = 0.06, p = 0.79; path length: $F_{(1,18)}$ = 0.26, p = 0.61]. On the last day the probe trial was performed to monitor the strength of memory (Figure 34D). In order to do so, the platform was removed and the time the animals spent in the former target quadrant was measured. The six-month alcohol treatment did not influence the memory strength in this setup [$F_{(1,18)}$ = 1.27, p = 0.27].

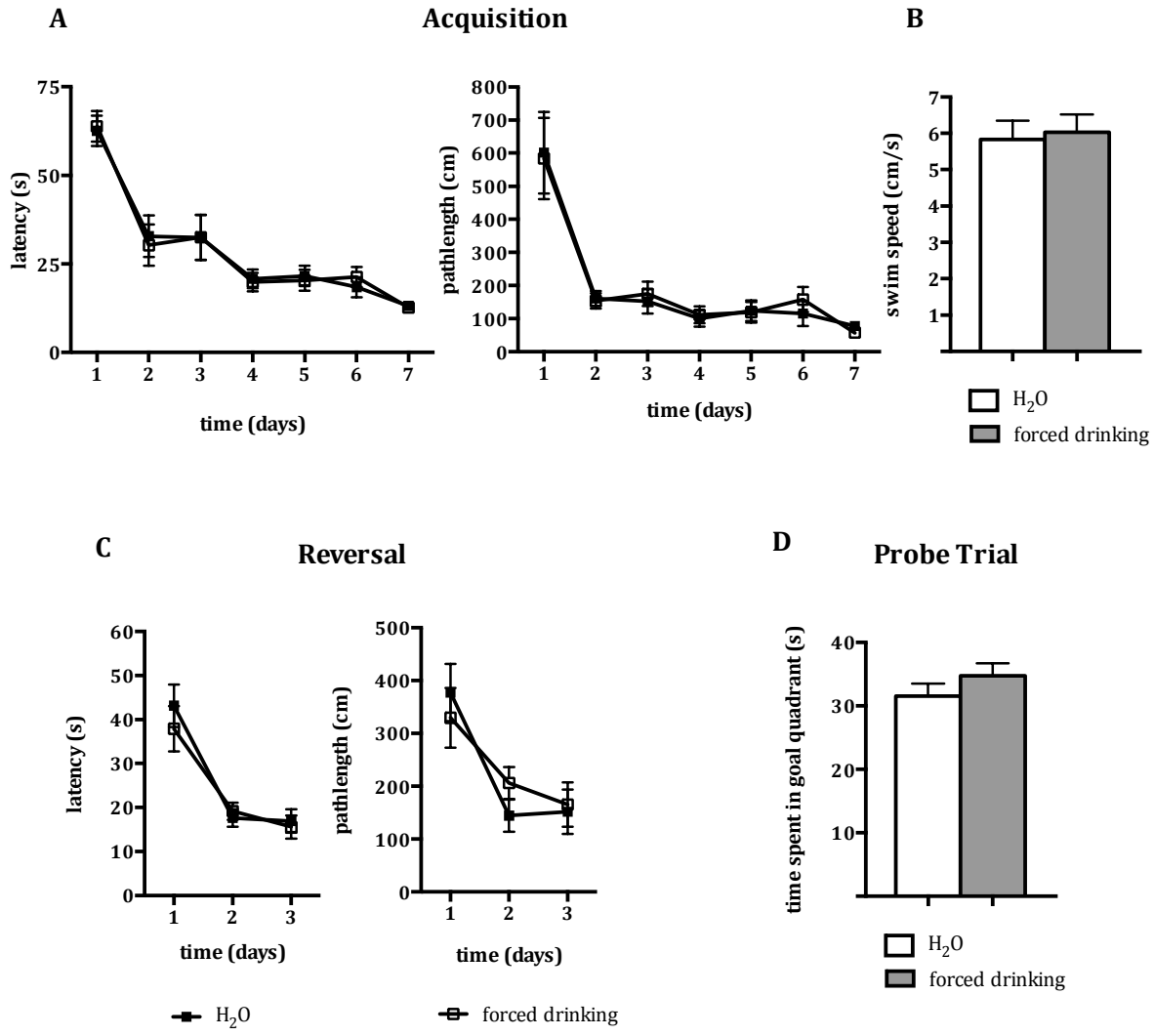



Figure 34. Spatial memory performance in the Morris-water maze of WT animals after six months in the FD model. (A) During the acquisition phase, water and alcohol-treated animals showed the same learning performance. (B) The swim speed was not affected by the treatment. (C) In the reversal phase, both groups showed the same learning flexibility. (D) After removal of

the platform in the probe trial, both groups spent the same time in the former target quadrant (n = 10 per group). Alcohol-treated animals were analysed without withdrawal. Data were analysed by (A and C) repeated measures one-way ANOVA (main factor: treatment, within effect: time) or (B and D) one-way ANOVA (main factor: treatment) and represented as mean value ± SEM.

### 4.3.3 Behavioural effects of $CB_2$ deletion after chronic alcohol drinking

Withdrawal of alcohol leads to an imbalanced emotional state thereby precipitating behavioural changes. We therefore took advantage of the regular withdrawal periods in the intermittent forced drinking model and performed the behavioural analysis three days after the beginning of withdrawal, respectively. Furthermore, we investigated the impact of $CB_2$ deletion on the animals' behaviour in two different housing conditions.

#### Single-housed animals

Open field activity was investigated at the age of four months when animals already had two months of intermittent alcohol access, which was similar to the setup in Figure 29. Surprisingly, the exploratory behaviour of WT mice was not changed by the intermittent alcohol treatment [$F_{(1,32)}$ = 0.31, p = 0.58] (Figure 35A). Deletion of $CB_2$ receptors did not alter open field activity either [$F_{(1,32)}$ = 0.88, p = 0.35]. Centre field activity was not affected by alcohol treatment either in WT or in $CB_2$ deficient mice [$F_{(1,32)}$ = 0.11, p = 0.74] (Figure 35B).

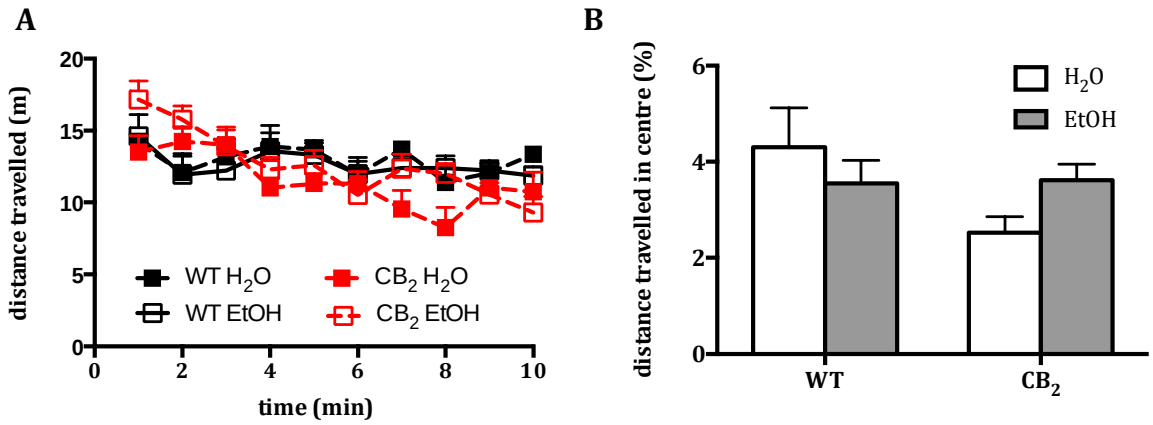
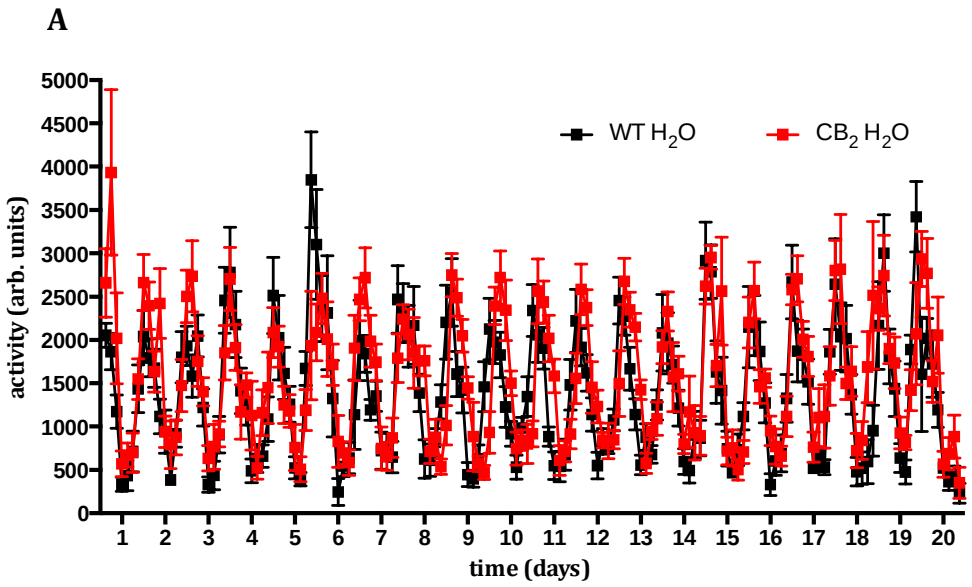



Figure 35. Activity in the open field of WT and $CB_2$ deficient animals after two months of alcohol treatment in the IFD model. (A) Neither alcohol treatment nor deletion of $CB_2$ affected the overall distance travelled per minute. (B) Distance travelled in percent in the centre of the open

field was not affected by the treatment (n = 13 - 14 per group). Data were analysed by (A) repeated measures two-way ANOVA (main factors: treatment and genotype, within effect: time) or (B) two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM.

To investigate phenotypes of the withdrawal syndrome including insomnia and increased irritability, the home cage activity of the animals was monitored during three periods of withdrawal and alcohol consumption. The overall home cage activity of water-treated animals is representatively shown in Figure 36A. Alcohol-treated WT and $CB_2$ deficient animals are shown in Figure 36B. These graphs demonstrate that the circadian rhythm was not changed after four months of chronic alcohol drinking.



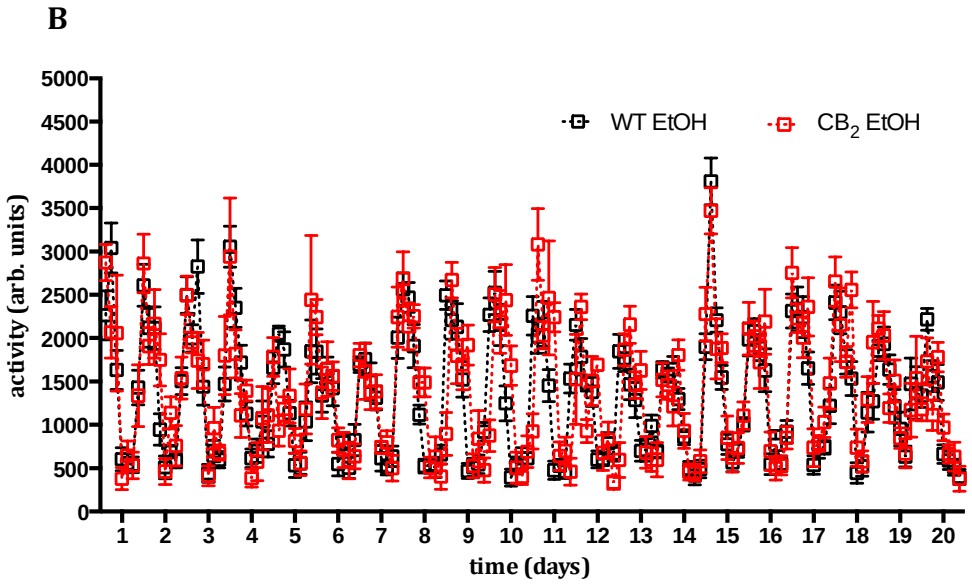
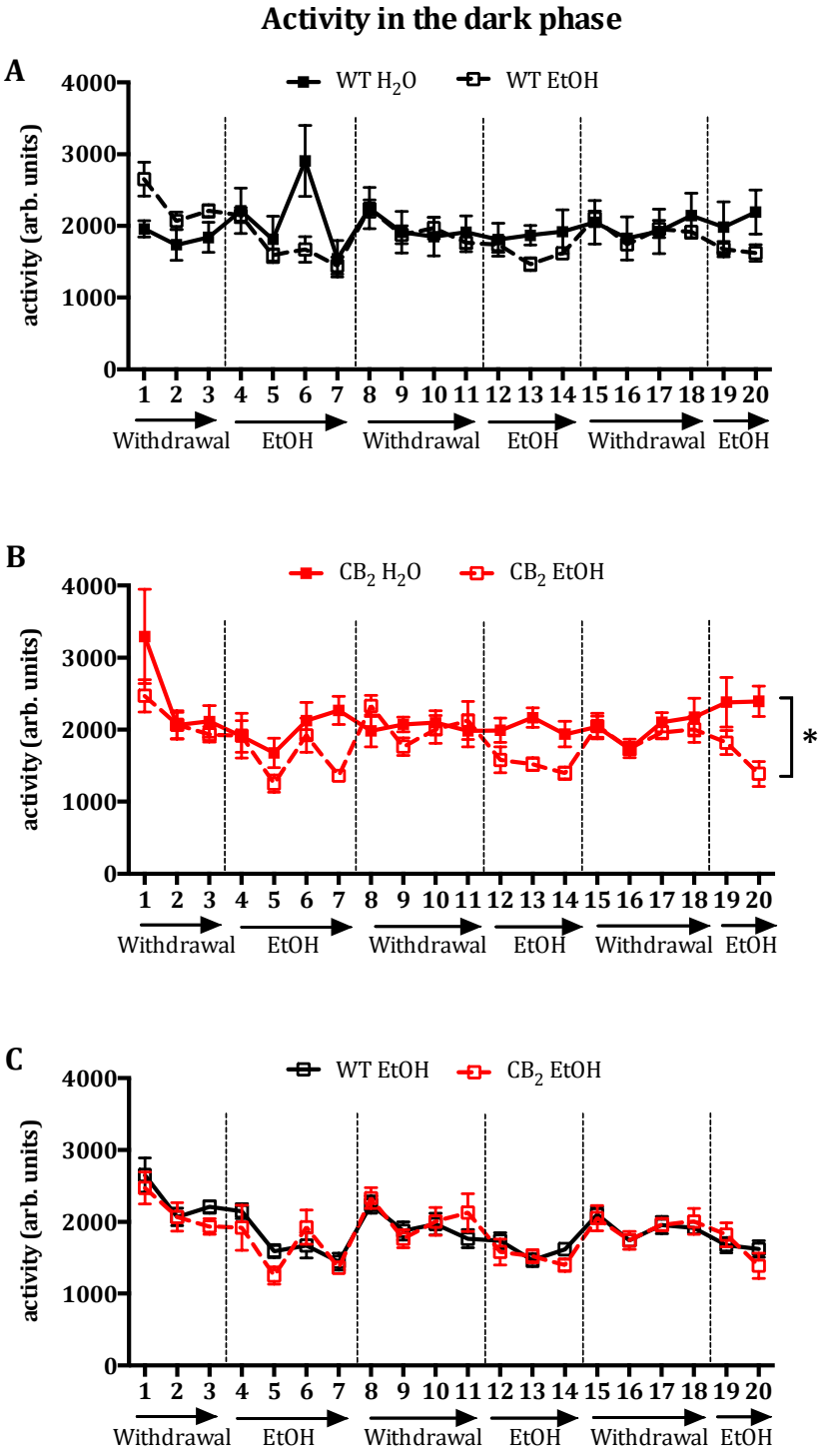



Figure 36. Activity in the home cage of WT and $CB_2$ deficient animals after four months of alcohol treatment in the IFD model. (A) Representative figure of the circadian rhythm in three hour intervals of water-treated control animals throughout 20 days. (B) Representative figure of the circadian rhythm in three hour intervals of alcohol-treated animals over 20 days ($H_2O$ n = 6 per group; EtOH n = 8 – 11 per group).

For a clear overview of the home cage activity data I devided the daily activity in two periods: the dark, active phase (Figure 37) and the light, inactive phase (Figure 38). During the active phase WT animals showed a slight decrease in activity during the alcohol periods compared to water controls (Figure 37A). Withdrawal of alcohol slightly enhanced the activity to the level of water-treated animals. However, this effect was not significant for the treatment [$F_{(1,12)} = 0.18$, $p = 0.67$]. In $CB_2$ deficient animals, the home cage activity was significantly reduced while animals had access to alcohol [$F_{(1,12)} = 5.69$, $p = 0.03$] (Figure 37B). The genotype did not further alter the activity of alcohol-treated animals [$F_{(1,13)} = 0.00$, $p = 0.99$], but there was a striking effect over time [$F_{(1,247)} = 9.86$, $p = 0.000$] (Figure 37C). In water-treated control animals, there were no significant changes [$F_{(1,9)} = 0.65$, $p = 0.43$] (Figure 37D).


Activity in the dark phase
A
WT H2O
WT EtOH
activity (arb. units)
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Withdrawal
EtOH
Withdrawal
EtOH
Withdrawal
EtOH
B
CB2 H2O
CB2 EtOH
activity (arb. units)
4000
3000
2000
1000
0
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Withdrawal
EtOH
Withdrawal
EtOH
Withdrawal
EtOH
C
WT EtOH
CB2 EtOH
activity (arb. units)
4000
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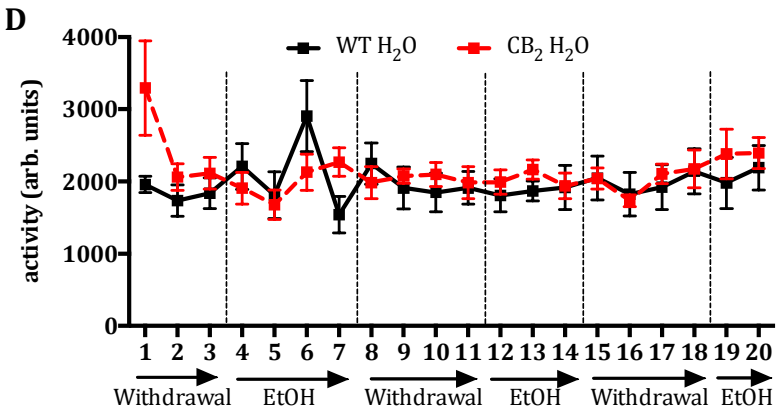
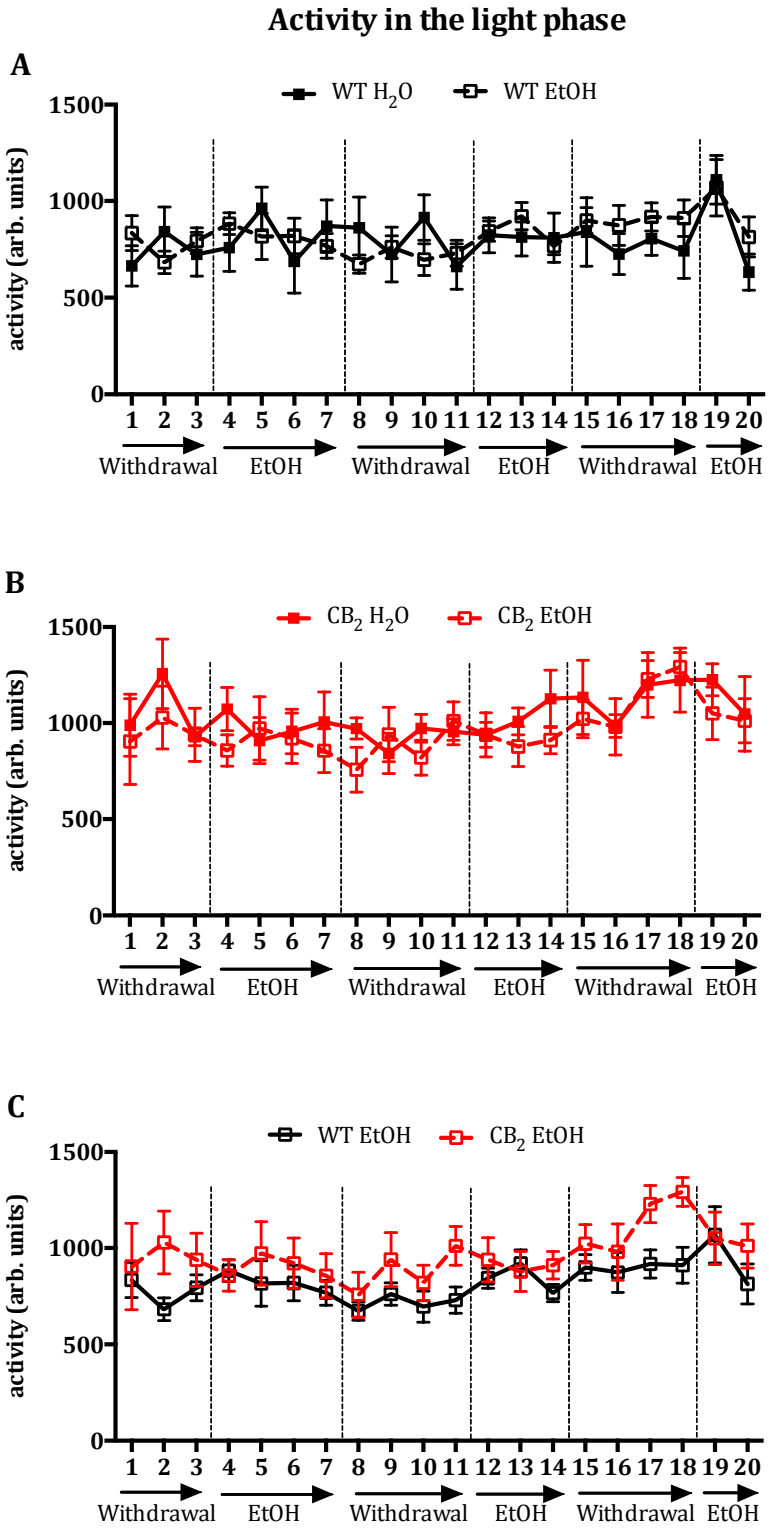



Figure 37. Home cage activity during the active phase of WT and $CB_2$ deficient animals after four months of alcohol treatment in the IFD model. (A) Alcohol treatment did not significantly affect the home cage activity in WT animals. (B) Deletion of the $CB_2$ receptor significantly reduced the home cage activity during the alcohol period. (C) The genotype did not influence the activity in alcohol-treated animals although it was decreased by alcohol treatment. (D) Water-treated controls did not show any difference between WT and $CB_2$ deficient animals ($H_2O$ n = 6 per group; EtOH n = 8 – 11 per group). Data were analysed by repeated measures one-way ANOVA (main factor: treatment (A and B) or genotype (C and D), within effect: time) and represented as mean value ± SEM. * indicates treatment effect. **p < 0.01, ***p < 0.001.

During the resting phase, home cage activity was decreased by more than 50 % compared to the active phase. Alcohol treatment neither affected activity in the sleeping phase in WT [$F_{(1,11)}$ = 1.06, p = 0.32] (Figure 38A) nor in $CB_2$ deficient animals [$F_{(1,9)}$ = 0.74, p = 0.41] (Figure 38B) compared to respective water controls. However, compared with alcohol-treated animals (Figure 38C), $CB_2$ deleted mice showed a trend to increased activity [$F_{(1,13)}$ = 3.22, p = 0.09]. This effect was enhanced when comparing water-treated controls (Figure 38D). So, deletion of $CB_2$ receptors resulted in a significantly increased activity during the sleeping phase [$F_{(1,9)}$ = 6.03, p = 0.03].


Activity in the light phase
A
WT H2O
WT EtOH
activity (arb. units)
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Withdrawal
EtOH
Withdrawal
EtOH
Withdrawal
EtOH
B
CB2 H2O
CB2 EtOH
activity (arb. units)
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Withdrawal
EtOH
Withdrawal
EtOH
Withdrawal
EtOH
C
WT EtOH
CB2 EtOH
activity (arb. units)
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Withdrawal
EtOH
Withdrawal
EtOH
Withdrawal
EtOH

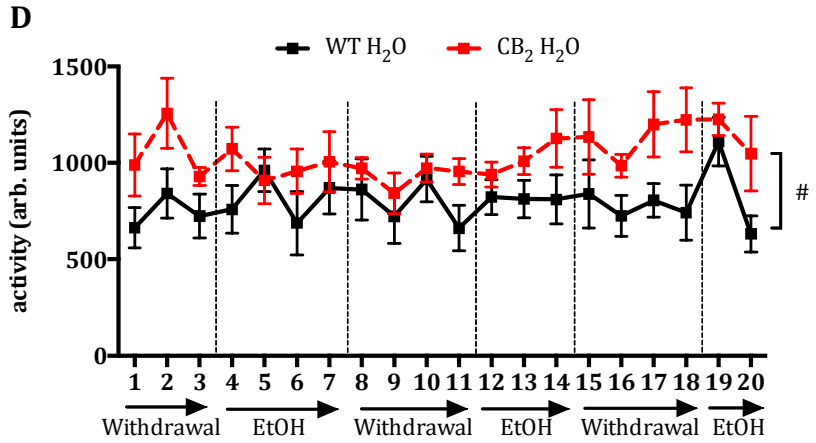
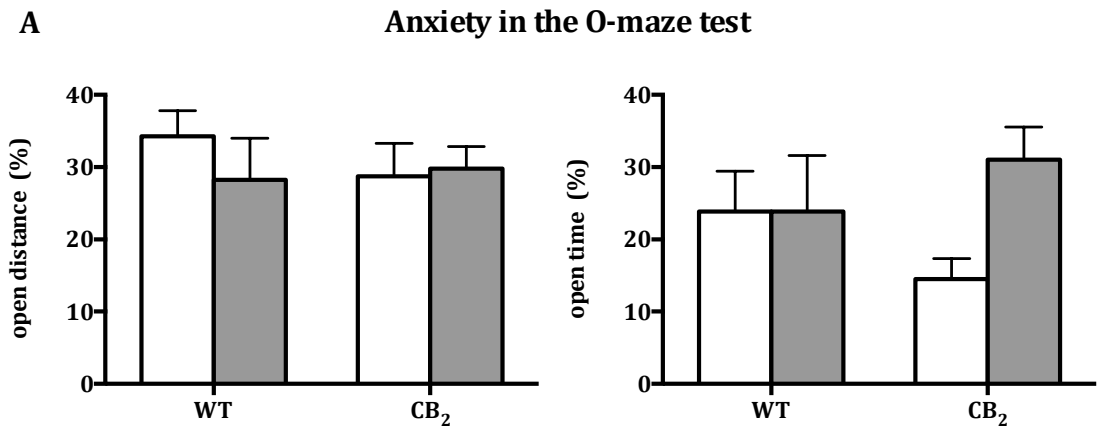



Figure 38. Home cage activity during the resting phase of WT and $CB_2$ deficient animals after four months of alcohol treatment in the IFD model. (A and B) Alcohol treatment did not alter the activity during the resting phase either in WT or $CB_2$ deficient animals. (C and D) $CB_2$ deficient animals showed increased activity during the resting phase. This effect is the strongest in water controls ($H_2O$ n = 6 per group; EtOH n = 8 – 11 per group). Data were analysed by repeated measures one-way ANOVA (main factor: treatment (A and B) or genotype (C and D), within effect: time) and represented as mean value ± SEM. # indicates genotype effect. #p < 0.05.

To investigate withdrawal-induced anxiety, I analysed the animals in the O-maze test (Figure 39A) and in the dark/light box test (Figure 39B). Distance and time spent in the open arm of the O-maze [distance: $F_{(1,28)} = 0.26$, p = 0.60; time: $F_{(1,28)} = 1.70$, p = 0.20] or in the open area of the dark/light box [distance: $F_{(1,19)} = 0.14$, p = 0.70; time: $F_{(1,19)} = 0.02$, p = 0.87], were not altered by alcohol withdrawal.



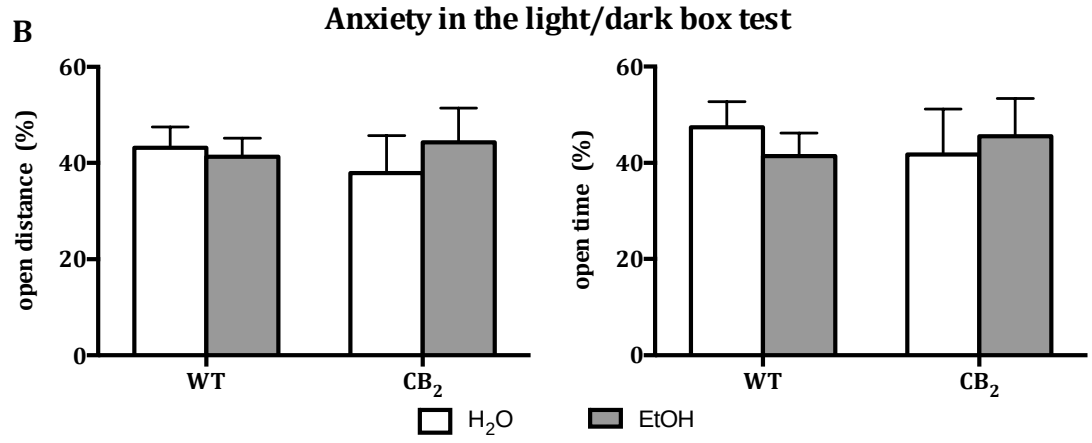



Figure 39. Anxiety in the O-maze (A) and in the dark/light box test (B) of WT and $CB_2$ deficient animals after five months of alcohol treatment in the IFD model (A) The distance and the time spent in the open parts of the O-maze were calculated in percent. The test was performed at 40 lux. Anxiety in the dark/light box test (B) The distance and the time spent in the open parts of the dark/light box were calculated in percent. The test was performed at 15 lux. Alcohol-treated animals were analysed during a withdrawal phase after six months of intermittent alcohol treatment (n = 6 - 10 per group). Data were analysed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM.

To investigate cognitive performance of mice after five months of alcohol drinking, I analysed the animals in the object and social recognition tests. Both tests are designed to measure the declarative memory. During the pre-test trial in the object recognition test, WT and $CB_2$ deficient animals did not show preference for a specific object (Figure 40A). When one of the familiar objects was removed and exchanged by a novel object, water-treated WT and $CB_2$ deficient animals showed a significant increase in the preference for the novel object [$F_{(1,33)}$ = 27.77, p = 0.000]. This effect was also present in alcohol-treated WT and $CB_2$ deficient mice.

A prerequisite for performing the social recognition test is that animals show social interaction with partner mice. For this purpose, the animals were subjected to the test for social preference. Here, both water and alcohol-treated animals showed preference for a partner mouse in a grid cage compared to an empty cage [$F_{(1,27)}$ = 39.71, p = 0.000] (Figure 40B). However, in the social recognition test WT, $CB_2$ deficient control animals as well as alcohol-treated $CB_2$ knockout mice did not show preference for a novel partner mouse (Figure 40C). Surprisingly, alcohol-treated WT animals spent

significantly more time with the novel compared to the familiar partner mouse [$F_{(1,18)}$ = 6.73, p = 0.01].

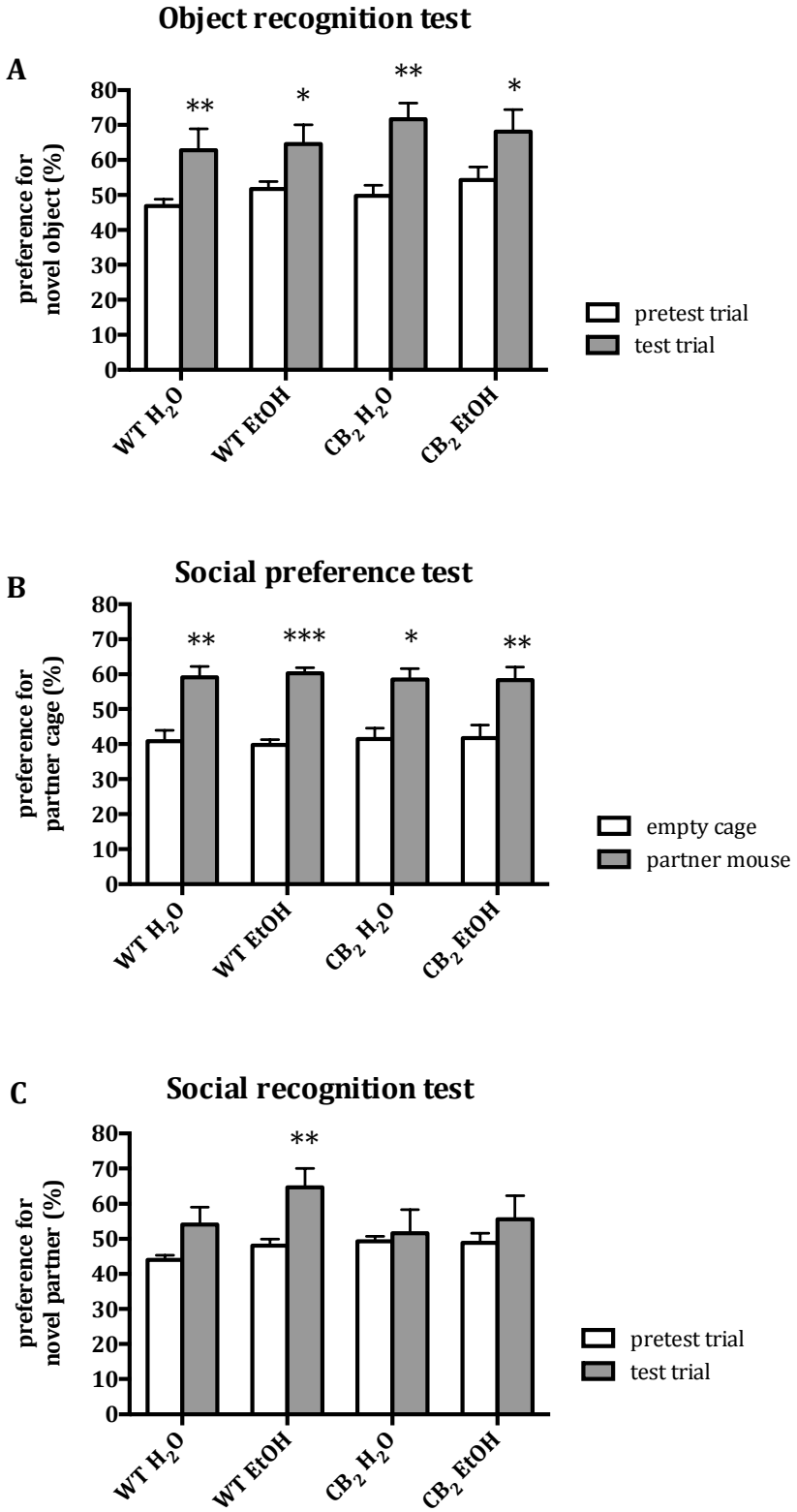


Figure 40. Declarative memory performance of WT and $CB_2$ deficient animals after five months of alcohol treatment in the IFD model. (A) Water- and alcohol-treated animals and alcohol-treated WT and $CB_2$ mice showed increased preference for the novel object in the object recognition test. (B) Both treatment groups showed preference for a partner mouse in the social preference test independent of the genotype. (C) Only alcohol-treated WT animals showed increased preference for the novel partner in the social recognition test (n = 9 – 12 per group). Data were analysed by repeated measures two-way ANOVA (main factors: genotype and treatment, within effect: test) and represented as mean value ± SEM.*p < 0.05, **p > 0.01, ***p > 0.001.

## Group-housed animals

After six months of intermittent alcohol access, the exploratory behaviour of group-housed animals in the open field arena was analysed. Chronic alcohol treatment significantly increased the activity in both of the genotypes (Figure 41A) [$F_{(1,54)}$ = 4.43, p = 0.03]. Deletion of $CB_2$ receptors decreased the exploratory behaviour of mice in the open field arena [$F_{(1,54)}$ = 6.16 p = 0.01]. The activity in the centre field of WT [$F_{(1,58)}$ = 1.73, p = 0.19] and $CB_2$ knock-out mice [$F_{(1,58)}$ = 3.05, p = 0.08] was not significantly altered (Figure 41B).

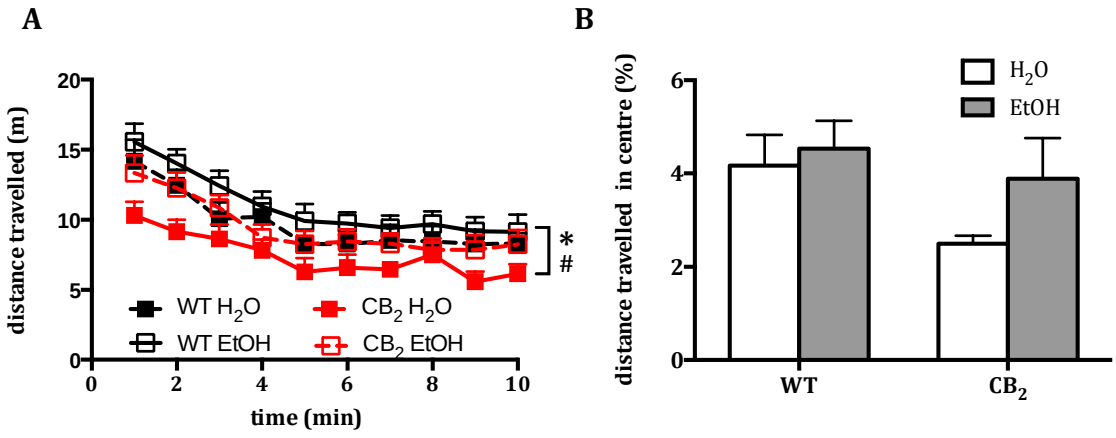



Figure 41. Activity in the open field of WT and $CB_2$ deficient animals after six months of alcohol treatment in the IFD model. (A) Treatment and genotype significantly influenced the overall distance travelled in the open field. (B) Distance travelled in percent in the center of the open field (n = 14 - 17 per group). The test was performed during an alcohol withdrawal phase. Data were analysed by three-way ANOVA and represented as mean value ± SEM. * indicates treatment effect, # indicates genotype effect. *p > 0.05.

Next, working memory performance was investigated in the Y-maze test. Working memory errors did not significantly increase in alcohol-treated WT animals [$F_{(1,58)}$ = 1.16, p = 0.28] (Figure 42). Lack of $CB_2$ receptors led to a higher level of returns compared to water-treated WT animals. Alcohol treatment did not further affect the working memory performance in $CB_2$ deficient mice. The percentage of correct alternations was similar among the different groups [$F_{(1,58)}$ = 0.09, p = 0.75].

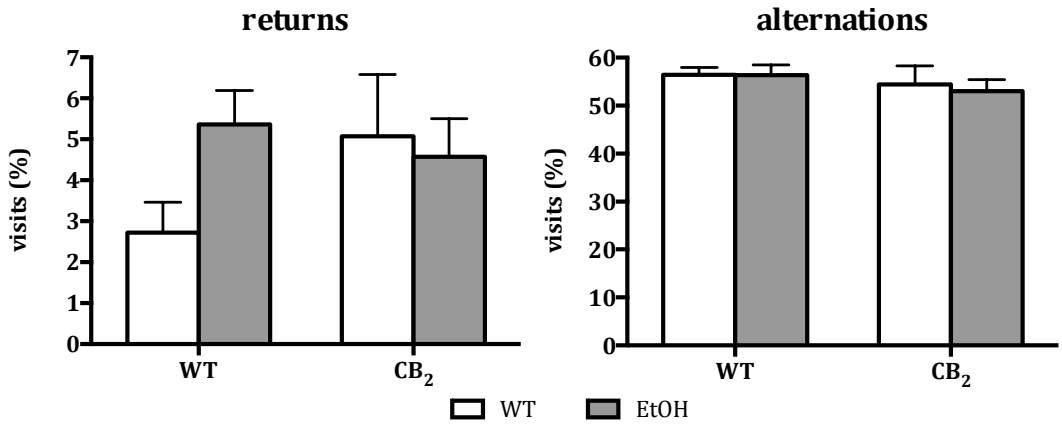



Figure 42. Working memory performance in the Y-maze test of WT and $CB_2$ deficient animals after six months of alcohol treatment in the IFD model. Different parameters were analysed including percentages of alternations and returns. The treatment did not affect the working memory performance (n = 8 per group). The test was performed during an alcohol withdrawal phase. Data were analysed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM.

Withdrawal-induced anxiety was investigated in the O-maze test (Figure 43). Both, distance [$F_{(1,59)}$ = 0.08, p = 0.77] and time [$F_{(1,59)}$ = 0.13, p = 0.71] spent in the open arms were not changed by the treatment or by the genotype.

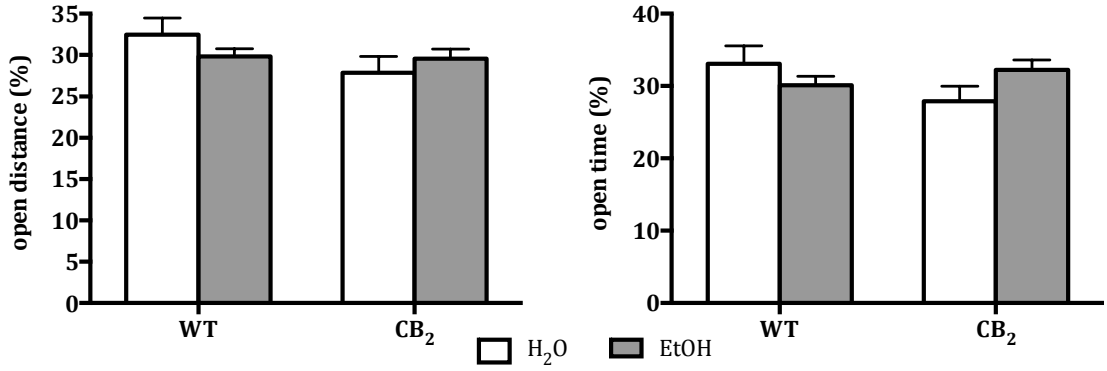



Figure 43. Anxiety-related behaviour in the O-maze test of WT and $CB_2$ deficient animals after six months of alcohol treatment in the IFD model. The distance and the time spent in the open parts of the O-maze were not changed by alcohol treatment or by the genotype (n = 12 - 19 per group). The test was performed at 40 lux. Alcohol-treated animals were analysed during a withdrawal phase after six months of alcohol treatment. Data were analysed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM.

## 4.4 Effects of the $CB_2$ receptor on alcohol-related behaviour

There is increasing evidence that the $CB_2$ receptor modulates rewarding effects of alcohol (Al Mansouri et al., 2014; Ishiguro et al., 2007; Ortega-Álvaro et al., 2013). Here, we investigated alcohol consumption in chronic alcohol models with different housing conditions. Furthermore, we analysed ethanol preference, as well as stress-induced drinking, development of tolerance through hypothermia, alcohol clearance and handling-induced convulsions.

### 4.4.1 Alcohol consumption, body weight and food intake in chronic models

In the FD model, single-housed WT animals drank more than their group-housed conspecifics [$F_{(1,39)}$ = 20.41, p = 0.000] (Figure 44A). This effect was similar in $CB_2$ deficient mice [$F_{(1,36)}$ = 19.92, p = 0.000] (Figure 44B). Correspondingly, in the IFD model, single-housed WT animals drank more ethanol than group-housed mice [$F_{(1,29)}$ = 22.52, p = 0.000] (Figure 44C). Interestingly, in the IFD model group-housed $CB_2$ deficient animals showed the same consumption of alcohol as single-housed animals [$F_{(1,21)}$ = 0.01, p = 0.89] (Figure 44D). In single-housed animals there was no difference between the genotypes in the FD [$F_{(1,17)}$ = 0.00, p = 0.98] and the IFD [$F_{(1,19)}$ = 1.69, p =

0.20] model (Figure 44A and B). Similarly, group-housed wild type and $CB_2$ deficient mice drank similar amounts of alcohol in the FD model [$F_{(1,58)}$ = 0.55, p = 0.46]. Surprisingly, group-housed $CB_2$ knockout animals showed increased alcohol consumption in the IFD model compared to WT controls [$F_{(1,31)}$ = 21.60, p = 0.000] (Figure 44C and D).

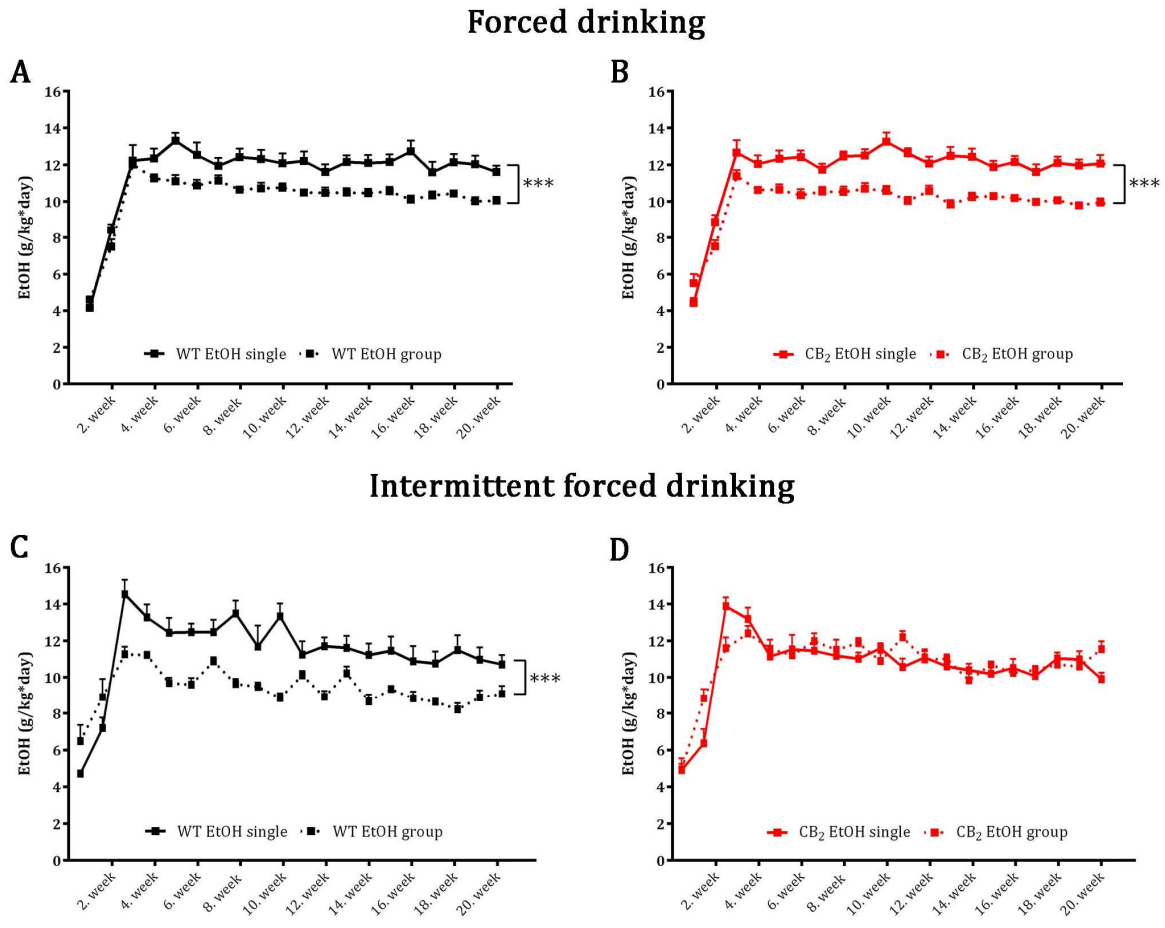



Figure 44. Alcohol consumption of single- and group-housed animals in the forced (A, B) and intermittent forced (C, D) drinking models. (A and B) In the forced drinking model single-housed WT and $CB_2$ mice drank more than their group-housed conspecifics. (C) In the intermittent forced drinking single-housed WT animals drank significantly more than group-housed WT mice. (D) Group-housed $CB_2$ animals drank the same amount of alcohol as single-housed mice in the intermittent forced drinking model. (WT FD single n = 12, group n = 30; WT IFD single n = 12, group n = 19; $CB_2$ FD single n = 10, group n= 31; $CB_2$ IFD single n = 9, group n= 14). Data were analysed by repeated measures one-way ANOVA (main factor: housing condition, within effect: time) and represented as mean value ± SEM. ***p < 0.001.

The body weight was increased in the FD model in group-housed animals compared to single-housed mice in both genotypes [WT: $F_{(1,38)} = 4.40$, $p = 0.04$; $CB_2$: $F_{(1,41)} = 4.49$, $p = 0.04$] (Figure 45A and B). The housing conditions did not significantly alter the body weight in the IFD model in either of the genotypes [WT: $F_{(1,29)} = 0.15$, $p = 0.69$; $CB_2$: $F_{(1,21)} = 0.98$, $p = 0.33$] (Figure 45C and D). In water control WT animals housing conditions did not affect the body weight gain [$F_{(1,68)} = 0.72$, $p = 0.40$] (Figure 45E). Interestingly, group-housed $CB_2$ deficient animals showed increased body weight compared to single-housed controls [$F_{(1,62)} = 11.14$, $p = 0.001$] (Figure 45F). In single-housed animals deletion of $CB_2$ did not affect the body weight [FD: $F_{(1,21)} = 2.28$, $p = 0.14$; IFD: $F_{(1,19)} = 0.00$, $p = 0.99$; $H_2O$: $F_{(1,37)} = 0.09$, $p = 0.75$]. However, peer-housing led to increased body weight in $CB_2$ deficient animals in the FD model [$F_{(1,58)} = 5.69$, $p = 0.02$] and in water controls [$F_{(1,93)} = 10.31$, $p = 0.001$], but not in the IFD model [$F_{(1,31)} = 0.62$, $p = 0.43$] compared to WT littermates.

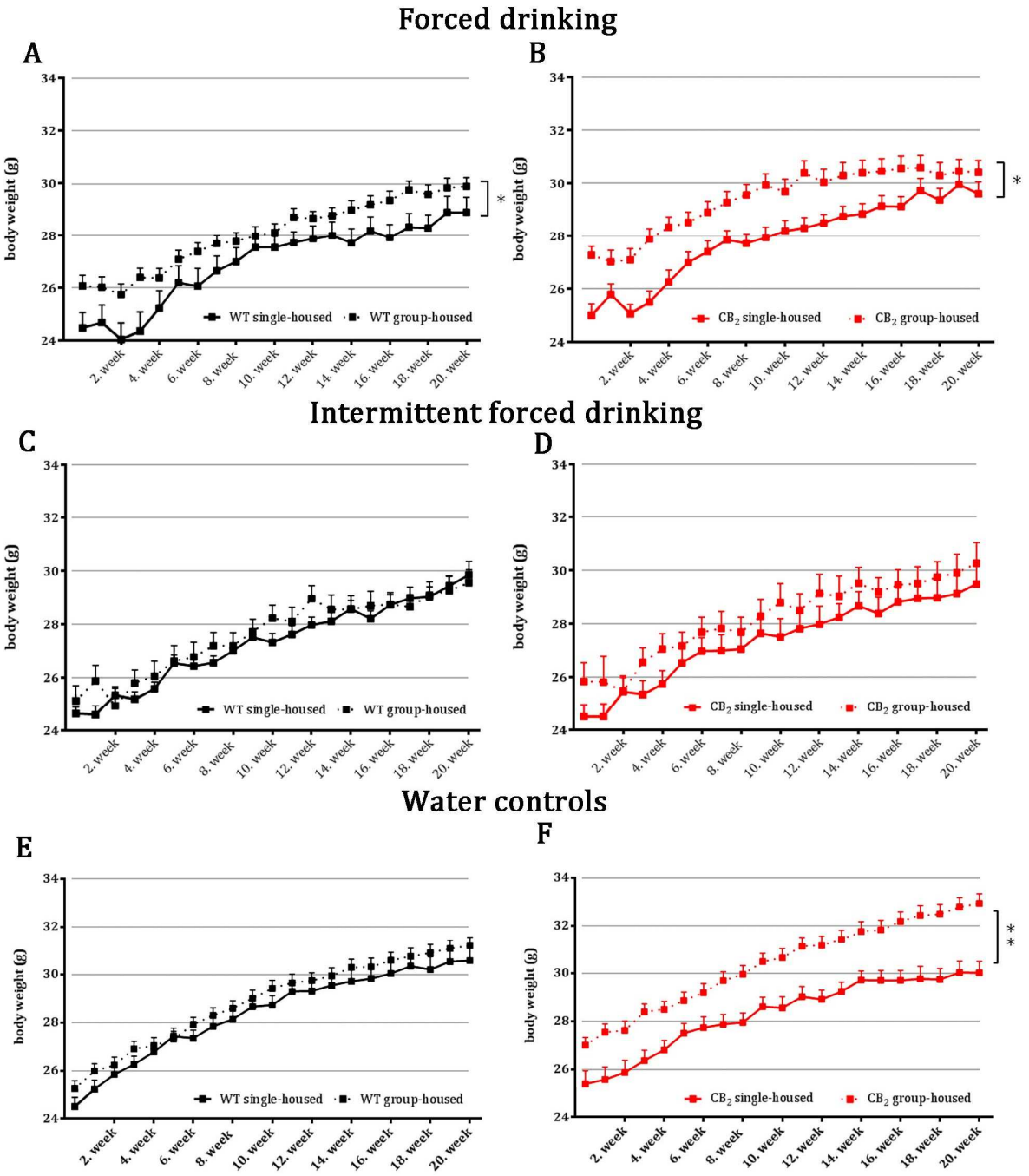



Figure 45. Body weight of single- and group-housed animals in the forced (A, B), intermittent forced (C, D) drinking models and in water control mice (E, F). (A and B) Group-housed WT and $CB_2$ mice showed increased body weight in the forced drinking model. (C and D) In the intermittent forced drinking model both WT and $CB_2$ animals showed the same body weight independent of the housing conditions. (E) Similarly, the housing conditions did not alter the body weight in water control WT animals. (F) Interestingly, water-treated, group-housed $CB_2$ animals showed a significant increase in body weight compared to single-housed animals. (n: WT FD single = 12, group = 30; WT IFD single = 12, group = 19; WT $H_2O$ single = 22, group = 48; $CB_2$ FD single = 10, group = 31; $CB_2$ IFD single = 9, group = 14; $CB_2$ $H_2O$ single = 18, group = 47).

Data were analysed by repeated measures one-way ANOVA (main factor: housing condition, within effect: time) and represented as mean value ± SEM. *p < 0.05, **p < 0.01.

As food consumption of the animals was constant during the entire experiments, we calculated mean values for each group (Table 4). $CB_2$ deficient mice ate generally more compared to WT animals [$F_{(1,262)} = 7.97$, $p = 0.005$]. We also found significant differences in food intake comparing the models [$F_{(2,262)} = 33.25$, $p < 0.0001$] and the housing conditions [$F_{(1,262)} = 13.08$, $p = 0.003$]. In the FD model WT mice showed a significantly elevated food consumption when they were single housed [$F_{(1,39)} = 4.08$, $p = 0.05$] (Table 4). This effect was enhanced in the IFD model [$F_{(1,29)} = 10.11$, $p = 0.003$] (Table 4). Group-housed $CB_2$ deficient animals showed a similar food consumption as single-housed mice in both drinking paradigms [FD: $F_{(1,41)} = 2.44$, $p = 0.12$; IFD: $F_{(1,21)} = 3.09$, $p = 0.09$] (Table 4). In water control animals of both genotypes we could not detect any differences in food intake between group- and single-housed animals [WT: $F_{(1,69)} = 2.24$, $p = 0.13$; $CB_2$: $F_{(1,63)} = 1.36$, $p = 0.24$] (Table 4). The FD paradigm led to strongly decreased food consumption compared to water control mice [WT single: $F_{(1,32)} = 10.28$, $p = 0.003$; WT group: $F_{(1,76)} = 38.16$, $p = 0.000$; $CB_2$ single: $F_{(1,27)} = 19.90$, $p = 0.000$; $CB_2$ group: $F_{(1,77)} = 17.59$, $p = 0.000$] (Table 4). This effect was similar in a comparison between FD and IFD models [WT single: $F_{(1,21)} = 43.61$, $p < 0.001$; WT group: $F_{(1,47)} = 6.30$, $p = 0.015$; $CB_2$ single: $F_{(1,19)} = 38.85$, $p < 0.001$; $CB_2$ group: $F_{(1,43)} = 20.31$, $p < 0.001$]. In the IFD model only group-housed WT animals showed decreased food intake compared to water controls [$F_{(1,65)} = 4.84$, $p = 0.03$].

| | WT | | | | $CB_2$ | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | single | | group | | single | | group | |
| FD | $3.35^{*,ww}$ | ± 0.09 | $3.19^{www}$ | ± 0.05 | $3.42^{www}$ | ± 0.08 | $3.30^{www}$ | ± 0.05 |
| IFD | $3.81^{**,mmm}$ | ± 0.09 | $3.41^{w,m}$ | ± 0.10 | $3.89^{mmm}$ | ± 0.08 | $3.68^{g,mmm}$ | ± 0.11 |
| H2O | 3.78 | ± 0.19 | 3.67 | ± 0.12 | 4.04 | ± 0.19 | 3.85 | ± 0.17 |

Table 4. The mean food consumption of alcohol-treated single- and group-housed animals in the FD and IFD models and water control mice. *Housing*: The food consumption was moderately, but significantly increased in single-housed WT animals in the FD model compared to group-housed mice *(*)*. This effect was more pronounced in the IFD model *(**)*. Single housing did not affect the food intake in water-treated WT animals. Single-housed $CB_2$ knockout mice ate similar amounts of food in all models compared to group-housed animals. *Model*: Alcohol treatment in

the FD model resulted in reduced food consumption in both genotypes and housing conditions compared to the IFD model *(mmm)* and to water controls *(www)*. Furthermore, group-housed WT animals in the IFD model ate significantly less compared to water controls (w) and to $CB_2$ knockout animals (g) in the same housing condition and model. n: WT FD single = 12, group = 30; WT IFD single = 12, group = 19; WT $H_2O$ single = 22, group = 48; $CB_2$ FD single = 10, group = 31; $CB_2$ IFD single = 9, group = 14; $CB_2$ $H_2O$ single = 18, group = 47. Data were analysed by one-way ANOVA (main factor: housing condition) and represented as mean value ± SEM. */w/m/g$p < 0.05$; **/ww/mm$p < 0.01$; www/mmm$p < 0.001$.

For a comprehensive statistical analysis alcohol consumption and body weight gain we used a repeated measures three-way ANOVA. The main factors were the following: genotype, alcohol model and housing conditions. The ethanol intake was significantly affected by the model [$F_{(1,131)} = 3.921$, $p = 0.049$] and the housing conditions [$F_{(1,131)} = 30.97$, $p = 0.000$], but not by the genotype [$F_{(1,131)} = 0.13$, $p = 0.71$] (Table 5). However, WT and knockout animals showed a significantly altered alcohol consumption, which was dependent on the housing conditions [$F_{(1,131)} = 3.842$, $p = 0.05$] and on the drinking paradigm together with the social environment [$F_{(1,131)} = 11.149$, $p = 0.001$] (Table 5). The body weight was influenced by all main factors examined [G: $F_{(1,260)} = 5.75$, $p = 0.01$; M: $F_{(1,260)} = 9.85$, $p < 0.001$; H: $F_{(1,260)} = 14.07$ $p < 0.001$] (Table 5). Moreover, the changes in body weight were not affected by interactions between genotypes, housing conditions and models.

| | | EtOH | | Body weight | |
|---|---|---|---|---|---|
| | | p-value | observed power | p-value | observed power |
| Main factors | Genotype (G) | **0.71** | **0.065** | **0.017** | **0.665** |
| | Model (M) | **0.049** | **0.052** | **0.000** | **0.982** |
| | Housing (H) | **0.000** | **0.999** | **0.000** | **0.962** |
| Interactions | G x H | **0.05** | **0.494** | **0.128** | **0.330** |
| | G x M | **0.22** | **0.226** | **0.611** | **0.130** |
| | M x H | **0.25** | **0.205** | **0.515** | **0.161** |
| | G x M x H | **0.001** | **0.912** | **0.476** | **0.175** |

Table 5. Statistical analysis of ethanol consumption and body weight in relation to genotype, alcohol drinking model and housing conditions. The ethanol consumption was significantly affected by two main factors: alcohol drinking model and housing conditions. Furthermore, we observed significant interactions for gene x housing and gene x model x housing. The body

weight was significantly affected by all main factors analyzed and did not show significant interactions. Data were analyzed by repeated measures three-way ANOVA (main factors: genotype, model and housing condition, within effect: time) and represented as p-value and observed power (alpha = 0.05).

The blood alcohol levels were not significantly different between the genotypes [FD single: $F_{(1,16)} = 0.09$, $p = 0.76$; FD group: $F_{(1,34)} = 0.39$, $p = 0.53$; IFD single: $F_{(1,9)} = 0.98$, $p = 0.34$; IFD group: $F_{(1,34)} = 0.39$, $p = 0.53$]. However, as blood was not sampled at the same point of time it is not possible to compare the BALs of the different alcohol models.

| | WT | | $CB_2$ | |
|---|---|---|---|---|
| FD single | 1.34 | ± 0.45 | 1.53 | ± 0.33 |
| FD group | 0.82 | ± 0.15 | 0.71 | ± 0.15 |
| IFD single | 0.42 | ± 0.06 | 0.60 | ± 0.21 |
| IFD group | 0.71 | ± 0.12 | 0.38 | ± 0.07 |

Table 6. Blood alcohol level after chronic alcohol access in the different alcohol models. Blood alcohol level (g / l) was not significantly altered by the genotype (n: WT FD single = 10, group = 18; WT IFD single = 7, group = 13; $CB_2$ FD single = 8, group = 18; $CB_2$ IFD single = 4, group = 9). Data were analyzed by one-way ANOVA and represented as mean value ± SEM.

### 4.4.2 Alcohol preference

In the two-bottle choice test WT animals showed a constant preference for alcohol of about 60 % [$F_{(1,24)} = 2.64$, $p = 0.11$] (Figure 46A), whereas $CB_2$ deficient animals significantly decreased their preference over time from 60 % to 36 % [$F_{(1,120)} = 2.95$, $p = 0.01$]. As the preference values of the animals were not normally distributed, we performed a nonparametric analysis of the first (Figure 46C) and the last two weeks (Figure 46D). Here, $CB_2$ knockout animals showed a significant decrease in alcohol preference after twelve weeks compared to the WT controls [$p < 0.05$]. The alcohol consumption was similar in both strains, although $CB_2$ deficient animals showed a stronger decrease in alcohol consumption than the WT controls [$F_{(1,24)} = 1.72$, $p = 0.20$] (Figure 46B).

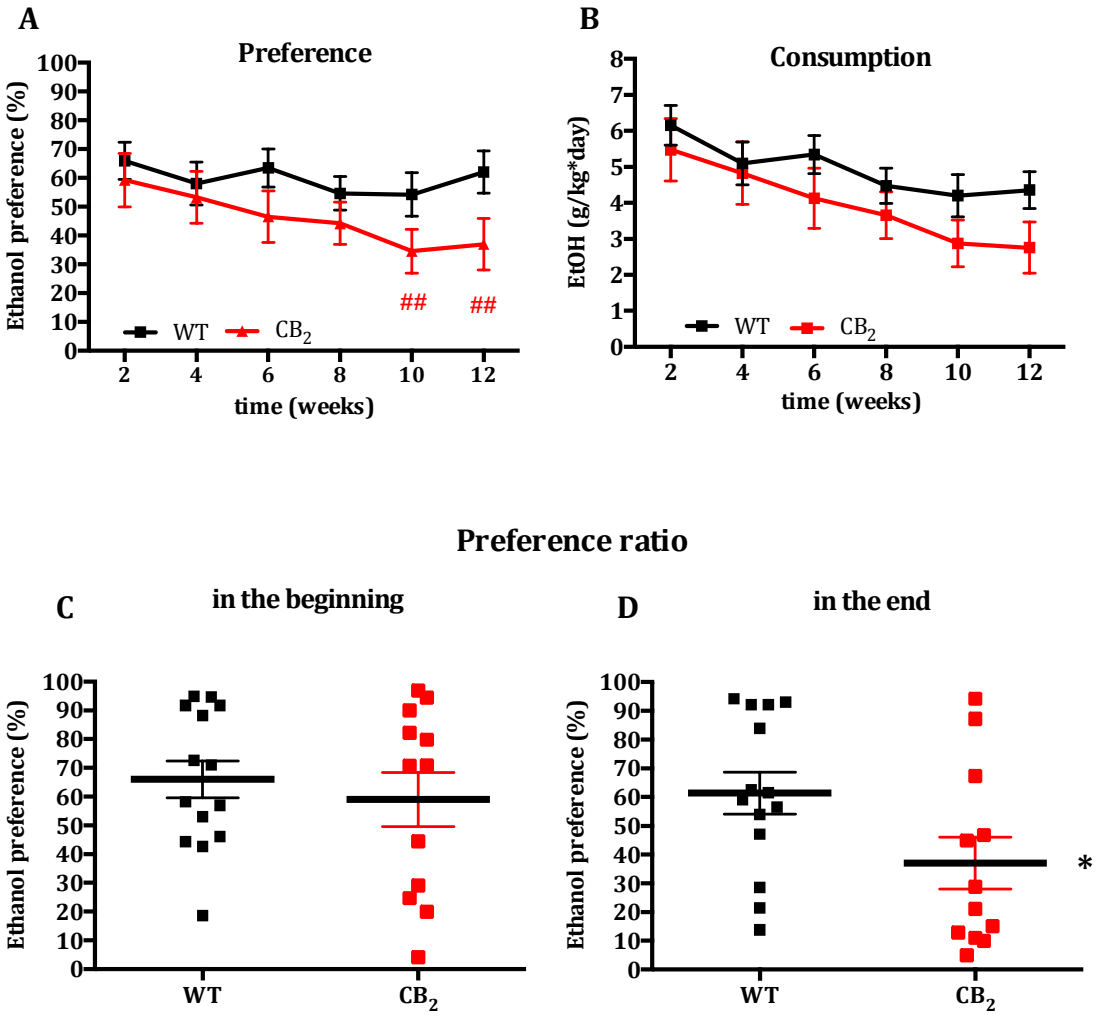



Figure 46. Alcohol drinking behaviour in the two-bottle choice test of WT and $CB_2$ deficient animals. (A) The alcohol preference in WT animals remained constant, whereas $CB_2$ deficient animals showed reduced alcohol preference in the end compared to the beginning. (B) Alcohol consumption was similar in WT and $CB_2$ deficient animals. (C) The alcohol preference in the first two weeks. (D) The alcohol preference in the last two weeks. Only at the end of the test did $CB_2$ deficient animals showe a significantly decreased preference (WT n = 14; $CB_2$ n = 12). (A and B) Data were analyzed by repeated measures one-way ANOVA (main factor: genotype, within effect: time) and represented as mean value ± SEM. (C and D) Data were analyzed by the nonparametric Kolmogorov-Smirnov test and represented as mean value ± SEM. *p < 0.05; ##p < 0.01. * indicates treatment effect; # indicates time effect in $CB_2$ deficient animals.

### 4.4.3 Stress-induced drinking

As the $CB_2$ receptor has been shown to modulate stress responses (García-Gutiérrez et al., 2010; Ishiguro et al., 2007), we investigated the alcohol preference and blood corticosterone levels after application of mild foot shocks in WT and $CB_2$ deficient mice at the end of the two-bottle choice test. The stress resulted only in a moderate increase in alcohol preference in WT animals, which was not significant (Figure 47A). However, the alcohol preference of $CB_2$ knockout mice did not increase at all after the stress and was significantly lower compared to WT animals [$F_{(1,18)}$ = 18.90, p = 0.000]. The foot shock induced a startle response (Figure 47B) and a robust humoral stress response, which was similar in both of the genotypes [$F_{(1,17)}$ = 116.68, p = 0.000] (Figure 47C). However, there was a significant stress x genotype interaction for the expression levels of corticosterone [$F_{(1,17)}$ = 12.22, p = 0.002].

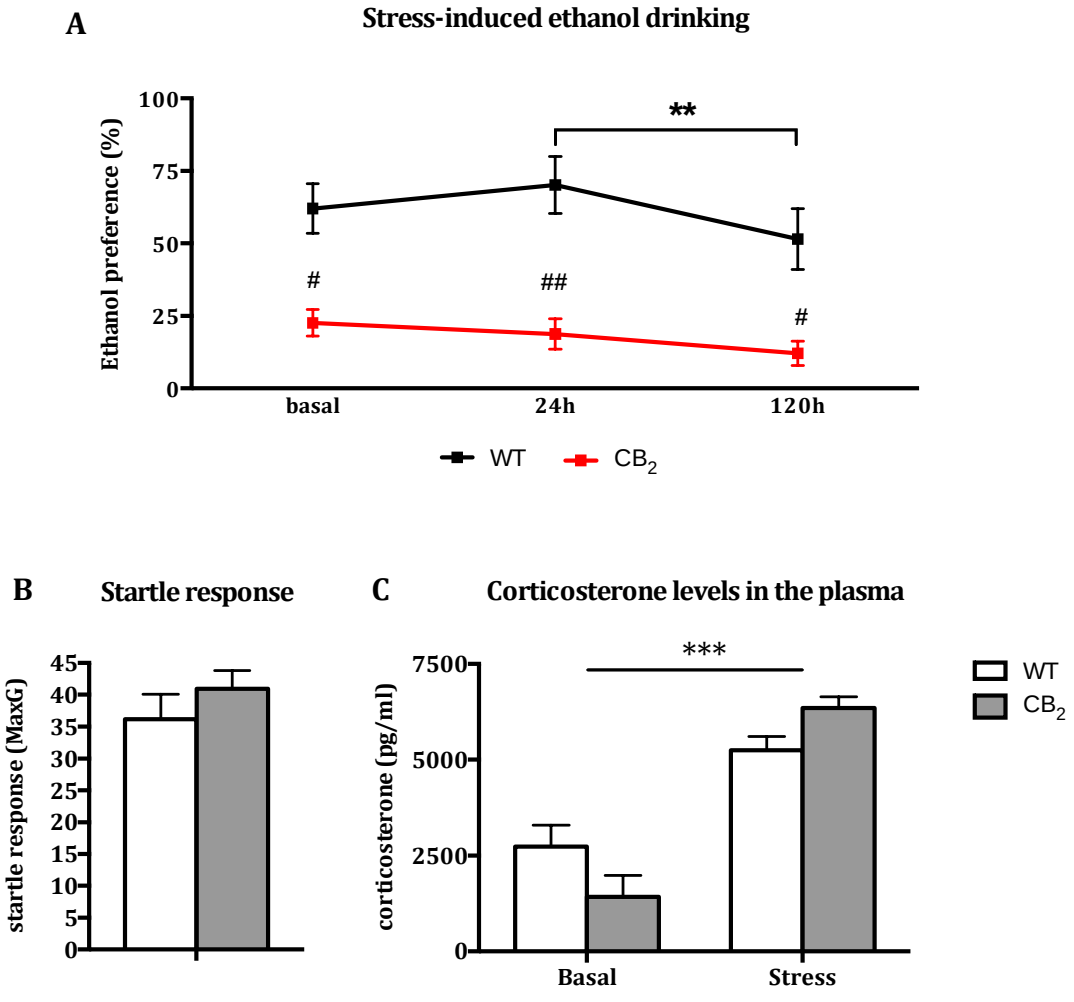


Figure 47. Stress-induced drinking of WT and $CB_2$ deficient animals. (A) Alcohol preference after the foot shock did not significantly increase in WT animals. $CB_2$ deficient animals showed lower alcohol preference compared to WT (WT n = 11; $CB_2$ n = 9). (B) The startle response to the foot shock was similar in both genotypes. (C) Plasma corticosterone levels showed a strong increase after the foot shock (n = 9 - 10). Data were analyzed by repeated measures one-way ANOVA (main factor: genotype, within effect: stress) and represented as mean value ± SEM. # indicates genotype effect, * indicates stress effect. #p < 0.05, ##/**p < 0.01, ###/***p < 0.001.

### 4.4.4 Development of tolerance

The development of tolerance was assessed by measuring hypothermia after acute injection of alcohol before and three weeks after chronic alcohol consumption. Acute injection of 2 g/kg alcohol in ethanol naïve WT animals decreased the body temperature by 2.6 °C (Figure 48A). This effect was less pronounced in $CB_2$ deficient animals, as the body temperature only decreased by 1.3 °C [$F_{(1,19)}$ = 9.37, p = 0.006]. Injection of 3.5 g/kg ethanol strongly decreased the body temperature in both genotypes by 2.8 °C. After chronic alcohol treatment, WT animals developed tolerance to alcohol as acute alcohol injection of 2 and 3.5 g/kg only decreased the body temperature by 1 °C (Figure 48B). $CB_2$ knockout animals showed tolerance to alcohol only after injection of 3.5 g/kg.

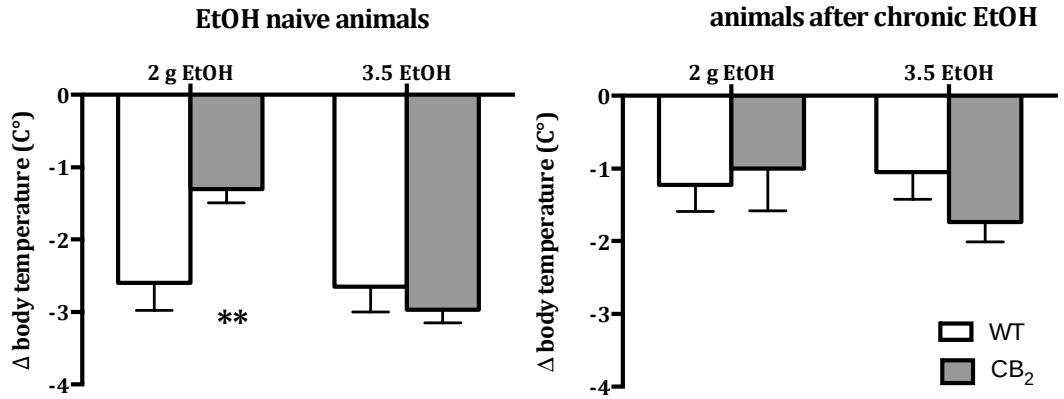



Figure 48. Alcohol-induced hypothermia and tolerance of WT and $CB_2$ deficient animals. (A) After injection of 2 g/kg EtOH $CB_2$ deficient animals showed less hypothermia than WT controls. This effect is saturated after injection of 3.5 g/kg EtOH. (B) After three weeks of chronic, continuous alcohol access both strains developed similarly tolerance to acute alcohol injections (n = 5 – 6 per group). Data were analyzed by two-way ANOVA (main factors: genotype and injection) and represented as mean value ± SEM. **p < 0.01.

During the measurement of body temperature, we observed a difference in this parameter in alcohol naïve WT and $CB_2$ animals. Therefore, we measured the body temperature in alcohol naive and chronic alcohol-treated animals (Figure 49). Alcohol naïve $CB_2$ knock-out animals showed a reduced body temperature compared to WT controls [$F_{(1,19)} = 4.5$, $p = 0.04$]. Deletion of the $CB_2$ receptor resulted in slightly reduced body temperature in alcohol naïve animals. Those animals did not show a change in their body temperature after chronic alcohol access, presenting increased body temperature compared to chronic alcohol treated WT mice [$F_{(1,19)} = 35.3$, $p = 0.000$].

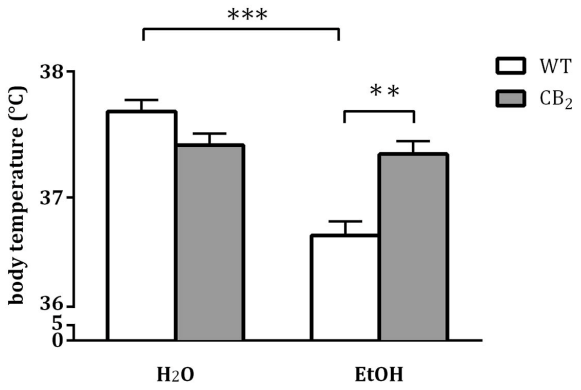



Figure 49. Effect of chronic alcohol treatment on the body temperature of WT and $CB_2$ deficient animals. The body temperature of WT animals was strongly decreased by chronic alcohol treatment, whereas $CB_2$ deficient animals did not show a decrease in body temperature ($H_2O$ n = 3; EtOH n = 8 – 9). Data were analyzed by two-way ANOVA (main factors: treatment and genotype) and represented as mean value ± SEM. **p < 0.01, ***p < 0.001.

### 4.4.5 Alcohol clearance

In the chronic alcohol models, we observed moderately altered blood alcohol levels in $CB_2$ deficient animals compared to WT controls. Therefore, we measured the rate of alcohol metabolism in alcohol naïve and chronically alcohol-treated animals (Figure 50). Application of 2 g/kg of ethanol yielded blood ethanol concentrations around 4 g/l. After three hours blood alcohol was reduced to 1 g/l (Fig. 5A). This effect was independent of the genotype [$F_{(1,37)} = 0.04$, $p = 0.82$]. The alcohol clearance was similar in chronic alcohol treated animals [$F_{(1,30)} = 0.05$, $p = 0.80$] (Fig. 5 B). Thus, the rate of alcohol metabolism was not affected by the genotype and chronic alcohol treatment.

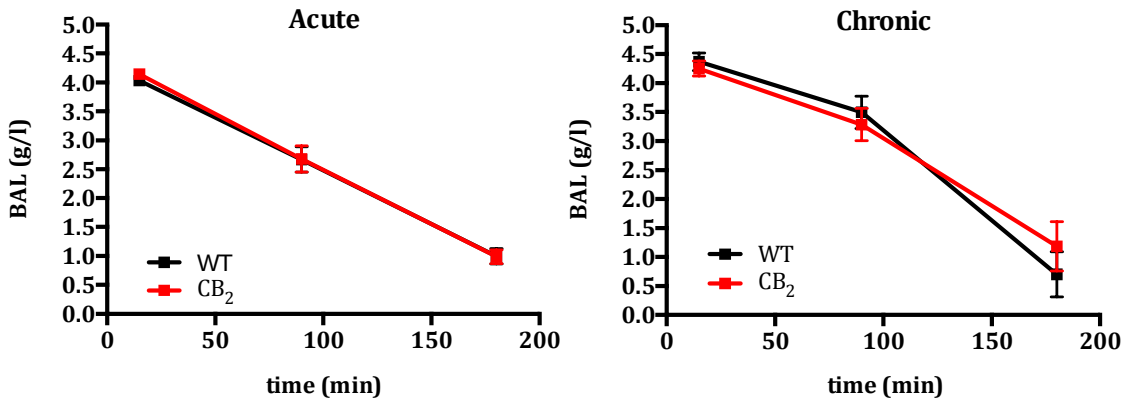



Figure 50. Alcohol clearance after acute and chronic alcohol administration of WT and $CB_2$ deficient animals. (A) Injection of 2 g/kg EtOH resulted in a similar alcohol clearance in WT and $CB_2$ animals. (B) After chronic alcohol treatment both strains metabolized alcohol in a similar way (n = 9 per group). Data were analyzed by two-way ANOVA (main factors: genotype and time point) and represented as mean value ± SEM.

### 4.4.6 Handling-induced convulsions

The severity of withdrawal seizures was assessed by scoring handling-induced convulsions. The animals were scored while they had free access to alcohol. They were scored again three hours after withdrawal was initiated. We could detect a strong increase in convulsions independent of the genotype [WT: $p < 0.001$; $CB_2$: $p < 0.01$] (Figure 51A and B).

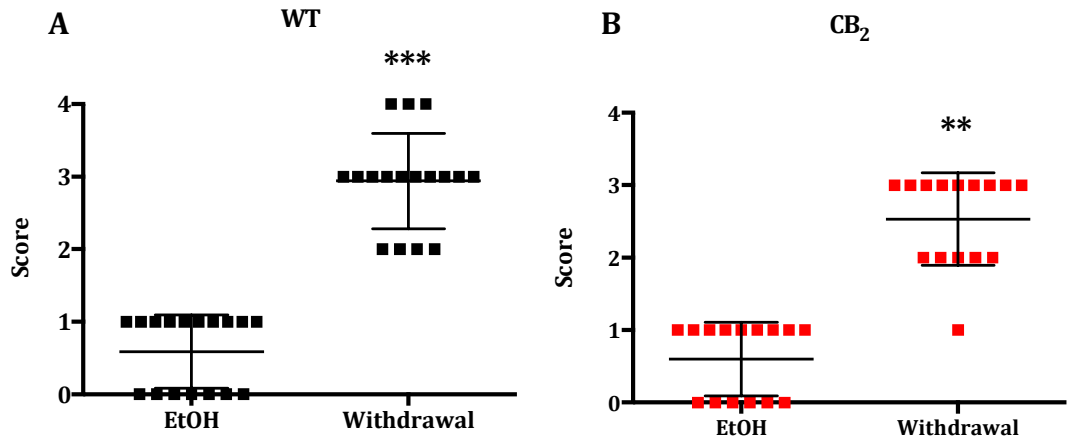



Figure 51. Scoring of handling-induced convulsions after alcohol withdrawal of WT and $CB_2$ deficient animals. (A) WT animals showed increased convulsions during the withdrawal. (B) $CB_2$

deficient mice also show increased handling-induced convulsions during the alcohol withdrawal. Data were analyzed by the nonparametric Kolmogorov-Smirnov test and represented as mean value ± SEM. **$p < 0.01$; ***$p < 0.001$.

# 5 Discussion

The results will be discussed in two parts: the first part is about alcohol-induced neuroinflammation, its modulation by the $CB_2$ and its effects on cognition. The second part focusses on the role of the $CB_2$ receptor in alcohol addiction and includes alcohol preference, modulation of alcohol drinking by the social environment and systemic effects of alcohol.

## 5.1 Alcohol-induced neuroinflammation

Recently it has been discovered that neuroinflammation develops after chronic alcohol consumption (He & Crews, 2008), which might lead to the cognitive decline (Sullivan & Pfefferbaum, 2005). This process is known to be mediated via the innate immune system; specifically the TLR4 has been shown to play a crucial role in alcohol-induced neuroinflammation (Alfonso-Loeches et al., 2010). As the $CB_2$ receptor is known to modulate the TLR4 pathway the hypothesis was tested whether deletion of the $CB_2$ would lead to decreased neuroinflammation and improve cognitive performance after chronic alcohol consumption in mice. Therefore, in the following part I discuss the potency of different alcohol drinking models to induce neuroinflammation and characterise the microglial activation state after long-term alcohol drinking. After that, I discuss the modulatory role of the $CB_2$ in alcohol-induced neuroinflammation and outline the effects of $CB_2$ deletion on cognitive performance after chronic alcohol drinking. Finally, I will evaluate the effect of alcohol in the long-term models on systemic liver inflammation.

### 5.1.1 Potency of different alcohol models to induce neuroinflammation

The models investigated in this study induced neuroinflammation only after prolonged alcohol consumption. Animals that underwent the forced or intermittent forced drinking protocol for two months did not show increased cytokine expression in the frontal cortex. However, extending the protocols to six months of alcohol consumption led to an enhanced cytokine expression in this region, which is in accordance with published reports where similar alcohol models were applied (Alfonso-Loeches et al., 2010). After six months of forced alcohol consumption WT animals showed similar pro-inflammatory responses in cytokine expression in the cortex,

independent of housing conditions. IL-1β was always significantly induced; TNF-α showed an enhanced increase in group-housed animals and IL-10 was stronger induced in single-housed mice. This expression profile is consistent with published reports (Alfonso-Loeches et al., 2010; Fernandez-Lizarbe et al., 2009). The expression of IL-6 was not altered by alcohol treatment, which is in contrast to the literature (Alfonso-Loeches et al., 2010). However, in the published study IL-6 was quantified at mRNA level, whereas in the present study protein levels were investigated. Therefore the difference is probably due to post-transcriptional regulation of IL-6 expression. Furthermore, the expression of chemokine CCL-2 was not changed after long-term alcohol drinking, although CCL-2 was shown to be increased in post-mortem brain samples of alcoholics and after binge alcohol exposure in C57BL/6J mice (Crews et al., 2011; He & Crews, 2008). The discrepancy about CCL-2 expression in the different studies might be related to the use of different models and species. Surprisingly, six-month-long alcohol consumption did not lead to an enhanced activation of microglia, which is contrary to published reports (Alfonso-Loeches et al., 2010; Pascual, Baliño, et al., 2011). This effect might be attributable to the use of different microglial markers. In these studies CD11b was used to characterise microglia, whereas Iba1 was used in this study. However, only a few reports showed Iba1 in alcohol-related studies: one showed mildly increased Iba1 expression in the hippocampus after a binge-drinking model in rodents (Marshall et al., 2013), whereas another showed Iba1 expression in post mortem tissue of alcoholics (He & Crews, 2008). In the present work Iba1-IR was shown to be increased not before twelve months of alcohol drinking, which may parallel the finding in human post mortem tissue (He & Crews, 2008). Therefore it is possible that expression of CD11b precedes the induction of Iba1 in microglia after chronic alcohol treatment, and Iba1 may thus be considered a more conservative marker for microglial activation in this model.

Evaluation of the effect of frequent alcohol withdrawals suggests that the induction of cytokine expression in WT animals is positively correlated with the amount of ingested alcohol. This is indicated by the enhanced inflammatory response in the FD models compared to the IFD models. This suggests that regular withdrawal did not enhance the expression of cytokines. Thus, repeated withdrawal of alcohol does not seem to promote a pro-inflammatory phenotype of microglia. However, alcohol

withdrawal has been shown to increase activation of astrocytes (revealed by enhanced GFAP-IR) as a consequence of increased glutamate signalling during periods of abstinence (Miguel-Hidalgo, 2006). Consistent with the literature GFAP expression was increased in the cortex after six months of continuous alcohol drinking. However, this effect was restricted to the cingulate cortex and could not be observed in the ectorhinal or piriform cortex.

### 5.1.2 Chronic alcohol leads to pro-inflammatory phenotype of microglia

Chronic alcohol intake increased expression of pro-inflammatory cytokines and enhanced expression of the microglial marker Iba1 after twelve months of forced drinking. These findings suggest that microglia shift towards a pro-inflammatory activation state although current literature lacks *in vivo* evidence for such a phenotypic switch. Therefore this is the first study showing co-localisation of IL-1β and Iba1 in the cortex after chronic alcohol consumption. Twelve month alcohol consumption increased the total number of pro-inflammatory microglia. As pro-inflammatory microglial cells are known to exert neurotoxic effects (Block et al., 2007), the neuronal density was investigated using NeuN-IR. Neither alcohol nor age affected the density of neurons in the cortex. This finding does not exclude minor neural degeneration after chronic alcohol consumption, as there are reports showing increased degeneration and apoptosis following alcohol treatment (Crews & Nixon, 2009; Sullivan & Zahr, 2008). Importantly, the majority of apoptotic cells seems to be glial and not neural cells (Sullivan & Zahr, 2008). Furthermore, imaging studies clearly show that alcohol leads to severely decreased brain volumes (Sullivan & Pfefferbaum, 2005). However, this effect does not consistently correlate with the cognitive impairment of alcoholics, thereby suggesting that the reduced cognitive abilities of alcoholics are more likely due to a reduced connectivity of neurons (Zahr & Sullivan, 2009). Microglia could be involved in this process, as their function also comprises the maintenance of synaptic integrity (Graeber, 2010). Thus, the phenotypic shift of microglia may directly (via enhanced phagocytosis) or indirectly (via pro-inflammatory cytokines) lead to a reduced synaptic integrity. However, further studies are necessary to investigate this hypothesis in more detail.

### 5.1.3 Modulatory role of $CB_2$ in alcohol-induced neuroinflammation

The neuroinflammatory response activated by alcohol is mostly triggered via the TLR4 pathway. As the $CB_2$ is a well-known modulator of the TLR4 signalling pathway (Gertsch, 2008), the hypothesis was tested whether $CB_2$ deletion would dampen the alcohol-induced neuroinflammation in the cortex. Having established a model that induces neuroinflammation after six months of chronic alcohol access, the modulatory role of $CB_2$ in this process was investigated in the FD and IFD models. Chronic alcohol treatment led to a mild increase of the *CNR2* expression in various brain regions, including the cortex, suggesting that the receptor is involved in long-term adaptations to alcohol, which possibly involves neuroinflammation. Indeed, alcohol-induced neuroinflammation appeared to develop differently in $CB_2$ deficient animals compared to their WT conspecifics. However, this effect is probably specific to housing conditions. Single-housed $CB_2$ deficient animals showed a blunted response in IL-1β and IL-10 expression levels after chronic alcohol consumption. Furthermore, deletion of $CB_2$ per se reduced IL-6 and IL-4 expression, but increased expression of CCL-2 and Iba1. On the other hand, group-housed animals showed an alcohol-induced increase in IL-1β and IL-10 expression levels. The expression of IL-6 and CCL-2 was similar to that of WT animals, but expression of TNF-α was increased by $CB_2$ deletion independent of the treatment. Together these data indicate that $CB_2$ deficiency by itself leads to a different activation state of microglia. Social isolation might further alter the immune response in these animals, as social stress is well accepted to modify immune functions (Salak-Johnson & McGlone 2007; Kelley & Dantzer 2011; Bartolomucci 2007). Activation of $CB_2$ is generally considered to be anti-inflammatory (Ashton & Glass, 2007) and lack of $CB_2$ signalling should therefore result in an exacerbated immune response. However, there are also pleiotropic effects reported for the receptor, which might depend on the inflammatory stimulus and ligands, as both $CB_2$ selective agonists and inverse agonists, inhibited activation of the TLR4 pathway (Gertsch, 2008). In conclusion, the $CB_2$ might play a pro-inflammatory role in alcohol-induced neuroinflammation possibly by modulating TLR4 signalling in microglia. However, this effect is probably specific to environmental conditions and needs further investigation.

### 5.1.4 Effects of chronic alcohol consumption on cognition

Chronic alcohol drinking has been associated with cognitive deficits in multiple rodent models, including impaired spatial and reversal learning (Cacace et al., 2012; Obernier et al., 2002) and object recognition (Pascual, Baliño, et al., 2011). In the present study, working, declarative and spatial memory performance was investigated after long-term alcohol drinking. However, clear alcohol effects could not be observed. Working memory performance, which is dependent on the prefrontal cortex activity (Jones, 2002), was assessed in the Y-maze test. Neither alcohol treatment nor genetic deletion of the $CB_2$ showed an effect in this test. Interestingly, animals that were single-housed for six months committed more working memory errors (Figure 33 8 – 10 %) compared to age-matched group-housed animals (Figure 42 3 – 5 %). This effect seemed to be elicited by social isolation; but this issue has been little addressed in the literature. It can only be speculated that social isolation influences animals' behaviour via changes in the dopaminergic system in the prefrontal cortex (Fitzgerald et al., 2013). DA signalling in the prefrontal cortex plays an important role in the cognitive process of working memory as reduced DR1 function leads to a decreased working memory performance (Sawaguchi & Goldman-Rakic, 1991). Therefore a reduced DA-activity induced by social isolation might parallel the reduced working memory performance in these animals.

The declarative memory performance was investigated in the object and social recognition tests following long-term alcohol treatment. Animals that were subjected to this test were single-housed for six months and alcohol treatment did not alter the cognitive performance. Importantly, the general memory performance of these animals was very low. Although they displayed an increased preference for the novel object this effect was only detectable after an interval of ten minutes. Analysis at later points in time (30 and 60 min) indicated that WT control animals did not recognise the previously encountered object (data not shown). Moreover, the interaction time was so short that the objects had to be odour-cued in order to increase the time spent with the objects. However, this is in line with published reports about decreased memory performance after single housing (Möller et al., 2013; Pereda-Pérez et al., 2013). Therefore these results do not permit a clear evaluation of alcohol effects as the cognitive performance is heavily restricted by social isolation. Interestingly, single-housed WT animals showed a

good spatial memory performance in the Morris-water maze and had escape latencies, which are consistent with the literature (Albayram et al., 2011). Mice that performed this task were of the same age, underwent the same treatment and were kept in the same housing conditions compared to those of the object recognition test. The difference between these tests is the environment: the object recognition test is performed in a non-aversive open field arena, whereas the Morris-water maze test is carried out in a water basin, which provides a highly aversive environment for the animals. This suggests that single housing did not affect memory performance per se but rather led to a strong deficit in motivation to perform the task. Surprisingly, alcohol treatment did not impair memory performance in the Morris-water maze test in WT mice. This is contrary to published results reporting an impaired memory acquisition and reversal learning in rats after a three-bottle choice regimen (Cacace et al., 2012). Furthermore, Obernier and co-workers reported a mild deficit in reversal learning after a four-day binge drinking procedure (Obernier et al., 2002). These discrepancies may be due to species and model differences, or more importantly, be related to the fact that mice used in the present study were analysed during alcohol access and not during withdrawal. Moreover, as neuroinflammation developed after six months of alcohol drinking, this finding also indicates that neuroinflammation per se does not affect spatial memory performance in the Morris-water maze.

### 5.1.5 Chronic alcohol-induced liver inflammation and modulation by $CB_2$

Twelve month forced alcohol drinking resulted in a robust pro-inflammatory response in the liver, which was indicated by strongly increased protein expression of TNF-α, IL-1β, IL-6 and CCL-2. The anti-inflammatory cytokines IL-4 and IL-10 were also strongly increased, possibly in order to counteract the pro-inflammatory environment. After six months, single-housed WT animals did not show altered cytokine expression. However, deletion of the $CB_2$ resulted in a robust pro-inflammatory response after six months of alcohol drinking, similar to that observed after twelve months in WT animals. This is consistent with previously published articles reporting an anti-inflammatory role of the $CB_2$ in the liver. Trebicka and co-workers found the most pronounced liver damage in $CB_2$ deficient single-housed female mice after eight months of alcohol treatment in the same model (Trebicka et al., 2011). Furthermore, Louvet et al. reported

a pro-inflammatory phenotypic shift of Kupfer cells in $CB_2$ deleted animals treated with liquid diet (Louvet et al., 2011).

Group housing resulted in a different hepatic cytokine expression profile in WT animals: in both models (FD and IFD) WT mice showed slightly increased expression of TNF-α and IL-1β. Furthermore, IL-6, IL-10 and GM-CSF were increased in WT animals with continuous alcohol access. These findings suggest that the onset of liver inflammation (revealed by cytokine expression) in this model appears to be earlier when animals are group-housed compared to single-housed animals, where hepatic inflammation starts after around eight months of alcohol drinking (see Trebicka, 2011). Chronic alcohol treatment leads to immunodeficiency and increases the risk of infectious diseases (Cook, 1998; Nelson & Kolls, 2002). Therefore, it is possible that long-term alcohol enhances the inflammatory response to pathogens from the faeces of littermates in peer-housed animals. Surprisingly, group-housed $CB_2$ deficient animals showed a blunted inflammatory response towards alcohol, which was indicated by unchanged cytokine expression. However, cytokine expression does not necessarily reflect liver fibrosis. Thus, fibrotic scoring of group-housed $CB_2$ deficient animals is required to state whether $CB_2$ also exerts an anti-inflammatory function in group-housed animals as described for single-housed $CB_2$ deficient mice (Trebicka et al., 2011).

## 5.2 Role of the $CB_2$ receptor in alcohol-related behaviour

The second part of the discussion focuses on the rewarding effects of alcohol in $CB_2$ deficient mice. Furthermore, interactions of the receptor with the environment, including the use of different alcohol models and housing conditions, will be highlighted. Finally, the effects of the $CB_2$ on withdrawal-induced anxiety and locomotion, the development of tolerance and alcohol clearance will be discussed.

### 5.2.1 Alcohol preference and stress-induced drinking

We detected a difference between the genotypes in the alcohol preference test. Single-housed $CB_2$ deficient animals showed a reduced preference for alcohol, which became significant over time. These data indicate that alcohol has no rewarding effect in $CB_2$ deficient animals under conditions that can be considered to be 'normal' or 'non-stressed'. In contrast to our finding, Ortega-Alvaro and colleagues found an increase in

alcohol preference in the two-bottle choice test using $CB_2$ knockout animals (Ortega-Álvaro et al., 2013). It is important to mention that these mice were on an outbred (CD1) background, which is known for its alcohol avoidance (Short et al., 2006). Mice used in our study were on a C57BL/6J inbred background that is known for its high alcohol preference (Short et al., 2006). Here, they showed a preference of around 60 %, which is consistent with earlier findings (Racz, Schürmann, et al., 2008; Yoneyama et al., 2008). Thus, the contradictory results between these two reports may be a consequence of background differences.

To investigate stress-induced alcohol drinking, the animals were stressed with mild electric foot shocks. The foot shock procedure did not result in an increase of either alcohol preference or intake, albeit the animals of both genotypes elicited a strong stress response as revealed by the startle response and the increase in corticosterone plasma levels. This diverges from recently published reports from our laboratory (Racz et al., 2003, 2012). However, this discrepancy is known in the literature as there are also reports showing no increase in alcohol preference after the foot-shock procedure (for review: Becker et al. 2011). In another study Ishiguro and co-workers found that chronic mild stress (CMS) increased the alcohol preference in one experiment, whereas they found no increase in another experiment (Ishiguro et al., 2007). These results thus illustrate the variability of stress-related alcohol consumption. Moreover, it can also be speculated that the gender of the experimenter plays an important role in this behaviour. Recently, it has been shown that olfactory male-related stimuli resulted in stress-induced analgesia in mice (Sorge et al., 2014). However, as WT animals did not react as expected we cannot draw conclusions from this experiment about the effect of the $CB_2$ receptor in this paradigm.

### 5.2.2 Effects of the $CB_2$ receptor on alcohol consumption

In this study, we investigated the effect of the $CB_2$ receptor on alcohol consumption in relation to the social environment. Group-housed WT animals showed reduced alcohol intake in the IFD model compared to single-housed controls, whereas this effect was absent in $CB_2$ knockout mice. The social environment plays an important role in alcohol drinking behaviour as group housing consequently reduced alcohol intake in WT animals, which is consistent with the literature (Lopez et al., 2011). However, if the

access to alcohol is limited, peer housing differently affected the ethanol consumption in WT and $CB_2$ knockout animals. Here, we used the IFD as a model for regular, but moderate social drinking with repeated phases of abstinence. Our data suggest that the $CB_2$ receptor plays an important role in the regulation of drinking behaviour, which integrates social environment together with withdrawal-induced stress. Interestingly, the involvement of the $CB_2$ receptor in gene x environment interactions has already been considered by Ishiguro et al. (2007). They reported that pharmacologic manipulation of $CB_2$ receptor activity modulated alcohol consumption only after chronic mild stress (Ishiguro et al., 2007). Additionally, they found a functional single nucleotide polymorphism (SNP) in the $CB_2$ receptor associated with alcoholism in a human study (Ishiguro et al., 2007). This SNP in the CNR2 gene locus, R63Q, leads to a missense mutation in the first intracellular domain, which results in a decreased cellular response to $CB_2$ receptor ligands (Ishiguro et al., 2007). Our results using $CB_2$ knockout animals are in line with this. Furthermore, a recent study investigated a novel natural $CB_2$ agonist, beta-caryophyllene (BCP), in alcohol-related behaviours (Al Mansouri et al., 2014). BCP-treated animals showed constantly low alcohol preference, whereas vehicle-treated mice increased preference. However, in this experiment animals were daily i.p. injected with BCP or vehicle. These daily injections can be considered as a constantly repeated stress factor, which is known to enhance ethanol preference (Little et al., 1999). Thus, the effect of BCP can be related to its stress relieving action as latest results revealed an anxiolytic- and anti-depressant-like effect of this compound (Bahi et al., 2014). In line with this, mice overexpressing the $CB_2$ receptor showed a reduced hormonal and behavioural stress reactivity (García-Gutiérrez & Manzanares, 2011). Altogether, these findings suggest that $CB_2$ receptors play an important role in stress-coping that is associated with alcohol-related behaviours.

### 5.2.3 Effects of the $CB_2$ receptor on body weight and food consumption

In our study we detected increased body weight gain in group-housed WT animals with continuous alcohol access. This finding is supported by the literature as several reports revealed that social housing conditions modulate weight gain and food consumption in mice (Guo et al., 2004; van Leeuwen et al., 1997; Yamada et al., 2000). Interestingly, $CB_2$ knockout mice were more sensitive to the social environment as group housing led to increased body weight gain. Furthermore, genetic deletion of the

$CB_2$ receptor increased body weight compared to WT in group-housed mice, which was accompanied by a slightly increased food intake. These data indicate a gene x environment interaction for the regulation of body weight in $CB_2$ knockout mice. We already reported increased body weight and food intake in single-housed female $CB_2$ deficient mice (Trebicka et al., 2011). In line with this, $CB_2$ overexpressing mice appeared to be leaner and also displayed reduced food intake (Romero-Zerbo et al., 2012). Furthermore, Agudo et al. showed that only old male $CB_2$ deficient animals displayed an increased body weight, which was associated with increased food intake (Agudo et al., 2010). Thus, it is likely that the $CB_2$ receptor regulates body weight gain and food consumption and that this modulatory effect is dependent on the social environment and gender.

We found that alcohol consumption was accompanied with a reduced food intake. Independent of the genotype, the food consumption was negatively correlated with the amount of ingested alcohol. This effect was the most pronounced in the FD model where animals drank the largest amount of alcohol and consumed the least food. The relationship between alcohol and food consumption has been little addressed in preclinical studies, but widely investigated in alcoholic patients. A detailed review analysing this interaction was based on a Medline database search for the period from 1984 to 2010. 31 studies were included and selected depending on relevance and quality of design (Sayon-Orea et al., 2011). They found positive, negative and no correlation between alcohol consumption and weight gain. However, this effect was highly dependent on the drinking pattern of the patients (heavy and light-to-moderate drinkers) and the type of alcoholic beverages consumed (beer, wine, spirits). Analogous to human studies our results suggest that the effect of alcohol on food consumption may depend on the genetic background and also on the alcoholic strength of ethanol solutions.

### 5.2.4 Withdrawal-induced anxiety and locomotion

The withdrawal syndrome is characterised by different phenotypes including anxiety, anorexia, insomnia, tremor, convulsions and sympathetic response (Koob & Le Moal, 2006). Withdrawal-induced anxiety can also be observed in mice (Racz et al., 2003). In the present study, withdrawal-induced anxiety was not detected either in the

O-maze, open field, or in the dark light box test. However, the level of anxiety of alcohol-treated animals was compared to water controls. Thus, it is possible that withdrawal-induced anxiety should be compared to animals that have alcohol access at the time of testing (Racz et al., 2003). Furthermore, deletion of $CB_2$ did not affect the anxiety-like behaviour. This result has been independently reproduced by colleagues in the same animal husbandry (Bilkei-Gorzo et al., unpublished data). Contrary to this, García-Gutiérrez and colleagues reported that pharmacologic blockade of the $CB_2$ resulted in increased anxiety and overexpression of $CB_2$ led to anxiolytic-like behaviour (García-Gutiérrez et al., 2012; García-Gutiérrez & Manzanares, 2011). However, anxiety is critically affected by the laboratory environment (like cages and noise level in the animal husbandry) thereby possibly masking subtle phenotypes (Bilkei-Gorzó, Otto, et al., 2008; Crabbe et al., 1999).

The locomotor activity in the open field was already extremely enhanced after two months of intermittent alcohol access in some WT animals compared to water controls. This effect was not observed in a second cohort of animals, suggesting that the initial observation was biased by a few animals that were extremely sensitive to alcohol treatment. However, after six months of intermittent alcohol treatment the exploratory behaviour was strongly increased in group-housed animals. The activity in the open field displays exploratory behaviour in a novel environment and is known to be largely mediated by the NMDA receptors (Castellani & Adams, 1981; Liljequist et al., 1991). As alcohol antagonises NMDA receptor function, long-term alcohol consumption results in compensatory effects leading to increased receptor expression (Holmes et al., 2013; Spanagel et al., 2014). Thus, alcohol withdrawal is characterised as a hyperglutamatergic state, which leads to increased locomotion. However, this effect becomes significant only after six months of alcohol treatment.

Deletion of the $CB_2$ receptor decreased exploratory behaviour in eight-month-old animals that were reared in groups, which is consistent with the literature (Ortega-Alvaro et al., 2011). However, four-months-old single-housed animals did not show decreased locomotion. This discrepancy might be due to the use of different housing conditions as social isolation is known to affect locomotor activity (Võikar et al., 2005). This probably indicates a novel G x E interaction for the $CB_2$. However, this effect may

also depend on the body weight as group-housed $CB_2$ deficient mice were shown to be much heavier, thereby reducing locomotion.

Monitoring of the home cage activity aimed at investigating aspects of the alcohol withdrawal syndrome, such as insomnia, increased irritability (hyperlocomotion) or a shifted circadian rhythm. This study revealed that the circadian rhythm was not altered after four months of repeated alcohol drinking and withdrawal cycles in single-housed animals. Furthermore, alcohol consumption resulted in a decreased home cage activity compared to water controls, which might be attributed to the sedative effect of alcohol (Koob & Le Moal, 2006). During withdrawal, the home cage activity was increased compared to the prior alcohol period. This suggests that alcohol withdrawal leads to hyperlocomotion similar to the activity in the open field. Furthermore, the activity during the inactive phase was not affected by alcohol withdrawal indicating that insomnia might be not reflected in this animal model. Surprisingly, $CB_2$ deficient water-treated animals displayed increased activity during the resting phase, which indicates that these animals sleep less. The endocannabinoid system is involved in the regulation of sleep. So far these effects have been attributed to $CB_1R$ signalling (Gates et al., 2014; Murillo-Rodríguez, 2008). These data suggest that the $CB_2$ might also play a role in the regulation of sleep. However, the exact mechanism needs further investigation.

### 5.2.5 Development of tolerance, handling-induced convulsions and alcohol clearance

We also analysed the effect of $CB_2$ receptors on development of alcohol-induced tolerance and physical signs of withdrawal. WT and $CB_2$ knockout animals similarly developed tolerance to alcohol. However, $CB_2$ deficient mice showed reduced hypothermia to acute injection of low-dose alcohol. Furthermore, we could not detect any difference between the genotypes in handling-induced convulsions after alcohol withdrawal. In contrast to this, Ortega-Alvaro et al. detected increased physical signs of withdrawal in $CB_2$ deficient animals (Ortega-Álvaro et al., 2013). As we already mentioned, this study has been performed with mice on a CD1 background. Furthermore, the experimental design was different as they scored the animals at different time points of the withdrawal. Furthermore, we analysed the clearance of ethanol after an acute injection of 2 g / kg (Figure 50). The rate of alcohol clearance was

not affected by the genotype in naïve or chronic alcohol treated mice. Thus, the $CB_2$ receptor does not modulate metabolism of alcohol.

# 6 Conclusion and Outlook

We can conclude that all models applied in this study led to neuroinflammation as revealed by cytokine expression and immuno-histochemistry. These changes were more pronounced when animals were continuously exposed to alcohol. Additionally we found a strong correlation between the duration of alcohol drinking and the severity of neuroinflammation. In line with this, long-term alcohol drinking led to a pro-inflammatory activation of microglia in the cortex. Furthermore, $CB_2$ deficiency dampens the inflammatory response in the cortex. However, this effect was strongly dependent on the housing conditions. Interestingly, we detected a similar environmental effect for the modulatory role of $CB_2$ receptors in alcohol drinking behaviour and in the regulation of body weight gain. Additionally, our data suggest that the $CB_2$ receptor is involved in the modulation of alcohol reward. However, several open questions remain that require further investigation. As the site of $CB_2$ expression is highly controversial further studies will have to elucidate through which cell type the receptor mediates its effects. Thus, use of conditional knockout mice might address the important question whether the behavioural phenotypes are mediated through neurons or immune cells. Additionally, the environmental interactions of the receptor in alcohol-related behaviour have to be investigated in more detail. Chronic treatment with $CB_2$ agonist 'BCP' in a model with intermittent alcohol access might further support the role of the receptor in alcohol addiction. Finally, $CB_2$ deficient animals might be analysed in a larger variety of alcohol models that include environmental factors like social or cue-induced stress.

# Appendix

## References


Agudo, J., Martin, M., Roca, C., Molas, M., Bura, A. S., Zimmer, A., Bosch, F., & Maldonado, R. (2010). Deficiency of CB2 cannabinoid receptor in mice improves insulin sensitivity but increases food intake and obesity with age. *Diabetologia*, *53*(12), 2629–2640.

Al Mansouri, S., Ojha, S., Al Maamari, E., Al Ameri, M., Nurulain, S. M., & Bahi, A. (2014). The cannabinoid receptor 2 agonist, β-caryophyllene, reduced voluntary alcohol intake and attenuated ethanol-induced place preference and sensitivity in mice. *Pharmacology, Biochemistry, and Behavior*, *124C*, 260–268. doi:10.1016/j.pbb.2014.06.025

Albayram, O. (2012). *Role of CB1 Cannabinoid Receptors in Brain Aging*. Universtiy of Bonn.

Albayram, O., Alferink, J., Pitsch, J., Piyanova, A., Neitzert, K., Poppensieker, K., Mauer, D., Michel, K., Legler, A., Becker, A., Monory, K., Lutz, B., Zimmer, A., & Bilkei-Gorzo, A. (2011). Role of CB1 cannabinoid receptors on GABAergic neurons in brain aging. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(27), 11256–11261.

Alfonso-Loeches, S., Pascual, M., Gómez-Pinedo, U., Pascual-Lucas, M., Renau-Piqueras, J., & Guerri, C. (2012). Toll-like receptor 4 participates in the myelin disruptions associated with chronic alcohol abuse. *Glia*.

Alfonso-Loeches, S., Pascual-Lucas, M., Blanco, A., Sanchez-Vera, I., & Guerri, C. (2010). Pivotal Role of TLR4 Receptors in Alcohol-Induced Neuroinflammation and Brain Damage. *Journal of Neuroscience*, *30*(24), 8285–8295.

Aracil-Fernández, A., Trigo, J. M., García-Gutiérrez, M. S., Ortega-Álvaro, A., Ternianov, A., Navarro, D., Robledo, P., Berbel, P., Maldonado, R., & Manzanares, J. (2012). Decreased Cocaine Motor Sensitization and Self-Administration in Mice Overexpressing Cannabinoid CB2 Receptors. *Neuropsychopharmacology*. doi:10.1038/npp.2012.22

Ashton, J. C., & Glass, M. (2007). The Cannabinoid CB2 Receptor as a Target for Inflammation-Dependent Neurodegeneration. *Current Neuropharmacology*, *5*(2), 73–80.

Aso, E., Juvés, S., Maldonado, R., & Ferrer, I. (2013). CB2 cannabinoid receptor agonist ameliorates Alzheimer-like phenotype in AβPP/PS1 mice. *Journal of Alzheimer's Disease : JAD*, *35*(4), 847–58. doi:10.3233/JAD-130137

Atwood, B. K., & Mackie, K. (2010). CB2: a cannabinoid receptor with an identity crisis. *British Journal of Pharmacology*, *160*(3), 467–79. doi:10.1111/j.1476-5381.2010.00729.x

Bahi, A., Al Mansouri, S., Al Memari, E., Al Ameri, M., Nurulain, S. M., & Ojha, S. (2014). β-Caryophyllene, a CB2 receptor agonist produces multiple behavioral changes relevant to anxiety and depression in mice. *Physiology & Behavior*, *135C*, 119–124. doi:10.1016/j.physbeh.2014.06.003

Barron, S., Mulholland, P. J., Littleton, J. M., & Prendergast, M. A. (2008). Age and gender differences in response to neonatal ethanol withdrawal and polyamine challenge in organotypic hippocampal cultures. *Alcoholism, Clinical and Experimental Research*, *32*, 929–936. doi:10.1111/j.1530-0277.2008.00649.x

Bartolomucci, A. (2007). Social stress, immune functions and disease in rodents. *Frontiers in Neuroendocrinology*, *28*(1), 28–49. doi:10.1016/j.yfrne.2007.02.001

Becker, A., Bilkei-Gorzo, A., Michel, K., & Zimmer, A. (2010). Exposure of mice to long-light: a new animal model to study depression. *European Neuropsychopharmacology*, *20*(11), 802–812.

Becker, H., Lopez, M., & Doremus-Fitzwater, T. (2011). Effects of stress on alcohol drinking: a review of animal studies. *Psychopharmacology*, *218*(1), 131–156.

Benito, C., Núñez, E., Tolón, R. M., Carrier, E. J., Rábano, A., Hillard, C. J., & Romero, J. (2003). Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. *Journal of Neuroscience*, *23*(35), 11136–11141.

Bilkei-Gorzó, A., Otto, M., & Zimmer, A. (2008). Environmental modulation of anxiety-related neuronal activity and behaviors. *Behavioural Brain Research*, *186*(2), 289–292.

Bilkei-Gorzó, A., Racz, I., Michel, K., Mauer, D., Zimmer, A., & Klingmüller, D. (2008). Control of hormonal stress reactivity by the endogenous opioid system. *Psychoneuroendocrinology*, *33*(4), 425–436.

Bilkei-Gorzó, A., Racz, I., Michel, K., & Zimmer, A. (2002). Diminished anxiety- and depression-related behaviors in mice with selective deletion of the Tac1 gene. *Journal of Neuroscience*, *22*(22), 10046–10052.

Bilkei-Gorzó, A., Racz, I., Michel, K., Zimmer, A., Klingmüller, D., & Zimmer, A. (2004). Behavioral phenotype of pre-proenkecphalin-deficient mice on diverse congenic backgrounds. *Psychopharmacology*, *176*(3-4), 343–352.

Bilkei-Gorzó, A., Racz, I., Valverde, O., Otto, M., Michel, K., Sastre, M., Sarstre, M., & Zimmer, A. (2005). Early age-related cognitive impairment in mice lacking cannabinoid CB1 receptors. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(43), 15670–15675.

Blanco, A., & Guerri, C. (2007). Ethanol intake enhances inflammatory mediators in brain: role of glial cells and TLR4/IL-1RI receptors Ana. *Frontiers in Bioscience*, *12*, 2616–2630.

Block, M., Zecca, L., & Hong, J. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature Reviews Neuroscience*, *8*(1), 57–69.

Boileau, I., Assaad, J.-M., Pihl, R. O., Benkelfat, C., Leyton, M., Diksic, M., Tremblay, R. E., & Dagher, A. (2003). Alcohol promotes dopamine release in the human nucleus accumbens. *Synapse (New York, N.Y.)*, *49*, 226–231. doi:10.1002/syn.10226

Bondolfi, L., Calhoun, M., Ermini, F., Kuhn, H. G., Wiederhold, K.-H., Walker, L., Staufenbiel, M., & Jucker, M. (2002). Amyloid-associated neuron loss and gliogenesis in the neocortex of amyloid precursor protein transgenic mice. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(2), 515–522.

Boon, F. S. Den, Chameau, P., Schaafsma-zhao, Q., Aken, W. Van, Bari, M., & Oddi, S. (2012). Excitability of prefrontal cortical pyramidal neurons is modulated by activation of intracellular type-2 cannabinoid receptors. *Proc Natl Acad Sci USA*, *109*(9). doi:10.1073/pnas.1118167109/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1118167109

Brown, A. J. (2007). Novel cannabinoid receptors. *British Journal of Pharmacology*, *152*, 567–575. doi:10.1038/sj.bjp.0707481

Buckley, N. E. (2008). The peripheral cannabinoid receptor knockout mice: an update. *British Journal of Pharmacology*, *153*(2), 309–18. doi:10.1038/sj.bjp.0707527

Buckley, N. E., McCoy, K. L., Mezey, E., Bonner, T., Zimmer, A., Felder, C. C., & Glass, M. (2000). Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *European Journal of Pharmacology*, *396*(2-3), 141–149.

Cabral, G. A., & Griffin-Thomas, L. (2010). Emerging Role of the CB2 Cannabinoid Receptor in Immune Regulation and Therapeutic Prospects. *Expert Reviews in Molecular Medicine*, (804), 1–31. doi:10.1017/S1462399409000957.Emerging

Cacace, S., Plescia, F., Sardo, P., & Cannizzaro, C. (2012). Alcohol preference, behavioural reactivity and cognitive functioning in female rats exposed to a three-bottle choice paradigm. *Behavioural Brain Research*, *234*(1), 11–19.

Castellani, S., & Adams, P. M. (1981). Acute and chronic phencyclidine effects on locomotor activity, stereotypy and ataxia in rats. *European Journal of Pharmacology*, *73*(2-3), 143–54.

Chiara, G. Di, & Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(14), 5274–5278.

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, *162*, 156–159. doi:10.1038/nprot.2006.83

Clarke, T.-K., Treutlein, J., Zimmermann, U. S., Kiefer, F., Skowronek, M. H., Rietschel, M., Mann, K., & Schumann, G. (2008). HPA-axis activity in alcoholism: examples for a gene-environment interaction. *Addiction Biology*, *13*(1), 1–14. doi:10.1111/j.1369-1600.2007.00084.x

Cluny, N. L., Reimer, R. A., & Sharkey, K. A. (2012). Cannabinoid signalling regulates inflammation and energy balance: the importance of the brain-gut axis. *Brain, Behavior, and Immunity*, *26*(5), 691–8. doi:10.1016/j.bbi.2012.01.004

Collins, M., & Neafsey, E. (2012). Neuroinflammatory pathways in binge alcohol-induced neuronal degeneration: oxidative stress cascade involving aquaporin, brain edema, and phospholipase A2 activation. *Neurotoxicity Research*, *21*(1), 70–78.

Cook, R. T. (1998). Alcohol abuse, alcoholism, and damage to the immune system--a review. *Alcoholism, Clinical and Experimental Research*, *22*, 1927–1942. doi:00000374-199812000-00007 [pii]

Crabbe, J. C., Phillips, T. J., Harris, R. A., Arends, M. A., & Koob, G. F. (2006). Alcohol-related genes: contributions from studies with genetically engineered mice. *Addiction Biology*, *11*(3-4), 195–269.

Crabbe, J. C., Wahlsten, D., & Dudek, B. C. (1999). Genetics of Mouse Behavior: Interactions with Laboratory Environment. *Science*, *284*(5420), 1670–1672. doi:10.1126/science.284.5420.1670

Crews, F. T., & Nixon, K. (2009). Mechanisms of neurodegeneration and regeneration in alcoholism. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *44*(2), 115–127.

Crews, F. T., & Vetreno, R. (2011). Addiction, adolescence, and innate immune gene induction. *Frontiers in Psychiatry*, *2*, 19.

Crews, F. T., Zou, J., & Qin, L. (2011). Induction of innate immune genes in brain create the neurobiology of addiction. *Brain, Behavior, and Immunity*.

Darvas, M., Morsch, M., Racz, I., Ahmadi, S., Swandulla, D., & Zimmer, A. (2009). Modulation of the Ca2+ conductance of nicotinic acetylcholine receptors by Lypd6. *European Neuropsychopharmacology*, *19*(9), 670–681.

Di Marzo, V. (2011). Endocannabinoid signaling in the brain: biosynthetic mechanisms in the limelight. *Nature Neuroscience*, *14*(1), 9–15. doi:10.1038/nn.2720

Di Marzo, V., Bifulco, M., & Petrocellis, L. De. (2004). The endocannabinoid system and its therapeutic exploitation. *Nat Rev Drug Discov*, *3*(9), 771–784. doi:10.1038/nrd1495

Fama, R., Pfefferbaum, A., & Sullivan, E. V. (2004). Perceptual learning in detoxified alcoholic men: contributions from explicit memory, executive function, and age. *Alcoholism, Clinical and Experimental Research*, *28*, 1657–1665. doi:00000374-200411000-00010 [pii]

Fernandez-Lizarbe, S., Pascual, M., & Guerri, C. (2009). Critical role of TLR4 response in the activation of microglia induced by ethanol. *The Journal of Immunology*, *183*(7), 4733–4744.

Fernández-Ruiz, J., Romero, J., Velasco, G., Tolón, R. M., Ramos, J. A., & Guzmán, M. (2007). Cannabinoid CB2 receptor: a new target for controlling neural cell survival? *Trends in Pharmacological Sciences*, *28*(1), 39–45.

Fitzgerald, M. L., Mackie, K., & Pickel, V. M. (2013). The impact of adolescent social isolation on dopamine D2 and cannabinoid CB1 receptors in the adult rat prefrontal cortex. *Neuroscience*, *235*, 40–50. doi:10.1016/j.neuroscience.2013.01.021

Gamaleddin, I., Zvonok, A., Makriyannis, A., Goldberg, S. R., & Le Foll, B. (2012). Effects of a selective cannabinoid CB2 agonist and antagonist on intravenous nicotine self administration and reinstatement of nicotine seeking. *PLoS ONE*, *7*(1).

García-Gutiérrez, M. S., García-Bueno, B., Zoppi, S., Leza, J. C., & Manzanares, J. (2012). Chronic blockade of cannabinoid CB2 receptors induces anxiolytic-like actions associated with alterations in GABA(A) receptors. *British Journal of Pharmacology*, *165*(4), 951–64.

García-Gutiérrez, M. S., & Manzanares, J. (2011). Overexpression of CB2 cannabinoid receptors decreased vulnerability to anxiety and impaired anxiolytic action of alprazolam in mice. *Journal of Psychopharmacology (Oxford, England)*, *25*(1), 111–20. doi:10.1177/0269881110379507

García-Gutiérrez, M. S., Pérez-Ortiz, J., Gutiérrez-Adán, A., & Manzanares, J. (2010). Depression resistant endophenotype in mice overexpressing cannabinoid CB2 receptors. *British Journal of Pharmacology*, *160*(7), 1773–1784.

Gates, P. J., Albertella, L., & Copeland, J. (2014). The effects of cannabinoid administration on sleep: a systematic review of human studies. *Sleep Medicine Reviews*. doi:10.1016/j.smrv.2014.02.005

Gertsch, J. (2008). Anti-inflammatory cannabinoids in diet Addendum, Article, *1*(1), 26–28.

Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J. Z., Xie, X. Q., Altmann, K. H., Karsak, M., & Zimmer, A. (2008). Beta-caryophyllene is a dietary cannabinoid. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(26), 9099–9104.

Godlewski, G., Offert??ler, L., Wagner, J. A., & Kunos, G. (2009). Receptors for acylethanolamides-GPR55 and GPR119. *Prostaglandins and Other Lipid Mediators*. doi:10.1016/j.prostaglandins.2009.07.001

Goldman, D., Oroszi, G., & Ducci, F. (2005). The genetics of addictions: uncovering the genes. *Nature Reviews. Genetics*, *6*(7), 521–32. doi:10.1038/nrg1635

Gong, J.-P., Onaivi, E. S., Ishiguro, H., Liu, Q.-R., Tagliaferro, P. a, Brusco, A., & Uhl, G. R. (2006). Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Research*, *1071*(1), 10–23. doi:10.1016/j.brainres.2005.11.035

Graeber, M. B. (2010). Changing face of microglia. *Science*, *330*(6005), 783–788.

Grathwohl, S. A., Kälin, R. E., Bolmont, T., Prokop, S., Winkelmann, G., Kaeser, S. A., Odenthal, J., Radde, R., Eldh, T., Gandy, S., Aguzzi, A., Staufenbiel, M., Mathews, P. M., Wolburg, H., Heppner, F. L., & Jucker, M. (2009). Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia. *Nat. Neurosci.*, *12*(11), 1361–1363.

Gundersen, H. J. (1986). Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. *Journal of Microscopy*, *143*(Pt 1), 3–45.

Guo, M., Wu, C. F., Liu, W., Yang, J. Y., & Chen, D. (2004). Sex difference in psychological behavior changes induced by long-term social isolation in mice. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *28*(1), 115–21. doi:10.1016/j.pnpbp.2003.09.027

Haorah, J., Ramirez, S. H., Floreani, N., Gorantla, S., Morsey, B., & Persidsky, Y. (2008). Mechanism of alcohol-induced oxidative stress and neuronal injury. *Free Radical Biology & Medicine*, *45*(11), 1542–50. doi:10.1016/j.freeradbiomed.2008.08.030

He, J., & Crews, F. T. (2008). Increased MCP-1 and microglia in various regions of the human alcoholic brain. *Experimental Neurology*, *210*, 349–358. doi:10.1016/j.expneurol.2007.11.017

Holmes, A., Spanagel, R., & Krystal, J. (2013). Glutamatergic targets for new alcohol medications. *Psychopharmacology*.

Ignatowska-Jankowska, B. M., Muldoon, P. P., Lichtman, A. H., & Damaj, I. M. (2013). The cannabinoid CB 2 receptor is necessary for nicotine-conditioned place preference , but not other behavioral effects of nicotine in mice. *Psychopharmacology*, (229), 591–601. doi:10.1007/s00213-013-3117-6

Ishiguro, H., Horiuchi, Y., Ishikawa, M., Koga, M., Imai, K., Suzuki, Y., Morikawa, M., Inada, T., Watanabe, Y., & Takahashi, M. (2010). Brain cannabinoid CB2 receptor in schizophrenia. *Biological Psychiatry*, *67*(10), 974–982.

Ishiguro, H., Iwasaki, S., Teasenfitz, L., Higuchi, S., Horiuchi, Y., Saito, T., Arinami, T., & Onaivi, E. (2007). Involvement of cannabinoid CB2 receptor in alcohol preference in mice and alcoholism in humans. *The Pharmacogenomics Journal*, *7*(6), 380–385.

Jones, M. W. (2002). A comparative review of rodent prefrontal cortex and working memory. *Current Molecular Medicine*, *2*, 639–647. doi:10.2174/1566524023361989

Karsak, M., Gaffal, E., Date, R., Wang-Eckhardt, L., Rehnelt, J., Petrosino, S., Starowicz, K., Steuder, R., Schlicker, E., Cravatt, B., Mechoulam, R., Buettner, R., Werner, S., Di Marzo, V., Tüting, T., & Zimmer, A. (2007). Attenuation of allergic contact dermatitis through the endocannabinoid system. *Science (New York, N.Y.)*, *316*(5830), 1494–7. doi:10.1126/science.1142265

Katona, I., & Freund, T. F. (2008). Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. *Nature Medicine*, *14*(9), 923–930.

Kelley, K. W., & Dantzer, R. (2011). Alcoholism and inflammation: neuroimmunology of behavioral and mood disorders. *Brain, Behavior, and Immunity*, *25 Suppl 1*, S13–20. doi:10.1016/j.bbi.2010.12.013

Klauke, A.-L., Racz, I., Pradier, B., Markert, A., Zimmer, a M., Gertsch, J., & Zimmer, A. (2014). The cannabinoid CB2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. *European Neuropsychopharmacology*, *24*(4), 608–20. doi:10.1016/j.euroneuro.2013.10.008

Koike, H., Ibi, D., Mizoguchi, H., Nagai, T., Nitta, A., Takuma, K., Nabeshima, T., Yoneda, Y., & Yamada, K. (2009). Behavioral abnormality and pharmacologic response in social isolation-reared mice. *Behavioural Brain Research*, *202*(1), 114–21. doi:10.1016/j.bbr.2009.03.028

Koob, G. F., Ahmed, S. H., Boutrel, B., Chen, S. A., Kenny, P. J., Markou, A., O'dell, L. E., Parsons, L. H., & Sanna, P. P. (2004). Neurobiological mechanisms in the transition from drug use to drug dependence. *Neuroscience and Biobehavioral Reviews*, *27*(8), 739–749.

Koob, G. F., & Le Moal, M. (2006). Alcohol. In G. F. Koob & M. Le Moal (Eds.), *Neurobiology of addiction* (1st ed., pp. 173–242). San Diego: Elsevier.

Koob, G. F., & Le Moal, M. (2008). Neurobiological mechanisms for opponent motivational processes in addiction. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences*, *363*(1507), 3113–3123.

Koob, G. F., & Volkow, N. D. (2009). Neurocircuitry of Addiction. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *35*(1), 217–238.

Krügel, U., Fischer, J., & Bauer, K. (2014). The impact of social isolation on immunological parameters in rats. *Archives of Toxicology*, *88*(3), 853–855.

Kwak, C., Lee, S.-H., & Kaang, B.-K. (2009). Social Isolation Selectively Increases Anxiety in Mice without Affecting Depression-like Behavior. *The Korean Journal of Physiology & Pharmacology : Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology*, *13*(5), 357–60. doi:10.4196/kjpp.2009.13.5.357

Lichtman, A., Blankman, J., & Cravatt, B. (2010). Endocannabinoid Overload. *Molecular Pharmacology*, *78*(6), 993–995.

Lightman, S. L., & Conway-Campbell, B. L. (2010). The crucial role of pulsatile activity of the HPA axis for continuous dynamic equilibration. *Nature Reviews Neuroscience*, *11*(10), 710–718.

Liljequist, S., Ossowska, K., Grabowska-Andén, M., & Andén, N. E. (1991). Effect of the NMDA receptor antagonist, MK-801, on locomotor activity and on the metabolism of dopamine in various brain areas of mice. *European Journal of …*, *195*, 55–61.

Lingford-Hughes, A., Watson, B., Kalk, N., & Reid, A. (2010). Neuropharmacology of addiction and how it informs treatment. *British Medical Bulletin*, *96*, 93–110.

Little, H. J., O'Callaghan, M. J., Butterworth, A. R., Wilson, J., Cole, J., & Watson, W. P. (1999). Low alcohol preference among the "high alcohol preference" C57 strain of mice; preference increased by saline injections. *Psychopharmacology*, *147*, 182–189. doi:10.1007/s002130051159

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif)*, *25*(4), 402–408.

Lopez, M. F., Doremus-Fitzwater, T. L., & Becker, H. C. (2011). Chronic social isolation and chronic variable stress during early development induce later elevated ethanol intake in adult C57BL/6J mice. *Alcohol (Fayetteville, N.Y.)*, *45*(4), 355–64. doi:10.1016/j.alcohol.2010.08.017

Louvet, A., Teixeira-Clerc, F., Chobert, M.-N., Deveaux, V., Pavoine, C., Zimmer, A., Pecker, F., Mallat, A., & Lotersztajn, S. (2011). Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating Kupffer cell polarization in mice. *Hepatology (Baltimore, Md.)*, *54*(4), 1217–26. doi:10.1002/hep.24524

Maresz, K., Carrier, E. J., Ponomarev, E. D., Hillard, C. J., & Dittel, B. N. (2005). Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. *Journal of Neurochemistry*, *95*, 437–445. doi:10.1111/j.1471-4159.2005.03380.x

Marshall, S. A., McClain, J. A., Kelso, M. L., Hopkins, D. M., Pauly, J. R., & Nixon, K. (2013). Microglial activation is not equivalent to neuroinflammation in alcohol-induced neurodegeneration: The importance of microglia phenotype. *Neurobiology of Disease*.

Martin, A. L., & Brown, R. E. (2010). The lonely mouse: verification of a separation-induced model of depression in female mice. *Behavioural Brain Research*, *207*(1), 196–207. doi:10.1016/j.bbr.2009.10.006

McAllister, S. D., & Abood, M. E. (2006). Endocannabinoids and Intracellular Signaling. In E. S. Onaivi, T. Sugiura, & V. Di Marzo (Eds.), *Endocannabinoids: The Brain and Body's Marijuana and Beyond* (1st ed., pp. 119–130). Boca Raton: CRC Press.

Miguel-Hidalgo, J. J. (2006). Withdrawal from free-choice ethanol consumption results in increased packing density of glutamine synthetase-immunoreactive astrocytes in the prelimbic cortex of alcohol-preferring rats. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *41*(4), 379–385.

Möller, M., Du Preez, J. L., Viljoen, F. P., Berk, M., Emsley, R., & Harvey, B. H. (2013). Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. *Brain, Behavior, and Immunity*, *30*, 156–67. doi:10.1016/j.bbi.2012.12.011

Morgan, N. H., Stanford, I. M., & Woodhall, G. L. (2009). Functional CB2 type cannabinoid receptors at CNS synapses. *Neuropharmacology*, *57*, 356–368. doi:10.1016/j.neuropharm.2009.07.017

Murillo-Rodríguez, E. (2008). The role of the CB1 receptor in the regulation of sleep. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *32*(6), 1420–7. doi:10.1016/j.pnpbp.2008.04.008

Navarrete, F., Pérez-Ortiz, J. M., & Manzanares, J. (2012). Cannabinoid CB2 receptor-mediated regulation of impulsive-like behaviour in DBA/2 mice. *British Journal of Pharmacology*, *165*(1), 260–73. doi:10.1111/j.1476-5381.2011.01542.x

Navarrete, F., Rodríguez-Arias, M., Martín-García, E., Navarro, D., García-Gutiérrez, M. S., Aguilar, M. a, Aracil Fernández, A., Berbel, P., Miñarro, J., Maldonado, R., & Manzanares, J. (2013). Role of CB2 Cannabinoid Receptor in the Rewarding, Reinforcing and Physical Effects of Nicotine. *Neuropsychopharmacology*, 1–10.

Nelson, S., & Kolls, J. K. (2002). Alcohol, host defence and society. *Nature Reviews Immunology*, *2*(3), 205–209.

Nestler, E. J. (2005). Is there a common molecular pathway for addiction? *Nature Neuroscience*, *8*(11), 1445–9. doi:10.1038/nn1578

Nutt, D. J., King, L. a, & Phillips, L. D. (2010). Drug harms in the UK: a multicriteria decision analysis. *Lancet*, *376*(9752), 1558–65. doi:10.1016/S0140-6736(10)61462-6

Obernier, J. A., White, A. M., Swartzwelder, H. S., & Crews, F. T. (2002). Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. *Pharmacology, Biochemistry, and Behavior*, *72*(3), 521–532.

Onaivi, E., Ishiguro, H., Gong, J., Patel, S., Meozzi, P., Myers, L., Perchuk, A., Mora, Z., Tagliaferro, P., & Gardner, E. (2008). Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects. *PLoS ONE*, *3*(2), e1640.

Ortega-Alvaro, A., Aracil-Fernández, A., García-Gutiérrez, M., Navarrete, F., & Manzanares, J. (2011). Deletion of CB2 Cannabinoid Receptor Induces Schizophrenia-Related Behaviors in Mice. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *36*(7), 1489–1504.

Ortega-Álvaro, A., Ternianov, A., Aracil-Fernández, A., Navarrete, F., García-Gutiérrez, M. S., & Manzanares, J. (2013). Role of cannabinoid CB2 receptor in the reinforcing actions of ethanol. *Addiction Biology*. doi:10.1111/adb.12076

Pacher, P., & Mechoulam, R. (2011). Is lipid signaling through cannabinoid 2 receptors part of a protective system? *Progress in Lipid Research*, *50*(2), 193–211. doi:10.1016/j.plipres.2011.01.001

Pascual, M., Baliño, P., Alfonso-Loeches, S., Aragón, C. M., & Guerri, C. (2011). Impact of TLR4 on behavioral and cognitive dysfunctions associated with alcohol-induced neuroinflammatory damage. *Brain, Behavior, and Immunity*, *25 Suppl 1*, S80–91.

Pascual, M., Boix, J., Felipo, V., & Guerri, C. (2009). Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. *Journal of Neurochemistry*, *108*(4), 920–31. doi:10.1111/j.1471-4159.2008.05835.x

Pascual, M., Fernández-Lizarbe, S., & Guerri, C. (2011). Role of TLR4 in ethanol effects on innate and adaptive immune responses in peritoneal macrophages. *Immunology and Cell Biology*, *89*(6), 716–27. doi:10.1038/icb.2010.163

Paxinos, G., & Franklin, K. B. J. (2001). *The mouse brain in stereotaxic coordinates*. (G. Paxinos & K. B. J. Franklin, Eds.) (second.). San Diego: Academic Press.

Pereda-Pérez, I., Popović, N., Otalora, B. B., Popović, M., Madrid, J. A., Rol, M. A., & Venero, C. (2013). Long-term social isolation in the adulthood results in CA1 shrinkage and cognitive impairment. *Neurobiology of Learning and Memory*, *106*, 31–9. doi:10.1016/j.nlm.2013.07.004

Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P. H., Marzo, V. Di, Elphick, M. R., Greasley, P. J., Hansen, H. S., & Kunos, G. (2010). Cannabinoid Receptors and Their Ligands : Beyond CB 1 and CB 2. *Pharmacological Reviews*, *62*(4), 588–631. doi:10.1124/pr.110.003004.588

Pfefferbaum, A., & Sullivan, E. V. (2005). Disruption of brain white matter microstructure by excessive intracellular and extracellular fluid in alcoholism: evidence from diffusion tensor imaging. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *30*, 423–432. doi:10.1038/sj.npp.1300623

Piomelli, D. (2003). The molecular logic of endocannabinoid signalling. *Nature Reviews Neuroscience*, *4*(11), 873–884.

Pradier, B., Jeub, M., Markert, A., Mauer, D., Tolksdorf, K., Van De Putte, T., Seuntjens, E., Gailus-Durner, V., Fuchs, H., Hrabě De Angelis, M., Huylebroeck, D., Beck, H., Zimmer, A., & Rácz, I. (2013). Smad-interacting protein 1 affects acute and tonic, but not chronic pain. *European Journal of Pain (London, England)*.

Qin, L., He, J., Hanes, R., Pluzarev, O., Hong, J., & Crews, F. (2008). Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. *Journal of Neuroinflammation*, *5*(1), 10.

Racz, I., Bilkei-Gorzo, A., Toth, Z. E., Michel, K., Palkovits, M., & Zimmer, A. (2003). A critical role for the cannabinoid CB1 receptors in alcohol dependence and stress-stimulated ethanol drinking. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *23*(6), 2453–8.

Racz, I., Markert, A., Mauer, D., Stoffel-Wagner, B., & Zimmer, A. (2012). Long-term ethanol effects on acute stress responses: modulation by dynorphin. *Addiction Biology*.

Racz, I., Nadal, X., Alferink, J., Banos, J., Rehnelt, J., Martin, M., Pintado, B., Gutierrez-Adan, A., Sanguino, E., Manzanares, J., Zimmer, A., & Maldonado, R. (2008). Crucial Role of CB2 Cannabinoid Receptor in the Regulation of Central Immune Responses during Neuropathic Pain. *Journal of Neuroscience*, *28*(46), 12125–12135.

Racz, I., Schürmann, B., Karpushova, A., Reuter, M., Cichon, S., Montag, C., Fürst, R., Schütz, C., Franke, P., Strohmaier, J., Wienker, T., Terenius, L., Ösby, U., Gunnar, A., Maier, W., Bilkei-Gorzó, A., Nöthen, M., & Zimmer, A. (2008). The Opioid Peptides Enkephalin and β-Endorphin in Alcohol Dependence. *BPS*, *64*(11), 989–997.

Ramírez, B. G., Blázquez, C., Gómez del Pulgar, T., Guzmán, M., & de Ceballos, M. L. (2005). Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*, 1904–1913. doi:10.1523/JNEUROSCI.4540-04.2005

Rodrigues, A., Leão, P., Carvalho, M., Almeida, O. F., & Sousa, N. (2011). Potential programming of dopaminergic circuits by early life stress. *Psychopharmacology*, *214*(1), 107–120.

Romero-Zerbo, S. Y., Garcia-Gutierrez, M. S., Suárez, J., Rivera, P., Ruz-Maldonado, I., Vida, M., Rodriguez de Fonseca, F., Manzanares, J., & Bermúdez-Silva, F. J. (2012). Overexpression of cannabinoid CB2 receptor in the brain induces hyperglycaemia and a lean phenotype in adult mice. *Journal of Neuroendocrinology*, *24*(8), 1106–19. doi:10.1111/j.1365-2826.2012.02325.x

Salak-Johnson, J. L., & McGlone, J. J. (2007). Making sense of apparently conflicting data: stress and immunity in swine and cattle. *Journal of Animal Science*, *85*(13 Suppl), E81–8. doi:10.2527/jas.2006-538

Sawaguchi, T., & Goldman-Rakic, P. S. (1991). D1 dopamine receptors in prefrontal cortex: involvement in working memory. *Science (New York, N.Y.)*, *251*(4996), 947–50.

Sayon-Orea, C., Martinez-Gonzalez, M. a, & Bes-Rastrollo, M. (2011). Alcohol consumption and body weight: a systematic review. *Nutrition Reviews*, *69*(8), 419–31. doi:10.1111/j.1753-4887.2011.00403.x

Schmöle, A.-C., Lundt, R., Albayram, Ö., Bano, D., Nicotera, P., Alferink, J., & Zimmer, A. (n.d.). Cannabinoid receptor 2 deficiency results in reduced neuroinflammation in an Alzheimer ' s disease mouse model, *49*(0), 1–32.

Shizgal, P. B., & Hyman, S. E. (2013). Homeostasis, Motivation, and Addictive States. In E. R. Kandel, J. H. Schwartz, T. M. Jessell, S. A. Siegelbaum, & A. J. Hudspeth (Eds.), *Principles of Neural Science* (Fifth., pp. 1095–1115).

Short, J. L., Drago, J., & Lawrence, A. J. (2006). Comparison of ethanol preference and neurochemical measures of mesolimbic dopamine and adenosine systems across different strains of mice. *Alcoholism: Clinical and Experimental Research*, *30*, 606–620. doi:10.1111/j.1530-0277.2006.00071.x

Sillaber, I., Rammes, G., Zimmermann, S., Mahal, B., Zieglgänsberger, W., Wurst, W., Holsboer, F., & Spanagel, R. (2002). Enhanced and delayed stress-induced alcohol drinking in mice lacking functional CRH1 receptors. *Science (New York, N.Y.)*, *296*, 931–933. doi:10.1126/science.1069836

Sinha, R. (2008). Chronic stress, drug use, and vulnerability to addiction. *Annals of the New York Academy of Sciences*, *1141*, 105–130.

Solas, M., Francis, P. T., Franco, R., & Ramirez, M. J. (2013). CB2 receptor and amyloid pathology in frontal cortex of Alzheimer's disease patients. *Neurobiology of Aging*, *34*, 805–808. doi:10.1016/j.neurobiolaging.2012.06.005

Sommer, W. H., Rimondini, R., Hansson, A. C., Hipskind, P. A., Gehlert, D. R., Barr, C. S., & Heilig, M. A. (2008). Upregulation of Voluntary Alcohol Intake, Behavioral Sensitivity to Stress, and Amygdala Crhr1 Expression Following a History of Dependence. *Biological Psychiatry*, *63*, 139–145. doi:10.1016/j.biopsych.2007.01.010

Sorge, R. E., Martin, L. J., Isbester, K. a, Sotocinal, S. G., Rosen, S., Tuttle, A. H., Wieskopf, J. S., Acland, E. L., Dokova, A., Kadoura, B., Leger, P., Mapplebeck, J. C. S., McPhail, M., Delaney, A., Wigerblad, G., Schumann, A. P., Quinn, T., … Mogil, J. S. (2014). Olfactory exposure to males, including men, causes stress and related analgesia in rodents. *Nature Methods*, *11*(6), 629–32. doi:10.1038/nmeth.2935

Spanagel, R. (2009). Alcoholism: a systems approach from molecular physiology to addictive behavior. *Physiological Reviews*, *89*(2), 649–705.

Spanagel, R., Vengeliene, V., Bilbao, A., & Molander, A. (2008). Neuropharmacology of alcohol addiction. *British Journal of Pharmacology*, *154*(2), 299–315.

Spanagel, Vengeliene, V., Jandeleit, B., Fischer, W.-N., Grindstaff, K., Zhang, X., Gallop, M. a, Krstew, E. V, Lawrence, A. J., & Kiefer, F. (2014). Acamprosate produces its anti-relapse effects via calcium. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *39*(4), 783–91. doi:10.1038/npp.2013.264

Stella, N. (2010). Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia*, *58*(9), 1017–1030.

Sullivan, E., & Pfefferbaum, A. (2005). Neurocircuitry in alcoholism: a substrate of disruption and repair. *Psychopharmacology*, *180*(4), 583–594.

Sullivan, E., & Zahr, N. (2008). Neuroinflammation as a neurotoxic mechanism in alcoholism: Commentary on: Increased MCP-1 and microglia in various regions of human alcoholic brain. *Experimental Neurology*, *213*(1), 10–17.

Trebicka, J., Racz, I., Siegmund, S. V, Cara, E., Granzow, M., Schierwagen, R., Klein, S., Wojtalla, A., Hennenberg, M., Huss, S., Fischer, H.-P., Heller, J., Zimmer, A., & Sauerbruch, T. (2011). Role of cannabinoid receptors in alcoholic hepatic injury: steatosis and fibrogenesis are increased in CB2 receptor-deficient mice and decreased in CB1 receptor knockouts. *Liver International : Official Journal of the International Association for the Study of the Liver*, *31*(6), 860–70. doi:10.1111/j.1478-3231.2011.02496.x

Tsai, G., & Coyle, J. T. (1998). The role of glutamatergic neurotransmission in the pathophysiology of alcoholism. *Annual Review of Medicine*, *49*, 173–184. doi:10.1146/annurev.med.49.1.173

Van Leeuwen, S. D., Bonne, O. B., Avraham, Y., & Berry, E. M. (1997). Separation as a new animal model for self-induced weight loss. *Physiology & Behavior*, *62*(1), 77–81.

Van Sickle, M. D., Duncan, M., Kingsley, P. J., Mouihate, A., Urbani, P., Mackie, K., Stella, N., Makriyannis, A., Piomelli, D., Davison, J. S., Marnett, L. J., Di Marzo, V., Pittman, Q. J., Patel, K. D., & Sharkey, K. a. (2005). Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science (New York, N.Y.)*, *310*(5746), 329–32. doi:10.1126/science.1115740

Võikar, V., Polus, a, Vasar, E., & Rauvala, H. (2005). Long-term individual housing in C57BL/6J and DBA/2 mice: assessment of behavioral consequences. *Genes, Brain, and Behavior*, *4*(4), 240–52. doi:10.1111/j.1601-183X.2004.00106.x

Watson, W. P., & Little, H. J. (1999). Correlation between increases in dihydropyridine binding in vivo and behavioural signs of ethanol withdrawal in mice. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, *34*(1), 35–42.

WHO. (2014a). *Country profiles*.

WHO. (2014b). *Global status report on alcohol and health 2014*.

Wu, J., Bie, B., Yang, H., Xu, J. J., Brown, D. L., & Naguib, M. (2013). Activation of the CB2 receptor system reverses amyloid-induced memory deficiency. *Neurobiology of Aging*, *34*(3), 791–804.

Xi, Z.-X., Peng, X.-Q., Li, X., Song, R., Zhang, H.-Y., Liu, Q.-R., Yang, H.-J., Bi, G.-H., Li, J., & Gardner, E. L. (2011). Brain cannabinoid CB(2) receptors modulate cocaine's actions in mice. *Nature Neuroscience*, *14*(9), 1160–1166.

Yamada, K., Ohki-Hamazaki, H., & Wada, K. (2000). Differential effects of social isolation upon body weight, food consumption, and responsiveness to novel and social environment in bombesin receptor subtype-3 (BRS-3) deficient mice. *Physiology & Behavior*, *68*(4), 555–61.

Yoneyama, N., Crabbe, J. C., Ford, M. M., Murillo, A., & Finn, D. A. (2008). Voluntary ethanol consumption in 22 inbred mouse strains. *Alcohol*, *42*(3), 149–160.

Zahr, N. M., & Sullivan, E. V. (2009). Translational Studies of Alcoholism: Bridging the Gap. *Alcohol Research & Health : The Journal of the National Institute on Alcohol Abuse and Alcoholism*, *31*(3), 215–230.

Zorrilla, E. P., Logrip, M. L., & Koob, G. F. (2014). Corticotropin releasing factor: a key role in the neurobiology of addiction. *Frontiers in Neuroendocrinology*, *35*(2), 234–44. doi:10.1016/j.yfrne.2014.01.001

## List of figures

## List of tables