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Targeting the Endocannabinoid System to Modulate
Neuroinflammation and Signaling Dysregulation in FASD

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Abstract

Background: Fetal Alcohol Spectrum Disorder (FASD) is one of the serious consequences of prenatal alcohol exposure (PAE). PAE influences and disrupts numerous metabolic and neuroimmune signalling pathways in the hippocampus. AMPK and mTOR pathways, dopaminergic signalling pathways and glial markers (Iba1, GFAP) are considered key mediators of this dysregulation. The endocannabinoid system can be modulated via cannabidiol (CBD) and the inverse cannabinoid receptor agonists AM251 (CB1R) and AM630 (CB2R), thereby influencing this dysregulation.

Methods: Female and male C57BL/6 mice were exposed to ethanol prenatally and during lactation. Depending on the respective group, CBD (20 mg/kg), AM251 (0.3 mg/kg) and AM630 (0.3 mg/kg) were administered subcutaneously 30 minutes prior to alcohol administration during pregnancy and lactation. On postnatal day 21, tissue samples were taken from the hippocampi of the offspring for Western blot analysis (pmTOR/mTOR, pAKT/AKT, pAMPK/AMPK, pPKA/PKA, DARPP-32, DAT) and immunohistochemistry (Iba1, GFAP).

Results: CBD and the inverse agonists were able to reduce microglial activation (Iba1). In males, Iba1 was reduced particularly by AM630; in females, the PAE-induced increases in Iba1 in the CA1 region and dentate gyrus were attenuated by CBD and AM630. GFAP showed region- and sex-specific modulation: in the DG, GFAP increased in males under CBD, whilst in females AM251/AM630 reduced GFAP; in CA1, GFAP signals decreased in both sexes under several treatments. AM630 increased pmTOR in males whilst total mTOR remained unchanged. pAKT showed selective increases in females under AM251 and CBD+AM251, whilst pAMPK and pPKA remained treatment-insensitive. DARPP-32 was generally treatment-sensitive, with increases under CBD in females, DAT remained unaffected.

Conclusion: Interventions targeting the endocannabinoid system modulate hippocampal neuroinflammation (Iba1, GFAP) and mTOR/AKT signalling pathways following PAE in a sex-specific and region-dependent manner. The effects of AM630 on Iba1 and pmTOR in males underscore the relevance of CB2R signalling for neuroinflammatory processes, whilst increases in pAKT under AM251 in females suggest CB1R-mediated neuroprotection. These results support the therapeutic potential of interventions that modulate the endocannabinoid system.

Zusammenfassung

Hintergrund: Die Fetale Alkoholspektrumstörung (FASD) zählt zu den fatalen Folgen der Pränatalen Alkoholexposition (PAE). PAE beeinflusst und stört zahlreiche metabolische und neuroimmune Signalwege im Hippocampus. AMPK und mTOR-Achsen, dopaminerge Signalwege und gliale Marker (Iba1, GFAP) gelten als relevante Mediatoren dieser Dysregulation. Über Cannabidiol (CBD) und die inversen Cannabinoid-Rezeptor-Agonisten AM251 (CB1) und AM630 (CB2) kann das Endocannabinoidsystem moduliert und damit Einfluss auf diese Dysregulation genommen werden.

Methoden: Weibliche und männliche C57BL/6-Mäuse wurden pränatal und während der Laktation Ethanol ausgesetzt. Je nach entsprechender Gruppe wurden CBD (20 mg/kg), AM251 (0.3 mg/kg) und AM630 (0.3 mg/kg) s.c. 30 min vor Alkoholgabe während Trächtigkeit und Laktation appliziert. Am postnatalen Tag 21 erfolgte die Gewebeentnahme aus den Hippocampi der Jungtiere für Western Blot (pmTOR/mTOR, pAKT/AKT, pAMPK/AMPK, pPKA/PKA, DARPP-32, DAT) und Immunhistochemie (Iba1, GFAP).

Ergebnisse: CBD und die inversen Agonisten konnten die Mikroglia-Aktivierung reduzieren (Iba1). Bei den Männchen wurde Iba1 besonders durch AM630 reduziert, bei den Weibchen konnten die PAE-bedingten Iba1-Anstiege in der CA1-Region und Gyrus Dentatus durch CBD und AM630 abgeschwächt werden. GFAP zeigte eine regions- und geschlechtsspezifische Modulation: Im DG stieg GFAP bei Männchen unter CBD an, während bei Weibchen AM251/AM630 GFAP senkten; in CA1 nahmen GFAP-Signale in beiden Geschlechtern unter mehreren Behandlungen ab. AM630 erhöhte pmTOR in Männchen bei unverändertem mTOR total. pAKT zeigte in Weibchen selektive Anstiege unter AM251 und CBD+AM251, während pAMPK und pPKA behandlungsunempfindlich blieben. DARPP-32 war insgesamt behandlungssensitiv mit Erhöhungen unter CBD bei Weibchen, während DAT unbeeinflusst blieb.

Schlussfolgerung: Die auf das Endocannabinoid-System gerichteten Interventionen modulieren nach PAE hippocampale Neuroinflammation (Iba1, GFAP) und mTOR/AKT-Signalwege in einer geschlechtsspezifischen und regionabhängigen Weise. AM630-Wirkungen auf Iba1 und pmTOR bei Männchen unterstreichen die Relevanz von CB2R-Signalen für neuroinflammatorische Prozesse, während pAKT-Anstiege unter AM251 in Weibchen auf eine CB1R-vermittelte Neuroprotektion hindeuten. Diese Ergebnisse sprechen für das therapeutische Potenzial von Interventionen, die das Endocannabinoid-System modulieren.

1. Introduction

1.1 Overview

Alcohol intake during gestation poses a significant public health issue. According to a comprehensive meta-analysis published in 2017, approximately 9.8% of pregnant women globally reported alcohol consumption during gestation. Exposure to alcohol in utero has been linked to the development of fetal alcohol spectrum disorders (FASD), a collective term encompassing a wide range of developmental impairments manifested through diverse clinical signs and symptoms. The global prevalence of FASD is estimated to be 0.77%, with higher rates observed in Europe and North America. While Figure 1 illustrates global prevalence based on data modeled for 2012, these findings remain the most authoritative global estimates published to date.¹

Diagnostic criteria for fetal alcohol spectrum disorders differ internationally and are regularly updated to reflect emerging research findings. According to global consensus, a diagnosis typically requires documented prenatal alcohol exposure (PAE) and/or the presence of specific facial dysmorphologies, such as reduced palpebral fissure length, a smooth philtrum, and a thin upper lip. Additionally, confirmation of microcephaly or deficits in at least three functional domains, such as motor skills, neuroanatomy, cognition, language, memory, attention, executive function, academic achievement, or adaptive behavior, is essential for a comprehensive assessment.²

People suffering from fetal alcohol spectrum disorder experience, among other things, hyperactivity, attention deficits, planning difficulties, lower IQ and social difficulties.³

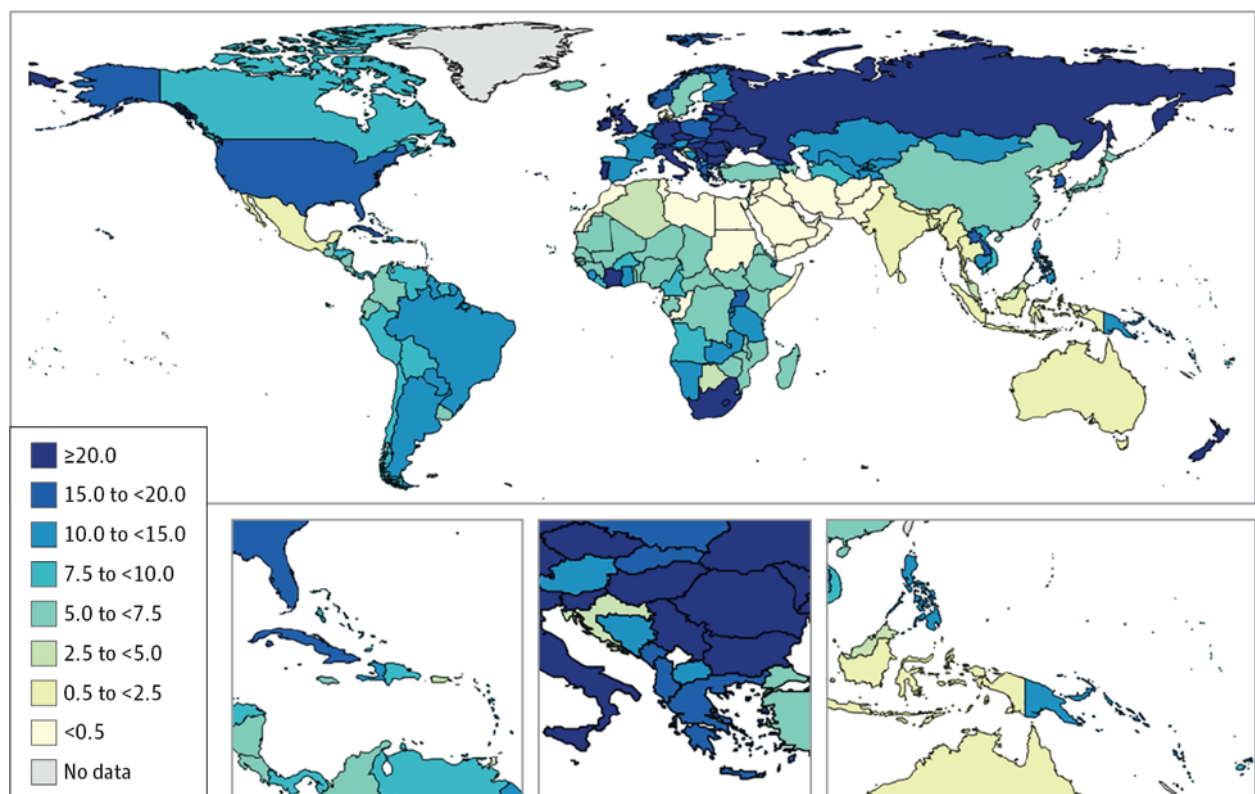


Figure 1: Global prevalence of fetal alcohol spectrum disorder among children and youth. Rates are shown per 1,000 population based on 2012 data models as reported in a 2017 global systematic review.⁴

Currently there is no specific pharmacologic therapy available. Attention-Deficit/Hyperactivity Disorder (ADHD) is a very common comorbidity of FASD (50-80 %). Therefore methylphenidate can be used to reduce hyperactivity and impulsivity.⁵

Research on therapeutic interventions for children and adolescents with FASD remains limited, with only a small number of randomized controlled trials available. However, the existing evidence suggests that interventions combining parent- and child-focused sessions may offer a particularly promising approach for addressing the challenges associated with FASD.⁶

An important barrier to effective intervention during pregnancy is the social stigma associated with prenatal alcohol consumption. This stigma often prompts pregnant women to hide their alcohol use, resulting in systematic underreporting that obstructs the implementation of necessary treatment and specialized care.⁷

1.2 Pathophysiology

Ethanol is recognized as a potent teratogenic agent, capable of crossing the placental barrier and rapidly distributing within the fetal compartment, where it accumulates in the amniotic fluid. Due to the fetus's significantly slower ethanol elimination rate, only 3–4% of the maternal rate, repeated ingestion of ethanol occurs, resulting in prolonged exposure.

In adults, ethanol metabolism is primarily mediated by alcohol dehydrogenase (ADH), which converts ethanol to acetaldehyde

Ethanol further disrupts neuronal cell-cell adhesion by upregulating proteins such as alpha- and beta-laminins 1, beta-integrins 3 and 5, secreted phosphoprotein-1, and sarcoglycan epsilon. The impaired transport of essential cofactors, including biotin and vitamin B6, may also play a significant role in the pathophysiology of fetal alcohol spectrum disorders (FASD).

Another critical factor is ethanol-induced placental vasoconstriction, mediated by reduced nitric oxide levels. This constriction compromises the delivery of oxygen and nutrients to the fetus, exacerbating developmental risks.

It was noticed that pre- and postnatal alcohol exposure could lead to a heightened DNA methyltransferase activity. This could cause epigenetic changes which impact brain development.³

1.2.1 Neuroinflammation

Exposure to alcohol during prenatal development induces a neuroinflammatory response within the central nervous system, resembling the inflammatory processes typically observed following tissue damage or infection. This inflammatory state is considered a key contributor to the neurotoxic pathways underlying fetal alcohol spectrum disorders.⁸ Alcohol intake elevates circulating concentrations of pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), interferon-gamma (IFN- γ), and tumor necrosis factor-alpha (TNF- α).⁹ In line with these findings, preclinical studies using animal models of prenatal alcohol exposure have demonstrated a pro-inflammatory shift in

cerebral gene expression profiles. Specifically, heightened levels of TNF- α , IL-6, interleukin-17 (IL-17), C-C motif chemokine ligand 2 (CCL2) and IFN- γ , interleukin-1 alpha (IL-1 α), interleukin-1 beta (IL-1 β), and cyclooxygenase-2 (COX2) have been observed.^{10,11} Concurrently, reductions in anti-inflammatory mediators, such as interleukin-10 (IL-10), and the neuroprotective chemokine CX3CL1, were noted.¹²

The precise mechanisms by which neuroinflammation contributes to neurodevelopmental impairments remain an active area of investigation. While neuroinflammation is known to be a central factor in neurodegenerative diseases¹³ emerging evidence suggests that FASD may involve overlapping pathways that underlie ethanol-induced neurotoxicity. These shared mechanisms are gradually being elucidated, offering new insights into the developmental consequences of prenatal alcohol exposure.¹⁴

E.g. elevated expression of IL-1 β , IL-6 and TNF- α is recorded in neurodegenerative diseases like Parkinson's disease and Alzheimer's disease. In these conditions, the overexpression of these cytokines contributes to pathological processes including demyelination, gliosis, and neurite degeneration. Specifically, IL-1 β triggers excitotoxic neurodegeneration by activating the apoptotic p53 pathway, while both IL-1 β and TNF- α amplify glutamate transmission, thereby increasing neuronal vulnerability to glutamate-induced excitotoxicity.^{15,16} Notably, IL-1 β , IL-6 and TNF- α serve as key initiators in microglial activation. While this process is essential for clearing cellular debris following brain injury, excessive or prolonged microglial activity can result in neuronal dysfunction. Such overactivation not only perpetuates neuroinflammation but may also induce neuronal loss by targeting stressed yet functional neurons through phagoptosis.¹⁷ A similar process can be observed with ethanol-induced neurotoxicity. Ethanol exposure triggers an excessive neuroinflammatory response. This dysregulated reaction causes microglia and astrocytes to exacerbate disease progression rather than mitigate damage. In their activated M1 state, microglia liberate pro-inflammatory cytokines, including IL-1, IL-6, TNF- α , and IFN- γ , which subsequently induce astrocytes to adopt a pro-inflammatory A1 phenotype. In this functional state, astrocytes not only contribute to the elimination of damaged neurons and oligodendrocytes, but also lose their ability to maintain synaptic function and perform phagocytosis.^{18,19} ²⁰ During pregnancy, microglia play a pivotal role in supporting neural precursor cell differentiation and survival, stimulating oligodendrogenesis, and facilitating vascular development. However, prenatal alcohol exposure disrupts these processes. Studies indicate that microglial activation due to PAE reduces neuronal survival and proliferation, resulting in hypoplasia and reduced dopaminergic connections.²¹

The ethanol-induced shift toward a pro-inflammatory M1 microglial phenotype is not merely a transient response but may represent a long-term reprogramming of the neuroimmune environment. This chronic inflammatory state, often sustained through epigenetic modifications²², creates a neurotoxic milieu that contributes to the persistent cognitive deficits observed in FASD.⁸ Consequently, therapeutic strategies aimed at promoting a phenotypic switch from the detrimental M1 state to the neuroprotective M2 state are of significant interest.¹⁴

Another hallmark of neuroinflammation is oxidative stress, characterized by a disparity between oxidative processes and antioxidant defenses. In the brain, ethanol is primarily metabolized by catalase (CAT) and aldehyde dehydrogenase (ALDH).²³ However, the fetal environment differs within the placenta, cytochrome P450 2E1 (CYP2E1) serves as the principal metabolizing enzyme. Ethanol exhibits a higher affinity for placental CYP2E1 compared to ADH. Through CYP2E1-mediated oxidation, ethanol generates hydroxyethyl or superoxide radicals. These radicals react with polyunsaturated fatty acid side chains in brain tissue membranes, leading to damage during critical periods of organogenesis.³ Figure 2 illustrates the link between ethanol exposure, oxidative stress and, central nervous system (CNS) dysfunction

Animal studies further reveal that prenatal and adult alcohol exposure reduces the levels of key antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, in brain tissue. Additionally, a single alcohol exposure has been observed to enhance NADPH oxidase (NOX) activity in both fetal and adult mouse brains, thereby amplifying reactive oxygen species (ROS) activity.²⁴ ²⁵ The resulting free radicals and ROS may trigger uncontrolled apoptosis of cranial neural crest cells, contributing to alterations in facial morphology. The brain's elevated lipid concentration renders it especially vulnerable to lipid peroxidation, a process of oxidative damage that compromises cellular membranes. This phenomenon is observed in neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, and Down syndrome.²⁶

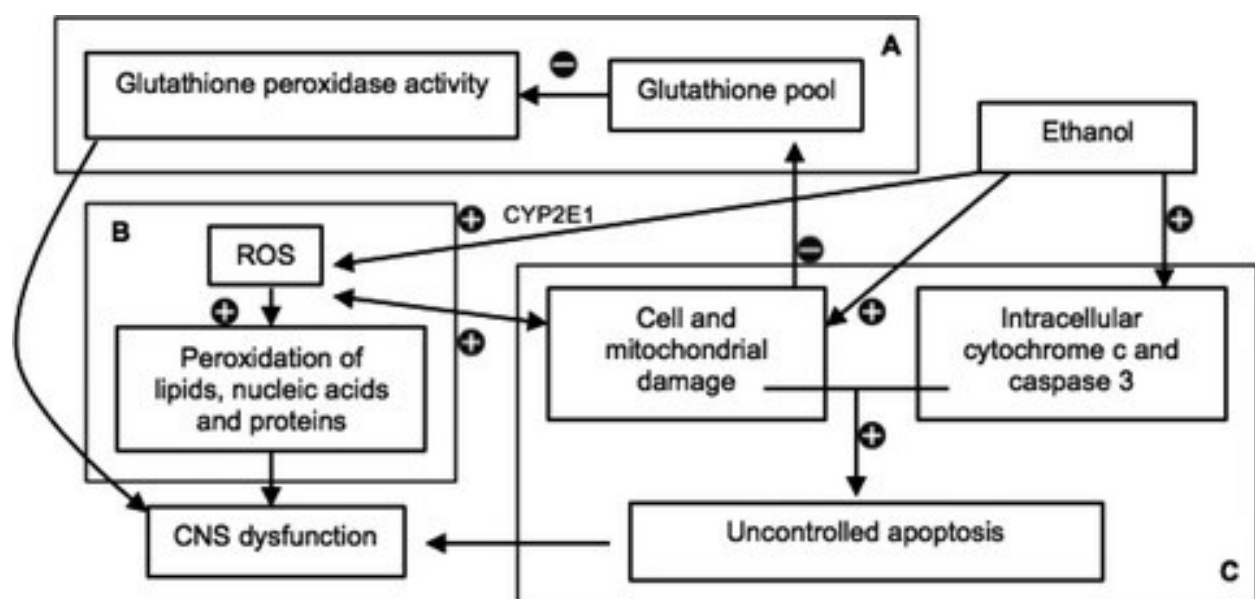


Figure 2: Overview of Ethanol-Mediated Mechanisms Contributing to Central Nervous System (CNS) Dysfunction: (A) Indirect Oxidative Stress Pathway: Ethanol exposure reduces the activity of glutathione peroxidase, a key endogenous antioxidant enzyme. This decline weakens the cellular defense against oxidative damage, thereby increasing susceptibility to oxidative stress. (B) Direct Oxidative Stress Pathway Ethanol metabolism via cytochrome P450 2E1 (CYP2E1) generates reactive byproducts, including hydroxyethyl and hydroxyl radicals. These free radicals and reactive oxygen species (ROS) induce peroxidation of lipids, nucleic acids, and proteins, leading to cellular damage. (C) Additional Cellular Effects of Ethanol: Beyond oxidative stress, ethanol disrupts cellular and mitochondrial integrity, triggering the release of cytochrome c and activation of caspase 3. This cascade ultimately results in uncontrolled apoptosis, contributing to fetal damage.⁶

Given the central role of neuroinflammation and synaptic dysfunction in FASD, identifying endogenous regulatory systems capable of modulating these processes is crucial.⁸ The endocannabinoid system (ECS)

has emerged as a key candidate due to its extensive involvement in neurodevelopment , immune regulation , and synaptic homeostasis.²⁷

1.3 The Endocannabinoid System

The endocannabinoid system comprises endocannabinoids, their corresponding receptors and the enzymes orchestrating their synthesis and degradation.

Current research confirms the extensive role of the endocannabinoid system across almost all physiological functions. These include digestion, reproductive activity, central nervous system regulation, endocrine balance, sleep modulation, and numerous additional processes.

1.3.1 Cannabinoid Receptors

Both cannabinoid receptors, the cannabinoid receptor 1 (CB1) and the cannabinoid receptor 2 (CB2) are under most physiological circumstances Gi/o-coupled and therefore modify adenylyl cyclases. The CB1 receptor is mainly expressed in the central nervous system, especially in the neurons of the cerebral cortex and the hippocampus where the receptor mediates its effects on among other things on memory and cognition. The presynaptic CB1 receptor lowers the release of neurotransmitters via stimulation of potassium channels and inhibition of calcium channels.^{27,28} Upon activation by endogenous cannabinoids or exogenous ligands, CB1 receptors initiate three waves of intracellular signaling: a first transient phase mediated by G proteins, followed by a β -arrestin-mediated phase, and a third wave occurring at intracellular compartments, which can be induced by either G proteins or β -arrestins. This complex signaling architecture allows CB1 receptors to take part in diverse physiological and pathophysiological processes.²⁹

The effect of the activation of both CB1 and CB2 on the adenylyl cyclases is context-dependent: adenylyl cyclases 1, 5, 6 and 8 are inhibited, adenylyl cyclases 2, 4, 7 are activated.³⁰ Under normal circumstances CB agonism leads to a reduction in cyclic adenosine monophosphate (cAMP) and a consequently a reduced activity of protein kinase A (PKA), as seen in Figure 3. Additionally, agonism of cannabinoid receptors can result in activation of mitogen-activated protein kinase (MAPK).²⁵

The CB2 receptor is densely localized in immune cells and peripheral tissues but is also distributed throughout the central nervous system (e.g. in the amygdala). In contrast to CB1 it does not typically modulate ion channels.³¹ CB2 receptors in neurons are predominantly localized postsynaptically, where its activation can cause hyperpolarization.^{32 33} The CB2 receptor takes part in the modulation of neuroinflammation, the immune system and neuroprotection. Therefore, it could play an important role in many neurodegenerative and psychiatric diseases.

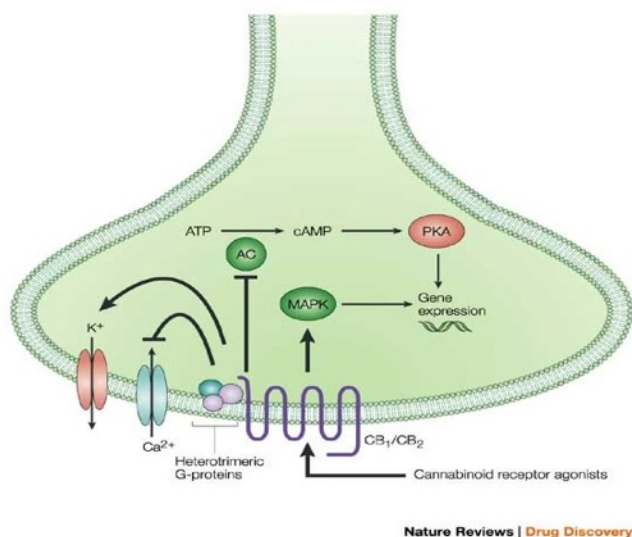


Figure 3: Signaling pathway of activated cannabinoid receptors²⁷ Activation of CB1R (cannabinoid receptor 1) and CB2R (cannabinoid receptor 2) leads either to inhibition of adenylyl cyclase (AC) and thereby inactivation of the protein kinase A (PKA) or to activation of mitogen-activated protein kinase (MAPK).

1.3.2 Endocannabinoids

The two most known and relevant endogen ligands of the cannabinoid receptors are anandamide (Figure 5) and 2-arachidonoglycerol (Figure 4). They modulate the synaptic function via retrograde signalling: Postsynaptic action activates the production of endocannabinoids. The endocannabinoid acts on the pre-synaptic CB1 receptor, where it inhibits the release of neurotransmitters via 2 mechanisms. They can induce short term plasticity via inhibition of presynaptic Ca²⁺ channels and long term plasticity via inhibition of adenylyl cyclases.³⁴

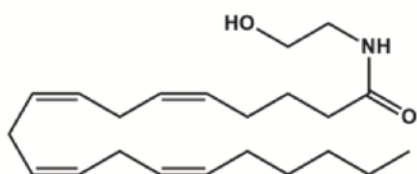


Figure 5: Anandamide³⁵

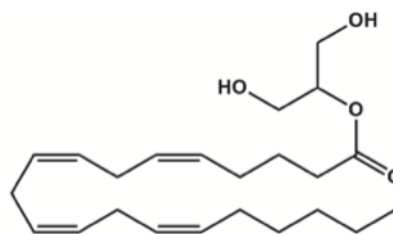


Figure 4: 2-Arachidonoylglycerol (2-AG)³³

Anandamide is a partial agonist for the CB1 and CB2 receptors, whereas 2-AG functions as a full agonist for CB1 and CB2 receptors, but with a higher affinity for CB1.^{36 37}

The biosynthesis and release of endocannabinoids, particularly anandamide, involve several enzymatic steps. N-acyltransferase catalyzes the transfer of an acyl group to phosphatidylethanolamine, generating N-acylphosphatidylethanolamine (NAPE). N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) then hydrolyzes NAPE to release anandamide and other N-acylethanolamines. Alternative biosynthetic pathways for anandamide include sequential actions of phospholipase A2 and lysophospholipase D, as well as phospholipase C-mediated hydrolysis followed by dephosphorylation by

specific phosphatases.²⁷ After its release, anandamide is primarily hydrolyzed and inactivated by fatty acid amide hydrolase (FAAH), which cleaves it into arachidonic acid and ethanolamine.³⁸

The endocannabinoid system is important for neurodevelopment, controlling processes such as neuronal proliferation, migration, differentiation, and synaptic refinement throughout embryogenesis and adolescence. Disruption of endocannabinoid signaling during critical developmental windows, such as by prenatal alcohol exposure, is increasingly recognized as a key contributor to the pathophysiology of fetal alcohol spectrum disorder. Alcohol alters endocannabinoid levels and CB1 and 2 receptor expression, leading to aberrant neurotransmitter signaling, impaired synaptic plasticity, and neuroinflammation, which underlie the cognitive, emotional, and behavioral deficits observed in FASD.^{39 40} These molecular and cellular disturbances are further modulated by epigenetic mechanisms, linking ECS dysregulation to long-term neurodevelopmental outcomes.²²

1.4 CBD and FASD

Various animal models have been created to simulate Fetal Alcohol Spectrum Disorder (FASD) and to investigate the molecular and behavioral changes associated with these conditions. These models employ diverse experimental methods, including administering different alcohol concentrations to pregnant rodents or their offspring via oral gavage or subcutaneous injection at various stages of gestation or postnatal development.⁴¹ Emerging evidence suggests that early and sustained administration of cannabidiol (CBD) may counteract the adverse effects of alcohol consumption through multimodal mechanisms targeting key molecular pathways.

Ethanol-exposed offspring exhibit pronounced anxiogenic and depressive-like phenotypes, alongside emotional hyperreactivity. CBD administration effectively restores many of these behavioral deficits in both sexes, through cognitive improvements, assessed via recognition and aversive memory tasks. These effects are more consistent in males. This discrepancy may reflect sex-specific vulnerability to ethanol's neurodevelopmental disruption, potentially linked to differential modulation of the HPA axis and endocannabinoid system.^{42 43}

CBD is a negative allosteric modulator of CB1⁴⁴ and an antagonist of CB2⁴⁵. CB1 and CB2, when activated by agonists, inhibit adenylyl cyclase, leading to decreased cAMP levels and reduced PKA activity. By negatively modulating CB1 and antagonizing CB2, CBD can reduce the inhibitory effect of cannabinoid agonists on adenylyl cyclase, resulting in elevated cAMP levels and increased PKA activity, which consequently activates cAMP response element-binding protein (CREB) and downstream gene transcription.⁴⁶ This could lead to increased expression of arginase-1⁴⁷, CD206⁴⁷, interleukin-10 (IL-10)⁴⁸, and interleukin-4 (IL-4)⁴⁷ through modulation of key transcriptional pathways in microglia. This is a protein expression pattern that is typical for the microglia M2 type and helps to inhibit neuroinflammatory effects via promotion of a shift from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype.⁴⁹ Arginase-1 catalyses the conversion of arginine to ornithine, which leads to a reduced

substrate for inducible nitric oxide synthase (iNOS). Nitric oxide (NO) production is lowered which has an antiinflammatroy effect.⁵⁰ CD206 is a mannose receptor which can recognize the surface of cell debris and pathogens and therefore is an important marker for neuroprotective M2 microglia.⁵¹ Heightened IL-10 inhibits the secretion of inflammatory cytokines TNF α and IL-1 β .⁵² IL-4 also lowers TNF α and IL-1 β and supports the expression of arginase-1 and CD206.⁵³

Figure 6 shows the antiinflammatory mechanism of CBD on the NLRP3 inflammasome via the PPAR γ (peroxisome proliferator-activated receptor γ) signaling pathway in LPS-triggered microglial cells. The inhibition of iNOS can also be mediated by PPAR γ .⁵⁴

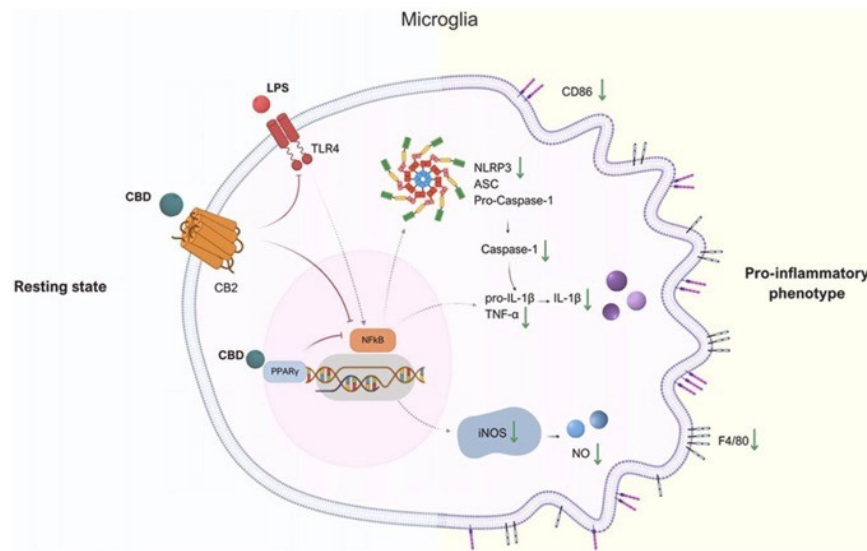


Figure 6: Anti-inflammatory effects of CBD via CB2R (cannabinoid receptor 2) and PPAR γ (peroxisome proliferator-activated receptor γ)⁵⁴ NF- κ B (Nuclear Factor kappa-light-chain-enhancer of activated B-cells) is inhibited which leads to several antiinflammatory effects. The inhibition of iNOS (inducible nitric oxide synthase) leads to a reduction in NO (nitric oxide).

In-vitro studies showed that CBD mediates anti-inflammatory effects in LPS-stimulated macrophages via transient receptor potential vanilloid receptor 1 (TRPV1).⁵⁵ CBD and anandamide act as TRPV1 agonists^{34,56}. For capsaicin, another TRPV1 agonist, a reduction in TNF α and IL-6 was shown.⁵⁶

Indirect mechanisms also play an essential role in the anti-inflammatory effects of CBD. Specifically, CBD inhibits the degradation of anandamide by FAAH, resulting in elevated systemic anandamide levels.⁵⁷ At first glance this seems paradoxical, because as an CB agonist anandamide can lower cAMP levels and therefore enhance proinflammatory effects. However, in the context of FASD, CB2 receptors may be overexpressed,⁵⁸ which might lead to a switch from Gi- to Gs coupling.⁵⁹ Therefore cAMP levels could be heightened. Furthermore, anandamide inhibits the degradation of I κ B, thereby reducing the translocation of the pro-inflammatory transcription factor NF- κ B (Nuclear Factor kappa-light-chain-enhancer of activated B-cells). Additionally, signaling through CB2 receptors, likely mediated via the Erk1/2 and JNK pathways, can promote the upregulation of the anti-inflammatory cytokine IL-10.⁶⁰

At the molecular level, ethanol exposure alters the expression of critical regulators within the HPA axis and endocannabinoid system. Under normal circumstances a spike in Crf (corticotropin-releasing factor) will lead to a downregulation of Nr3c1 (glucocorticoid receptor) in the hippocampus. Under ethanol

exposure this regulation mechanism is impaired in males. In males, CBD normalizes the upregulation of Crf in the paraventricular nucleus and Nr3c1 in the hippocampus, aligning with reduced emotional dysregulation. Conversely, females show limited responsiveness to CBD in these pathways, suggesting a dose-dependent or receptor-mediated divergence in therapeutic efficacy. Ethanol increases CB1 expression in males, a change reversed by CBD, while females exhibit baseline reductions in CB1 and CB2 and their ethanol-induced alterations (a reduction in CB1 and an increase in CB2) remain unmodulated by treatment. These findings imply that CBD's indirect agonism of CB1, via anandamide elevation, may underlie its neuroprotective effects in males, whereas alternative mechanisms (e.g., CB2 antagonism) could contribute to partial benefits in females.⁴²

Ethanol compromises hippocampal architecture, reducing neuronal survival (NeuN+ cells) and impairing synaptic plasticity. CBD mitigates these deficits by enhancing neurogenesis and restoring brain-derived neurotrophic factor (BDNF) signaling, a critical mediator of memory consolidation and glutamatergic transmission. Immunohistochemical analyses reveal that CBD reinstates the laminar distribution of Vesicular Glutamate Transporter 1 (VGLUT1), markers of excitatory synapses, in the CA3 and CA1 subfields, indicating repaired connectivity within the hippocampal circuit.

Metabolomic profiling reveals that perinatal ethanol exposure disrupts several key metabolic pathways involved in purine ribonucleosides conversion to ribose-1-phosphate, sirtuin signaling pathway, purine nucleotides degradation (aerobic), Nicotinamide adenine dinucleotide (NAD) biosynthesis (from tryptophan), phenylalanine degradation, and ferroptosis signaling. All these pathways could be modulated via CBD administration.

Notably, mitochondrial targets, including oxidative phosphorylation, remain unaffected by both ethanol and CBD, highlighting the selective neuroprotective profile of CBD.

Peroxisome proliferator-activated receptor β/δ (PPAR β/δ), a nuclear receptor implicated in neuronal differentiation, is downregulated in ethanol-exposed offspring. CBD restores its expression in males, potentially contributing to cognitive recovery. Given PPAR β/δ 's role in attenuating alcohol-induced neuronal damage, this interaction may represent a novel therapeutic axis for FASD.⁴²

1.5 Molecular Markers of Neurodevelopmental and Signaling Integrity

To evaluate the efficacy of therapeutic interventions such as CBD, specific molecular markers are utilized to assess the biochemical alterations induced by prenatal alcohol exposure. These proteins serve as critical indicators of the underlying pathophysiology of FASD, reflecting disruptions in neurodevelopment, cellular homeostasis, and neurotransmission.

1.5.1 mTOR (mechanistic target of Rapamycin)

The mTOR serine/threonine kinase is a central regulator of essential cellular processes, including growth, protein homeostasis, and neurodevelopmental stages such as neuronal differentiation and proliferation. Chronic ethanol exposure has been shown to activate the mTOR system while simultaneously impairing autophagy, a vital process for maintaining cellular homeostasis. Specifically, fetal alcohol exposure can lead to a reduction in the phosphorylation of mTOR itself, while increasing the phosphorylation of downstream targets such as 4E-BP1 and p70 S6K. This dysregulation contributes to the generation of reactive oxygen species (ROS) and subsequent neuroapoptosis. Research indicates that these effects, including oxidative stress and associated behavioral deficits, can be reversed by the mTORC1 antagonist rapamycin.^{61 62}

1.5.2 AKT (Protein kinase B)

AKT is a key component of a neuroprotective signaling pathway situated downstream of tropomyosin receptor kinase B (TrkB) activation. Consequently it can activate mTOR.⁶³ Neonatal alcohol exposure disrupts this signaling cascade, resulting in a significant reduction in the phosphorylation of AKT (pAKT). This decrease in pAKT levels is closely associated with increased neurotoxicity and compromised neuronal survival.⁶⁴

1.5.3 DARPP-32 and DAT

- Dopamine- and cAMP-regulated phosphoprotein (DARPP-32, 32 kDa) is a critical component of the downstream signaling of dopamine receptors. Alcohol-induced activation of DARPP-32 can shift the balance between dopamine D1 and D2 receptors, leading to characteristic behavioral abnormalities.^{65 66}
- Dopamine Transporter (DAT) is frequently upregulated in FASD. This upregulation impairs dopamine signaling, which is linked to the affective dysregulation and mood instability observed in affected individuals.⁶⁷

1.5.4 AMPK (Adenosine monophosphate-activated kinase)

AMPK serves as a crucial marker for metabolic and mitochondrial dysfunction. Ethanol exposure inhibits both mitochondrial proliferation and the synthesis of adenosine triphosphate (ATP). The resulting ATP deficiency triggers the activation of AMPK, which subsequently can inhibit mTOR and the synthesis of fatty acids and cholesterol, further disrupting neural metabolism.^{68 69}

1.5.5 Iba1 (Ionized Calcium-binding adapter molecule 1)

Iba1 is expressed specifically in microglia and used as an immunohistochemical marker to identify microglial cells and study their activation status and morphological changes in brain tissue. Alcohol exposure

during development induces microglial activation and apoptosis as a neuroinflammatory reaction, which is a key indicator of neuroimmune responses and pathological changes in the developing brain associated with FASD.⁷⁰

1.5.6 GFAP (Glial fibrillary acidic protein)

GFAP is a type III intermediate filament and serves as a definitive marker for mature astrocytes. Predominantly expressed as the α -isoform, GFAP constitutes the primary cytoskeletal component and provides the structural scaffold essential for maintaining astrocytic morphology.⁷¹ Prenatal alcohol exposure can lead to significant reduced GFAP expression, which indicates that prenatal alcohol exposure impairs astroglial precursor cells, suggesting that dysregulated astrogliogenesis may serve as a mechanistic basis for the disrupted neuronal migration and other CNS abnormalities associated with fetal alcohol exposure.⁷² In contrast to that heightened GFAP is a sign of reactive astrocytes and neuroinflammation which shows the bidirectional and complex role of GFAP in FASD.⁷³

Building on the discussed pathophysiological links between ethanol exposure and the endocannabinoid system, an experimental study was conducted to evaluate the therapeutic potential of CBD. The following chapter outlines the structure of this investigation, including the animal model used and the specific analytical procedures applied to obtain the results.

2. Materials and Methods

2.1 Animal model

Ethics statements

The protocols for animal care and use were approved by the Ethics and Research Committee at the University of Malaga (CEUMA registry no. 128-2022-A, date: November 20, 2022). All experimental procedures were performed in strict accordance with the Directive 2010/63/EU of the European Parliament and the EU Council (22 September 2010) and the Spanish regulation (RD 53/2013) on the protection of animals used for scientific purposes. We carried out the experiments in strict terms to minimize animal suffering and to reduce the number of animals used. Animal studies comply with the ARRIVE guidelines.

2.1.1 Animals and drugs

For this study, male and female C57BL/6 mice at reproductive age (45 days) were used. The rodents were accommodated at the Animal Experimentation Center of the University of Málaga, which adheres to all relevant regulations. The animals were kept in groups of four, with unlimited access to standard chow (2.9 kcal/g) and water. The environment was controlled with an ambient temperature of 20 ± 2 °C, relative humidity of $40 \pm 5\%$, and a 12-hour light/dark cycle (from 9:00 to 21:00 hours) that includes a sunrise/sunset effect. Food and water were provided ad libitum. The experimental design comprised multiple groups: a control group (Agua Veh) receiving only isotonic saline solution, a group administered ethanol, a group treated with ethanol and cannabidiol (CBD, PhytoPlant Research S.L.), a group receiving ethanol and the CB₁ receptor inverse agonist AM251 (Tocris Bioscience, #1117), a group treated with ethanol and the CB₂ receptor inverse agonist AM630 (Tocris Bioscience, #1120), a group administered ethanol, CBD and AM251, and a final group receiving ethanol, CBD, and AM630.

2.1.2 Experimental design

The maternal mice were administered ethanol four days per week, with alcohol availability lasting two hours per day on three consecutive days and four hours on the fourth day. This regimen was maintained for two weeks prior to gestation, continued throughout gestation, and extended into the lactation period. The remaining treatments—including cannabidiol (20 mg/kg), AM251 (0.3 mg/kg), and AM630 (0.3 mg/kg)—were administered subcutaneously 30 minutes before alcohol consumption, both during gestation and lactation. An overview of the different groups is presented in Figure 7 At postnatal day 21 (PND21), the offspring was euthanized, and the brains were harvested for subsequent analysis.

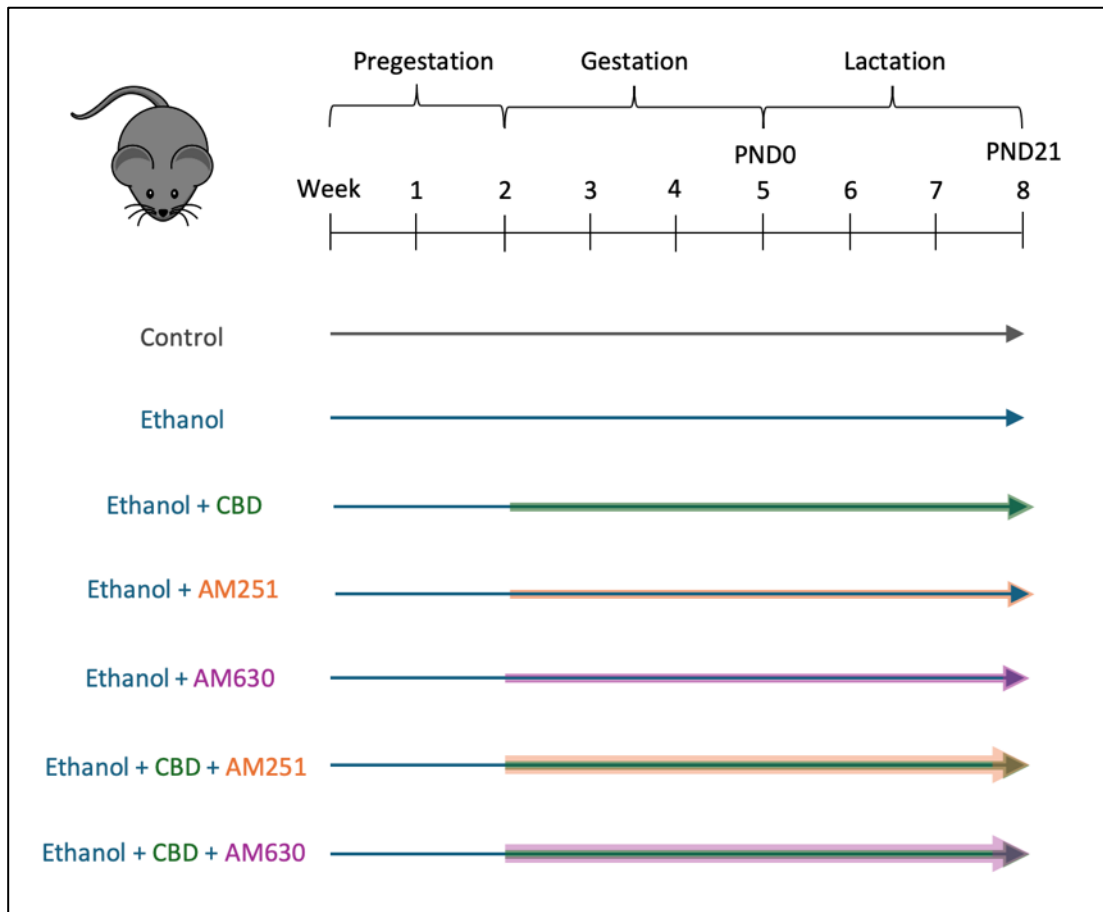


Figure 7: Animal model: different trial groups

2.2 Extraction of proteins

For protein extraction, the hippocampi (of one hemisphere, the hippocampus of the second one was used for the immunohistochemistry) were initially homogenized in 1 mL Trizol (Invitrogen, #15596026) followed by centrifugation.

The pink phenol phase was separated and after 5 minutes 200 μ L chloroform was added. After a second centrifugation the pink phase was transferred into a 2 mL protein LoBind[®] tube. Cold ethanol (300 μ L) was added, mixed by inversion, and incubated for 2–3 minutes at room temperature. After centrifugation, the supernatant was transferred to a new tube, and proteins were precipitated with 1 mL of cold isopropanol. Following a 10-minute incubation at room temperature, the sample was centrifuged again.

The pellet was washed twice with 2 mL of guanidine hydrochloride in 96% ethanol, incubating for 20 minutes at room temperature each time, followed by centrifugation. After a final wash with 2 mL of 100% ethanol, the pellet was dried in a fume hood for 10 minutes. The pellet was resuspended in 50–100 μ L of protein buffer (and vortexed until dissolved). The samples were incubated at 50°C for 1.5 hours, followed by the addition of dithiothreitol (DTT) and heating at 99°C for 5 minutes. Samples were stored at -20°C until Western blot analysis.

Table 1: Inhibitors added to protein buffer, 5 μ L each of protease inhibitor mix and phosphatase inhibitor mix were added

Inhibitor	Final concentration	Company	Code
Phenylmethyl-sulfonylfluoride	1 mM	ThermoFisher Scientific	36978
Pepstatin	1 µg/mL	Sigma-Aldrich	P4266
Leupeptin	5 µg/mL	Santa Cruz	Sc-215243A
Aprotinin	2 µg/mL	Santa Cruz	Sc-3595
Trypsin inhibitor	10 µg/mL	Roche	10109886001
Orthovanadate	1 mM	Sigma-Aldrich	56508
Sodium fluoride	1 mM	Fuica	7519
Protease inhibitor mix		Milipore	535140
Phosphatase inhibitor mix		Milipore	524627

2.3 Western blot

For Western blot analysis, the extracted proteins were initially denatured at 99°C for 5 minutes, followed by vortex homogenization. Protein separation was subsequently performed using gel electrophoresis. 15 µL of protein sample were loaded per well. To indicate molecular weights, 1.5 µL and 3 µL of Full-Range Rainbow™ Molecular Weight Markers (Cytiva, RPN800E) were added to the first and last wells, respectively. The gel (Bio-Rad Criterion™ XT Precast Gel, #3450124) was supplemented with MES (2-(N-morpholino)ethanesulfonic acid) buffer (MES 1:20) or alternatively, MOPS (3-(N-morpholino)propanesulfonic acid) buffer (MOPS 1:10), and proteins were resolved at 140 V in a Criterion™ Cell (Bio-Rad,

135BR0023699, USA). After electrophoretic separation, the resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad, 0.2 μ m, #1620112) using a Criterion™ Blotter (Bio-Rad, 560BR25089, USA). The transfer was conducted in transfer buffer (1x) at a constant voltage of 100 V for 45 minutes. To confirm efficient protein transfer, the membrane was stained with Ponceau Red solution, allowing visualization of the transferred bands. Following verification, the membranes were cut into strips to enable the targeted analysis of individual proteins of interest (Figure 8 and 9).

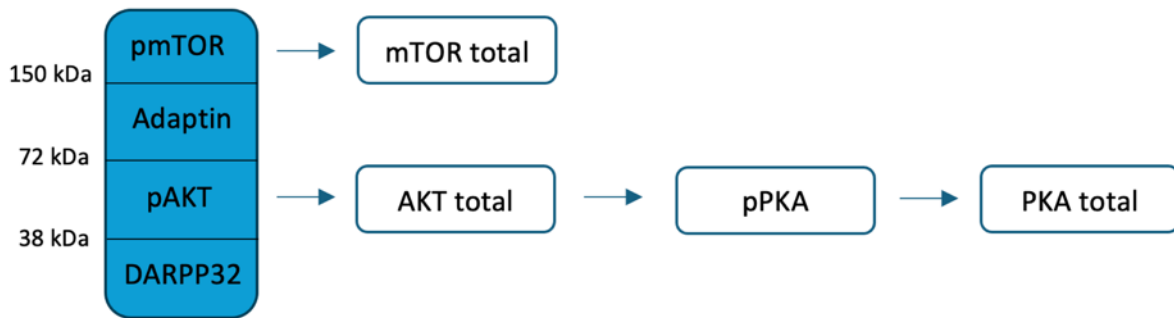


Figure 9: Revealed proteins of the membrane of gel 1 and location of performed cuts

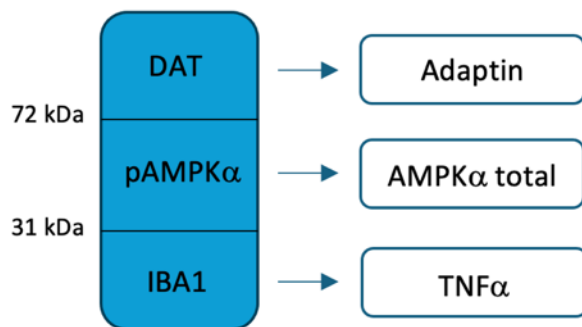


Figure 8: Revealed proteins of the membrane of gel 2 and location of performed cuts

Following membrane excision, the strips were blocked for 1 hour in 2% bovine serum albumin (BSA, Serva® albumin bovine fraction V, Protease-free, 9048-46-8) dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Subsequently, the membranes were incubated overnight at 4°C with the respective primary antibodies.

The following day, the membranes underwent three washing steps in TBS-T, each lasting 10 minutes. They were then incubated for 1 hour with species-specific secondary antibodies (anti-rabbit or anti-mouse, diluted 1:10,000). For the detection of dopamine transporter (DAT), a rat-derived primary antibody was used, necessitating the application of a donkey anti-rat secondary antibody. Additionally, this required a 1-hour incubation with ExtraVidin® (Sigma-Aldrich, #019M4817V; 1:1000 in 2% BSA), a step not required for the other secondary antibodies.

Afterward, the membranes were subjected to three additional washing steps in TBS-T, each lasting 10 minutes. For signal detection, the membranes were treated with either Amersham ECL™ Prime Western

Blotting Detection Reagent (Cytiva, RPN2236) or, if required, Clarity Max™ Western ECL Substrate (Bio-Rad, #1705062), and visualized using an imaging system (ChemiDoc™ MP Imaging System, Bio-Rad, USA). Should subsequent detection of an additional protein be required, the membranes were stripped of bound antibodies. For this purpose, the membranes were incubated with 80 µL β-mercaptoethanol in 10 mL stripping buffer for 30 minutes at 60°C. If the membrane was stripped more than one time, incubation time was lowered to 15 minutes. Following stripping, the membranes were rinsed thoroughly with water for 40 minutes, after which the procedure was repeated starting with the blocking step. However, if a different secondary antibody was to be applied (e.g., switching from anti-rabbit to anti-mouse), a full stripping procedure was not necessary. Instead, the membranes were washed for 15 minutes with TBS-T containing a spatula tip of sodium azide (NaN₃). Under these conditions, no additional blocking was required, and the next primary antibody could be applied directly.

Table 2: Buffers used for Western Blot

Buffer	Recipe
TBS	24,2 g tris base + 80 g NaCl up to 1000 mL with water (pH = 7.6)
TBS-T	TBS 1:10 with water + 1 mL Tween™ 20
Transfer buffer (1x)	100 mL Transfer buffer (10x) + 200 mL methanol + 700 mL water
Transfer buffer (10x)	30.3 g Tris + 144 g glycine up to 1000 mL with water
Stripping buffer	14.2 mL Tris (0.5 M) + 10 mL SDS (20%) up to 100mL with water

Table 3: Primary Antibodies used for Western Blot

Target	Host	Company	Code	Dilution	Molecular weight (target)
Adaptin	Mouse	BD Biosciences	610385	1:2000	104
AKT total	Rabbit	Cell Signaling	9272S	1:1000	60
pAKT	Rabbit	Cell Signaling	4060S	1:1000	60
AMPKα total	Rabbit	Cell Signaling	4150P	1:1000	62
pAMPKα	Rabbit	Cell Signaling	2535S	1:1000	62
DARPP32	Rabbit	Cell Signaling	2302G	1:500	33
DAT	Rat	Chemicon	MAB369	1:1000	80
IBA 1	Rabbit	Cell Signaling	17198S	1:500	18
mTOR total	Rabbit	Cell Signaling	2972S	1:1000	200
pmTOR	Rabbit	Cell Signaling	2971S	1:1000	200
PKA total	Rabbit	Santa Cruz	SC-903	1:1000	43
pPKA	Rabbit	Santa Cruz	SC-32968	1:200	43
TNFα*	Rabbit	Cell Signaling	3707	1:1000	26

Table 4: Secondary Antibodies used for Western Blot, *no quantifiable results could be achieved for TNF alpha.

Target	Company	Code
Rabbit	Promega	W401B
Mouse	Promega	W402B
Rat	Novex	A18743

Quantification of phosphorylated proteins was executed by normalizing phospho-specific signals to their associated total protein signals, and total protein signals were normalized to Adaptin. All results are expressed relative to the control group.

2.4 GFAP immunohistochemistry

Tissue Preparation: Mouse brains were perfused with 4% paraformaldehyde and stored at 4°C in a 30% sucrose solution. The brains were then cut into 30 µm sections using a freezing microtome and stored in PBS (Phosphate-Buffered Saline) with 0.02% NaN₃ at 4°C.

Protocol Steps: If not stated differently, the steps were performed at room temperature and with agitation. Sections were washed 3 times with PBS-Tx (Phosphate-Buffered Saline with Triton X100) for 10 minutes each. Following this, sections were incubated with 10% H₂O₂ and 10% methanol in PBS for 20 minutes to block endogenous peroxidase. After another wash with PBS-Tx for 10 minutes, sections were incubated with 10% goat serum (Sigma-Aldrich, G9023-5ML), 0.3% Triton X-100 (Sigma-Aldrich, T92884), and 0.1% NaN₃ in PBS for 60 minutes. Next, sections were incubated with the primary antibody (Anti-GFAP made in mouse, Dako, #20063373; 1:500 in PBS-Tx with 0.01% azide) overnight at 4°C.

Following primary antibody incubation, sections were washed 3 times with PBS-Tx for 10 minutes each at room temperature. Sections were then incubated with IgG-anti-mouse secondary antibody made in goat (Sigma-Aldrich, B7254-2ML) for 1 hour. After another 3 washes with PBS-Tx for 10 minutes, sections were incubated with ExtrAvidin®–Peroxidase (1:2000 in PBS Tx) for 1 hour in darkness. Sections were then washed 3 times with PBS (no Tx) for 10 minutes each in darkness. For visualization, sections were developed with DAB (3,3'-Diaminobenzidine, Sigma-Aldrich, #BCCC2073; 0.05% in PBS) for 10 minutes with agitation and 0.03% H₂O₂ (1 µL per mL DAB-Solution) was added. The color development was monitored under a microscope. After about 5-10 minutes (no agitation) sections were washed with PBS (no Tx) for 5 minutes, mounted on slides, allowed to dry overnight. The next day they were immersed in 70%, 95%, 100% ethanol and xylol. After that they were glued with DPX new (Sigma-Aldrich, 1005790500).

Table 5: Buffers and reactives used for immunohistochemistry.

Buffer	Recipe
PB	13.8 g NaH ₂ PO ₄ *H ₂ O + 53.4 g Na ₂ HPO ₄ *2 H ₂ O up to 1000 mL with water (pH = 7.3)
PBS	250 mL PB + 750 mL water + 9 g NaCl (pH = 7.3)

PBS-Tx	1 L PBS + 2 mL Triton X100
Citrate buffer	2.69 g sodium citrate up to 200 mL with water (pH = 6)

2.5 Iba1 immunohistochemistry

Tissue Preparation: Mouse brains were perfused with 4% paraformaldehyde and stored at 4°C in PB (Phosphate buffer) with 0.02% NaN₃. The brains were then cut into 30 µm sections using a microtome.

Protocol Steps: If not stated differently, the steps were performed at room temperature and with agitation. Sections were washed with PBS-Tx 100 (0.2%) 3 times for 10 minutes each. Following this, sections were incubated with citrate buffer pH 6 for 20 minutes at 80°C. After another 3 washes with PBS for 10 minutes each, sections were incubated with 10% methanol and 10% H₂O₂ in PBS for 20 minutes to block endogenous peroxidase. After another wash with PBS for 10 minutes (3x), sections were incubated with 10% donkey serum (Sigma-Aldrich, 00002800319) in PBS for 1 hour. Next, sections were incubated with the primary antibody (Anti-Iba1 made in rabbit, Wako, WTE6260; 1:1000 in PBS-Tx with 0.01% azide) overnight at 4°C.

Following the primary antibody incubation, sections were washed with PBS-Tx for 10 minutes (3x). Sections were then incubated with the secondary antibody (IgG-anti-rabbit made in donkey, AffiniPure, 711-167-003; 1:2000 in PBS-Tx) for 1 hour at room temperature. After another 3 washes with PBS-Tx for 10 minutes, sections were incubated with ExtrAvidin®-Peroxidase (1:2000 in PBS and Triton X-100) for 1 hour in darkness. Following 3 washes with PBS (no Tx) for 10 minutes each, sections were developed with DAB (0.05% in PBS) for 20 minutes in darkness and 0.03% H₂O₂ (1 µL per mL DAB-Solution) was added. After about 5-10 minutes (no agitation) sections were washed with PBS (no Tx) for 5 minutes, mounted on slides and allowed to dry overnight. The next day they were dehydrated in 70%, 95%, 100% ethanol and xylol. After that they were glued with DPX new.

2.6 Statistics

Data are presented as the mean ± standard error of the mean (SEM), with sample sizes (n) specified for each experiment. Group differences were evaluated using two-way and three-way analyses of variance (ANOVA). For the two-way ANOVA, alcohol exposure (saline or alcohol) and treatment (e.g., vehicle, CBD, or antagonists) were considered as independent variables. For the three-way ANOVA, the factors sex (male or female), alcohol exposure, and treatment were included in the model. Significant effects were further analyzed using Tukey's post-hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$. All analyses were performed using IBM SPSS Statistics 22 and GraphPad Prism 9.

3. Results and Discussion

3.1 Western blot

TNF- α could not be quantified due to low signal intensity, and Western blot data for Iba1 were not included in the analysis because the immunohistochemistry-derived measures were more informative.

3.1.1 Phosphorylated mTOR (pmTOR)

We analysed pmTOR using a factorial model as prespecified. The three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) revealed a significant main effect of Treatment ($F(5, 83) = 2.658, p = 0.028$), with no main effects of Sex or Alcohol and no interactions (all $p > 0.05$), consistent with the planned analysis framework. To increase power and verify robustness of the Treatment effect, we ran a two-way ANOVA collapsing Sex (Alcohol $\times$ Treatment). Treatment remained significant ($F(5, 90) = 2.574, p = 0.032$), while Alcohol was not. Tukey post-hoc tests showed higher pmTOR for AM630 versus Veh ($p = 0.029$), CBD ($p = 0.035$), and AM251 ($p = 0.016$), and for CBD+AM251 versus Veh ($p = 0.033$), CBD ($p = 0.038$), and AM251 ($p = 0.018$); all other contrasts were not significant. Given the incomplete factorial under saline for the antagonist/combination levels, we conducted the prespecified two-way ANOVAs within the EtOH condition, separately for males and females.

In males (EtOH groups only, Figure 10), Treatment was significant ($F(5, 46) = 3.792, p = 0.006$). Tukey post-hoc tests indicated that AM630 exceeded Veh ($p = 0.004$) and CBD ($p = 0.012$) and was also higher than AM251 ($p < 0.001$). In addition, CBD+AM251 exceeded AM251 ($p = 0.012$), and CBD+AM630 exceeded AM251 ($p = 0.048$). All other pairwise contrasts were not significant ($p \geq 0.05$). In females (Figure 11), Treatment was not significant, and no Alcohol main effect was detected.

Table 6: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	8	1 $\pm$ 0.205
Ethanol	Male	8	1.039 $\pm$ 0.165
Ethanol + CBD	Male	7	1.088 $\pm$ 0.458
Ethanol + AM251	Male	8	0.940 $\pm$ 0.295
Ethanol + AM630	Male	8	1.471 $\pm$ 0.306
Ethanol + CBD + AM251	Male	8	1.312 $\pm$ 0.290
Ethanol + CBD + AM630	Male	6	1.251 $\pm$ 0.146
Control	Female	6	1.254 $\pm$ 0.215
Ethanol	Female	7	1.035 $\pm$ 0.218
Ethanol + CBD	Female	4	0.912 $\pm$ 0.070
Ethanol + AM251	Female	5	1.097 $\pm$ 0.167
Ethanol + AM630	Female	8	0.907 $\pm$ 0.350

Ethanol + CBD + AM251	Female	7	1.234 ± 0.209
Ethanol + CBD + AM630	Female	7	1.138 ± 0.084

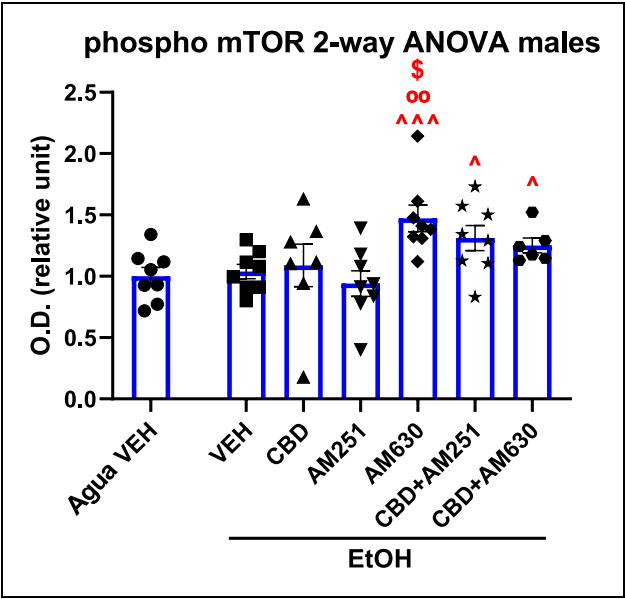


Figure 10: Optical density of pmTOR/mTOR total in males
SPSS Two-way ANOVA: Tukey post-hoc: Significant effect of treatment $p<0.01$; Comparisons in the same sex: $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.001$ vs EtOH AM251; $^{\circ}p<0.01$ vs EtOH VEH; $^{\$}p<0.05$ vs EtOH CBD
Agua VEH = Control

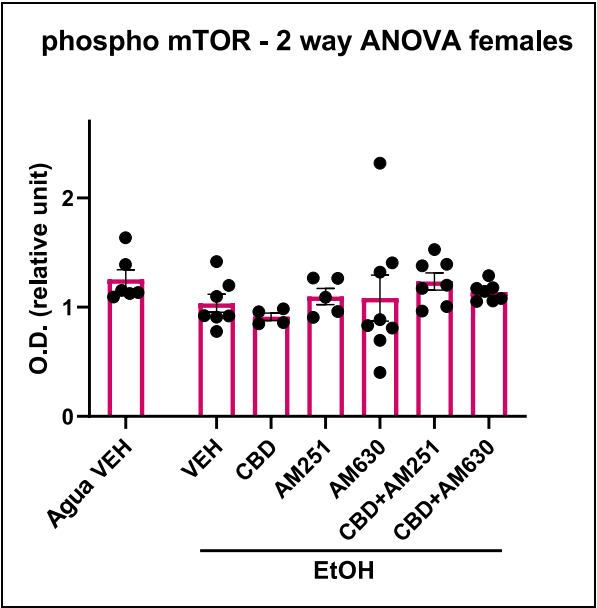


Figure 11: Optical density of pmTOR/mTOR total in females
SPSS Two-way ANOVA: Tukey post-hoc

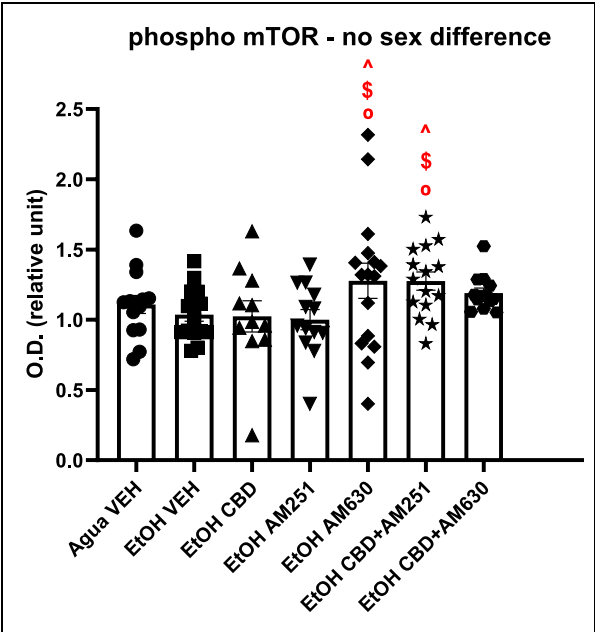


Figure 12: Optical density of pmTOR/mTOR total in males and females
SPSS Two-way ANOVA:
Tukey post-hoc
Significant effect treatment ($p<0.05$)
Comparisons:
 $^{\wedge}p<0.05$ vs EtOH AM251
 $^{\circ}p<0.05$ vs EtOH VEH

$\$p < 0.05$ vs EtOH CBD

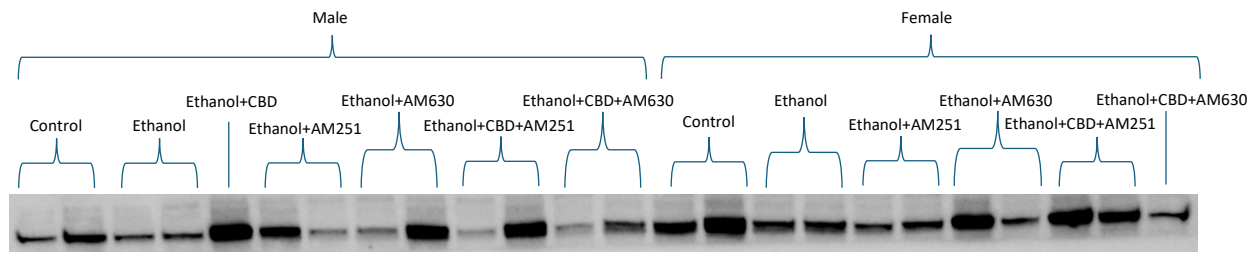


Figure 13: Representative immunoblot showing pmTOR.

The overall Treatment effect and the male-specific elevation of pmTOR with AM630 (and combinations relative to AM251) indicate that modulation of cannabinoid targets can shift mTOR signaling after prenatal alcohol exposure. This aligns with the notion that PAE perturbs mTOR pathway regulation and that pharmacological modulation can influence this axis.⁶¹ The absence of significant effects in females is compatible with the sex-dependent differences in ECS/HPA responsiveness to CBD and related manipulations which may constrain treatment effects in females under these conditions.⁴²

3.1.2 mTOR total

The omnibus model did not show main effects of Sex, Alcohol or Treatment and no interactions were detected.

To complement the factorial model, we ran the prespecified pooled two-way ANOVA (Alcohol × Treatment) collapsing Sex. Treatment was not significant, while Alcohol showed a trend that did not reach the significance threshold ($F(1, 94) = 3.040$, $p = 0.084$).

In males (Figure 14), Treatment did not reach significance. In females (Figure 15) the omnibus Treatment test showed a non-significant trend ($F(5, 39) = 2.111$, $p = 0.085$).

Table 7: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean ± SEM
Control	Male	8	1 ± 0.714
Ethanol	Male	8	0.422 ± 0.328
Ethanol + CBD	Male	7	0.777 ± 0.496
Ethanol + AM251	Male	8	0.884 ± 0.536
Ethanol + AM630	Male	8	0.736 ± 0.507
Ethanol + CBD + AM251	Male	8	0.803 ± 0.489
Ethanol + CBD + AM630	Male	8	0.752 ± 0.604
Control	Female	7	1.005 ± 0.573
Ethanol	Female	7	0.986 ± 0.442
Ethanol + CBD	Female	4	0.586 ± 0.412
Ethanol + AM251	Female	6	0.741 ± 0.340
Ethanol + AM630	Female	8	0.768 ± 0.454

Ethanol + CBD + AM251	Female	7	0.916 ± 0.310
Ethanol + CBD + AM630	Female	7	1.230 ± 0.396

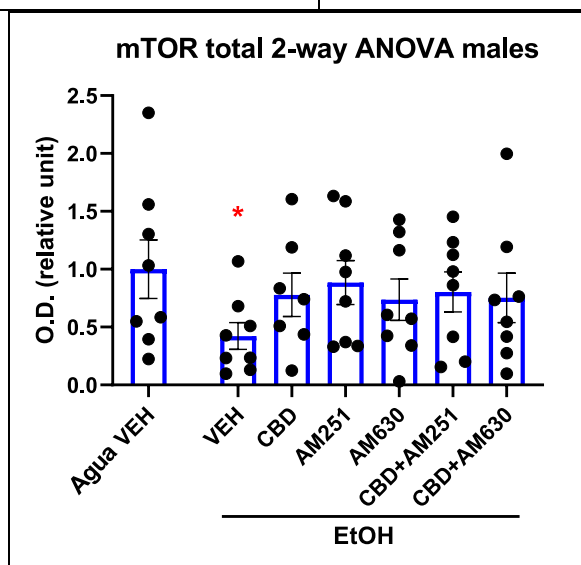


Figure 14: Optical density of total mTOR/Adaptin in males.
SPSS Two-way ANOVA:
Tukey post-hoc
Significant effect of EtOH ($p < 0.05$)
Comparisons in the same sex:
* $p < 0.05$ vs AGUA Veh

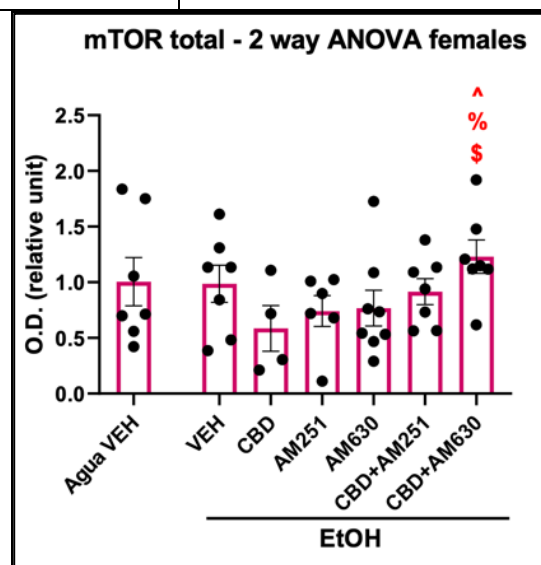


Figure 15: Optical density of total mTOR/Adaptin in females.
SPSS Two-way ANOVA:
Tukey post-hoc
Comparisons in the same sex:
\$ $p < 0.05$ vs EtOH CBD
^ $p < 0.05$ vs EtOH AM251
% $p < 0.05$ vs EtOH AM630

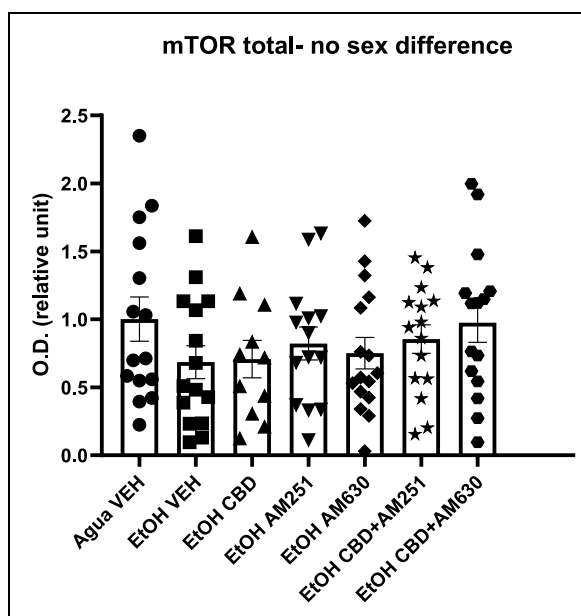


Figure 16: Optical Density of total mTOR/Adaptin in males and females SPSS Two-way ANOVA: .
Tukey post-hoc

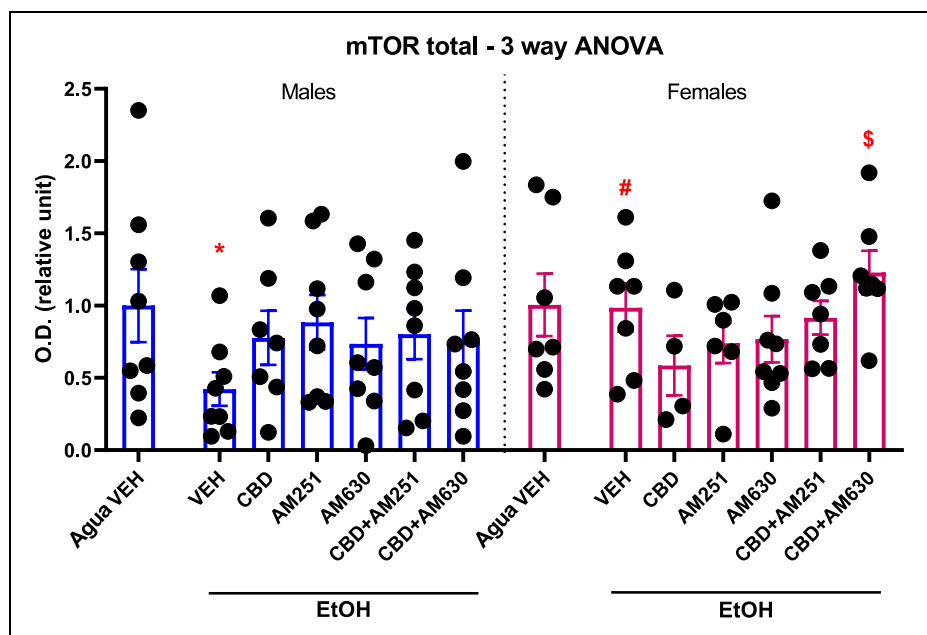


Figure 17: Optical density of total mTOR/Adaptin in males compared with females.

SPSS three-way ANOVA:

Tukey post-hoc

Comparisons in the same sex:

* $p < 0.05$ vs AGUA VEH

\$ $p < 0.05$ vs EtOH CBD

Comparisons in-between sexes:

$p < 0.05$ vs male with same treatment

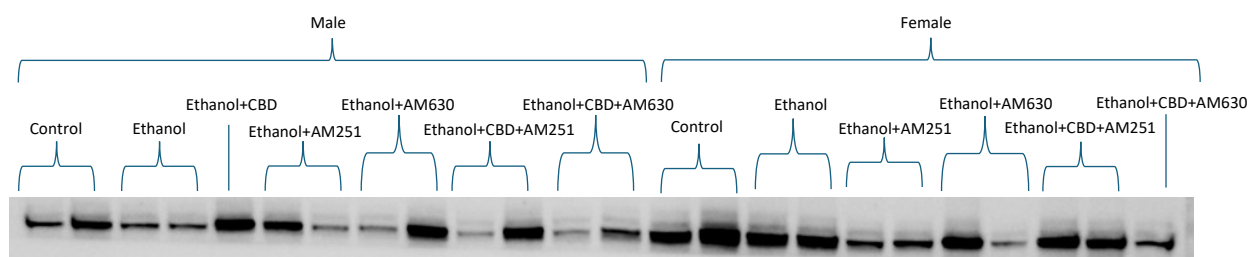


Figure 18: Representative immunoblot showing mTOR total

Taken together, total mTOR was not detectably altered by the cannabinoid-targeted conditions. This supports the view that modulation of the mTOR axis occurs predominantly at the phosphorylation level (pmTOR), rather than via changes in total protein expression. Remarkably mTOR was significantly lowered by ethanol in males, but not in females.

3.1.3 Phosphorylated AKT (pAKT)

Three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) detected a main effect of Treatment ($F(5, 77) = 2.380$, $p = 0.046$), whereas neither Sex nor Alcohol showed main effects and no interactions emerged.

Within EtOH, males (Figure 19) showed no Treatment effect. In contrast, females (Figure 20) exhibited a significant Treatment effect ($F(5, 36) = 2.898$, $p = 0.027$). Tukey post-hoc tests (females, EtOH) indicated that AM251 was higher than Veh ($p = 0.013$), CBD ($p = 0.013$), and AM630 ($p = 0.014$); CBD+AM251 also exceeded Veh ($p = 0.032$), CBD ($p = 0.030$), and AM630 ($p = 0.035$). A two-way ANOVA collapsing Sex (Alcohol $\times$ Treatment) showed a Treatment trend ($F(5, 84) = 2.194$, $p = 0.062$).

Table 8: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	8	1 $\pm$ 0.395
Ethanol	Male	7	1.188 $\pm$ 0.636
Ethanol + CBD	Male	7	0.783 $\pm$ 0.489
Ethanol + AM251	Male	8	1.036 $\pm$ 0.274
Ethanol + AM630	Male	6	0.993 $\pm$ 0.269
Ethanol + CBD + AM251	Male	7	1.192 $\pm$ 0.447
Ethanol + CBD + AM630	Male	5	0.853 $\pm$ 0.228
Control	Female	7	1.024 $\pm$ 0.309
Ethanol	Female	6	0.794 $\pm$ 0.210
Ethanol + CBD	Female	5	0.767 $\pm$ 0.323
Ethanol + AM251	Female	5	1.363 $\pm$ 0.639
Ethanol + AM630	Female	7	0.822 $\pm$ 0.323
Ethanol + CBD + AM251	Female	6	1.260 $\pm$ 0.456
Ethanol + CBD + AM630	Female	7	1.098 $\pm$ 0.165

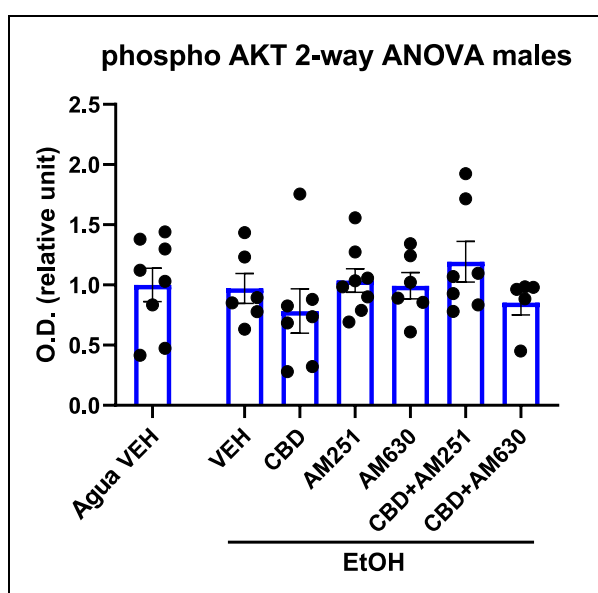


Figure 19: Optical density of pAKT/AKT total in males SPSS Anova: Tukey post-hoc

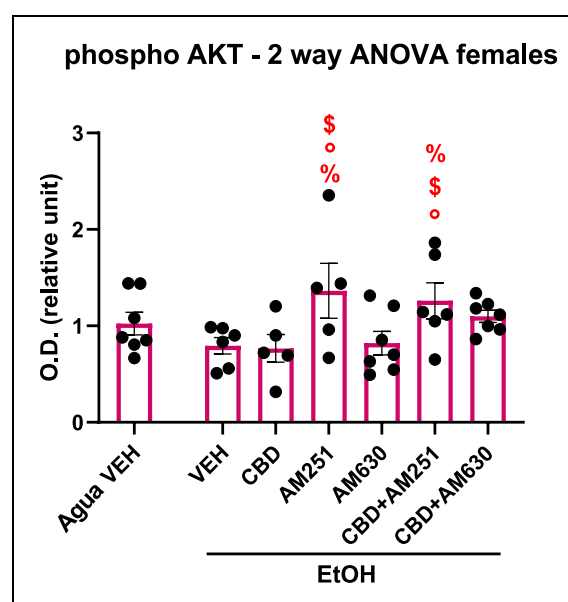


Figure 20: Optical density of pAKT/AKT total in females SPSS Two-way ANOVA: Significant effect of treatment ($p < 0.05$). Tukey post-hoc; Comparisons in the same sex: \$ $p < 0.05$ vs EtOH CBD; ^ $p < 0.05$ vs EtOH AM251; % $p < 0.05$ vs EtOH AM630

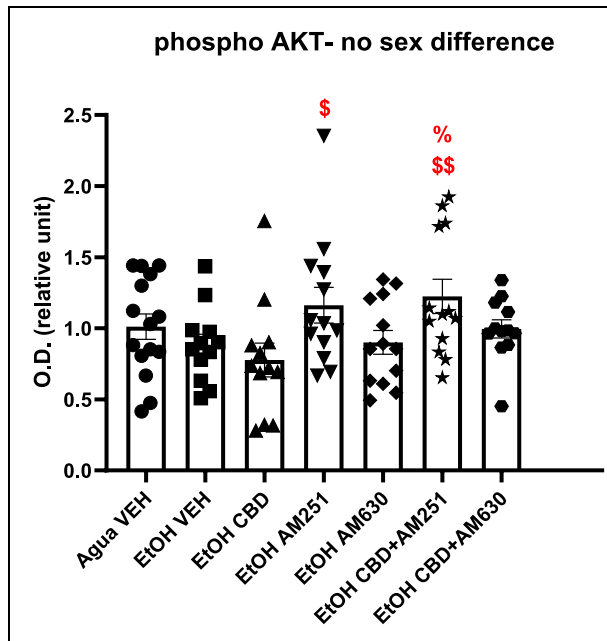


Figure 21: Optical density of pAKT/AKT total in males and females.

SPSS Two-way ANOVA;

No Effect of treatment but $p=0.062$

Tukey post-hoc

Comparisons:

% $p<0.05$ vs EtOH AM630

\$ $p<0.05$, \$\$ $p<0.01$ vs EtOH CBD

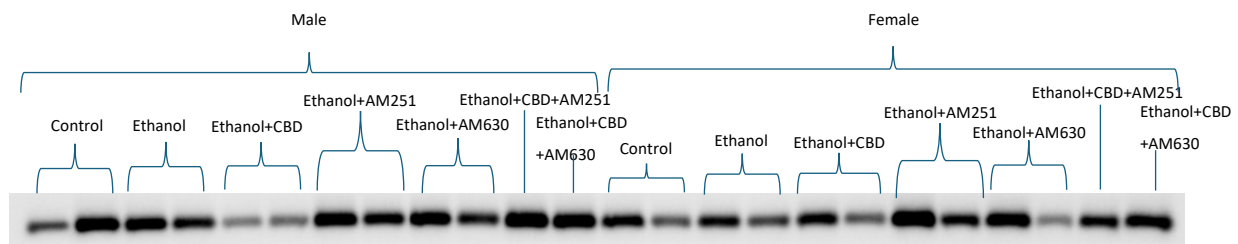


Figure 22: Representative immunoblot showing pAKT

Together, the omnibus Treatment effect and the selective increases in pAKT among EtOH-exposed females under AM251 and CBD+AM251 suggest that cannabinoid-targeted conditions can modify AKT signaling after prenatal alcohol exposure. This pattern is compatible with the background that neonatal alcohol exposure disrupts $\text{TrkB} \rightarrow \text{AKT}$ signaling and lowers pAKT, linking AKT phosphorylation to CB1R-mediated neuroprotective capacity in this context.⁶⁴

3.1.4 DARPP-32

Using the prespecified factorial approach, the three-way ANOVA ($\text{Sex} \times \text{Alcohol} \times \text{Treatment}$) identified a robust main effect of Treatment ($F(5, 87) = 4.355$, $p = 0.001$), with no main effects of Sex or Alcohol and no interactions.

Within EtOH, males (Figure 23) did not show a significant Treatment effect ($F(5, 43) = 2.036$, $p = 0.093$). In females (Figure 24), Treatment was significant ($F(5, 44) = 2.784$, $p = 0.029$). Post-hoc tests (EtOH, females) indicated higher DARPP-32 for CBD versus Veh ($p = 0.006$), AM251 ($p = 0.007$), and AM630 ($p = 0.006$),

and lower DARPP-32 for CBD+AM251 versus CBD ($p = 0.002$); CBD+AM630 also exceeded Veh ($p = 0.041$). The pooled (no Sex, Figure 25) two-way ANOVA (Alcohol $\times$ Treatment) confirmed a main effect of Treatment ($F(5, 94) = 4.487$, $p = 0.001$), while Alcohol was not significant.

Table 9: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	8	1 $\pm$ 0.508
Ethanol	Male	8	1.267 $\pm$ 0.746
Ethanol + CBD	Male	7	2.012 $\pm$ 1.768
Ethanol + AM251	Male	8	1.192 $\pm$ 1.124
Ethanol + AM630	Male	6	0.871 $\pm$ 0.471
Ethanol + CBD + AM251	Male	5	0.355 $\pm$ 0.170
Ethanol + CBD + AM630	Male	8	1.608 $\pm$ 1.038
Control	Female	7	1.185 $\pm$ 0.747
Ethanol	Female	7	0.864 $\pm$ 0.724
Ethanol + CBD	Female	6	1.947 $\pm$ 0.699
Ethanol + AM251	Female	8	0.900 $\pm$ 0.685
Ethanol + AM630	Female	8	0.889 $\pm$ 0.695
Ethanol + CBD + AM251	Female	8	0.716 $\pm$ 0.393
Ethanol + CBD + AM630	Female	6	1.151 $\pm$ 0.778

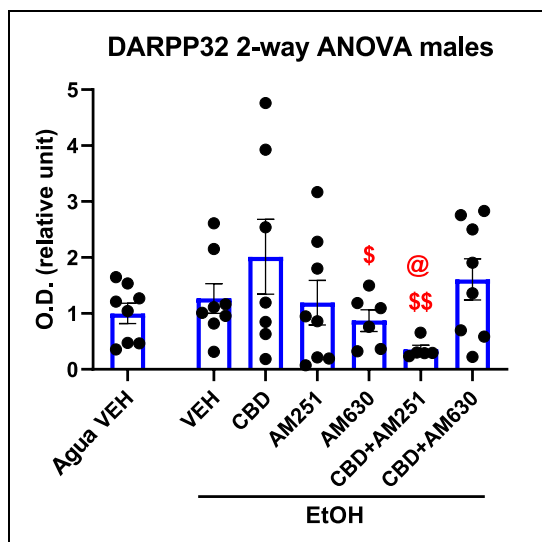


Figure 23: Optical density of DARPP-32/Adaptin in males
SPSS Two-way ANOVA:
Tukey post-hoc
Comparisons in the same sex:
\$ $p < 0.05$, \$\$ $p < 0.01$ vs EtOH CBD
@ $p < 0.05$ vs EtOH CBD AM630

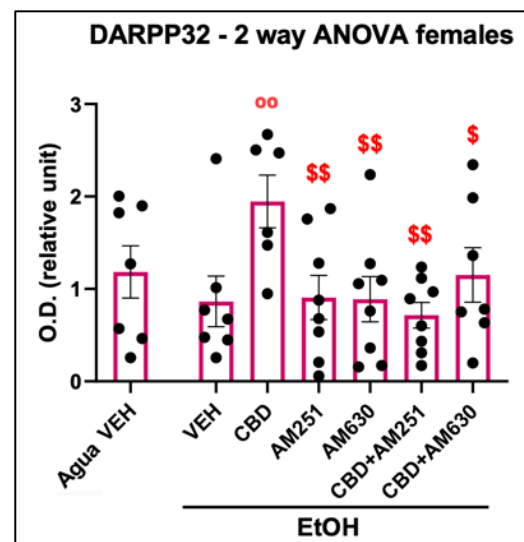


Figure 24: Optical density of DARPP-32/Adaptin in females
SPSS Two-way ANOVA:
Significant effect of treatment ($p < 0.05$)
Tukey post-hoc
Comparisons in the same sex:
\$ $p < 0.05$, \$\$ $p < 0.01$ vs EtOH CBD
oo $p < 0.01$ vs EtOH Veh

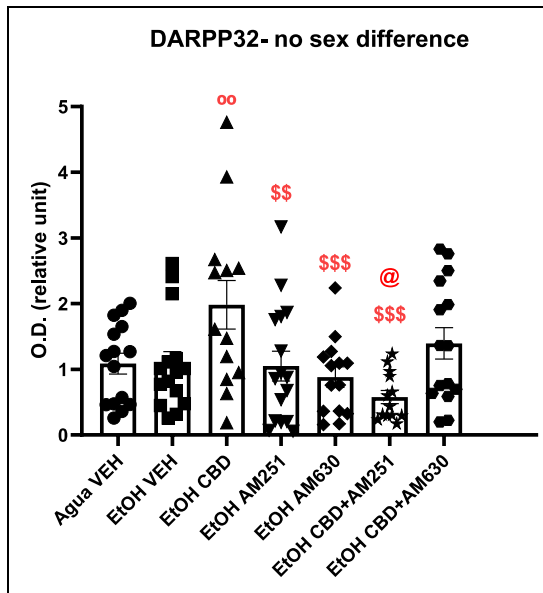


Figure 25: Optical density of DARPP-32/Adaptin in males and females: SPSS Two-way ANOVA:

Significant effect of treatment $p < 0.01$.

Tukey post-hoc

$$$$p < 0.01$, $$$$$p < 0.001$ vs EtOH CBD

$^^p < 0.05$ vs EtOH Veh

@ $p < 0.05$ vs EtOH CBD AM630

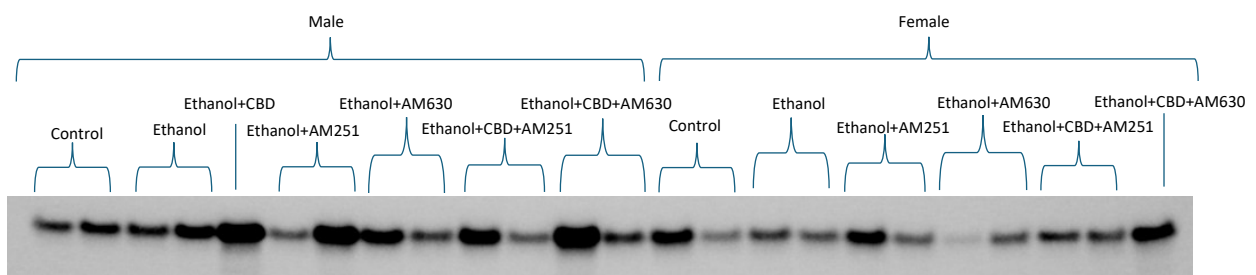


Figure 26: Representative immunoblot showing DARPP-32

The global Treatment effect and the female-specific elevations under CBD and CBD+AM251 suggest that cannabinoid-targeted conditions can modulate DARPP-32 signaling after prenatal alcohol exposure. This fits with the background that DARPP-32 is a key downstream effector of dopamine receptor signaling and is altered by alcohol, linking these molecular changes to characteristic behavioral phenotypes in FASD.

3.1.5 DAT

Adhering to the prespecified factorial framework, the three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) yielded a significant main effect of Sex ($F(1, 93) = 4.204$, $p = 0.043$), whereas Alcohol and Treatment were not significant.

Within EtOH, Treatment was not significant in males (Figure 27) and likewise not significant in females (Figure 28). For completeness, a pooled (no Sex, Figure 29) two-way ANOVA (Alcohol $\times$ Treatment) also showed no Treatment effect and no Alcohol effect.

Table 10: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	8	1 $\pm$ 0.154
Ethanol	Male	8	1.019 $\pm$ 0.208
Ethanol + CBD	Male	7	1.128 $\pm$ 0.191
Ethanol + AM251	Male	8	1.028 $\pm$ 1.768
Ethanol + AM630	Male	8	0.977 $\pm$ 0.121
Ethanol + CBD + AM251	Male	8	1.012 $\pm$ 0.110
Ethanol + CBD + AM630	Male	8	0.933 $\pm$ 0.118
Control	Female	7	0.916 $\pm$ 0.120
Ethanol	Female	7	0.949 $\pm$ 0.104
Ethanol + CBD	Female	6	0.999 $\pm$ 0.113
Ethanol + AM251	Female	8	0.988 $\pm$ 0.124
Ethanol + AM630	Female	8	0.871 $\pm$ 0.139
Ethanol + CBD + AM251	Female	8	0.936 $\pm$ 0.127
Ethanol + CBD + AM630	Female	8	0.950 $\pm$ 0.222

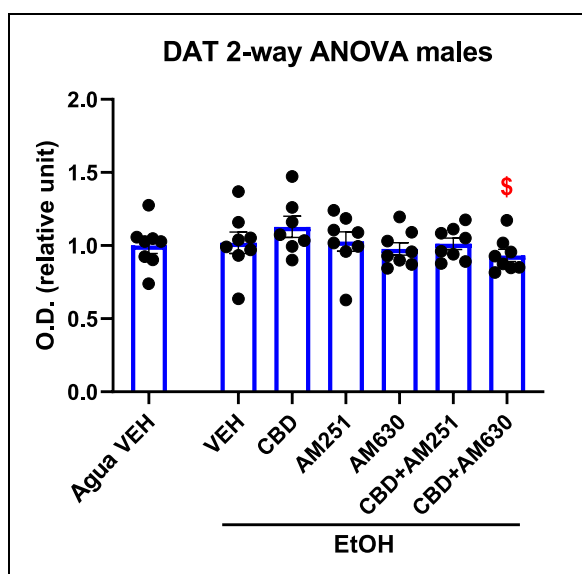


Figure 27: Optical density of DAT/Adaptin in males
SPSS Two-way ANOVA:
Tukey post-hoc
\$p < 0.05 vs EtOH CBD

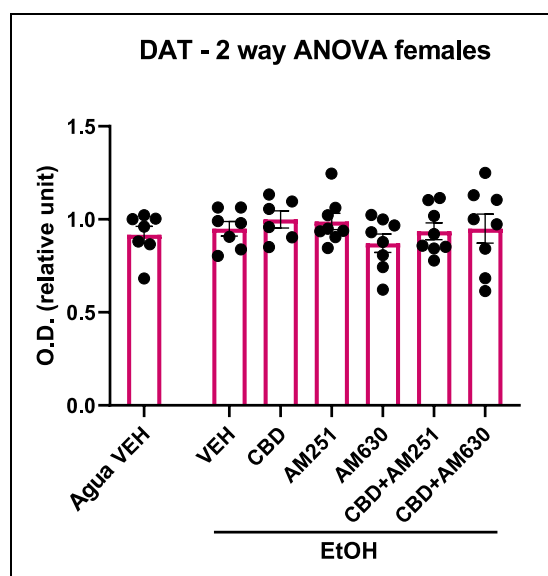


Figure 28: Optical density of DAT/Adaptin in females
SPSS Two-way ANOVA: Tukey post-hoc

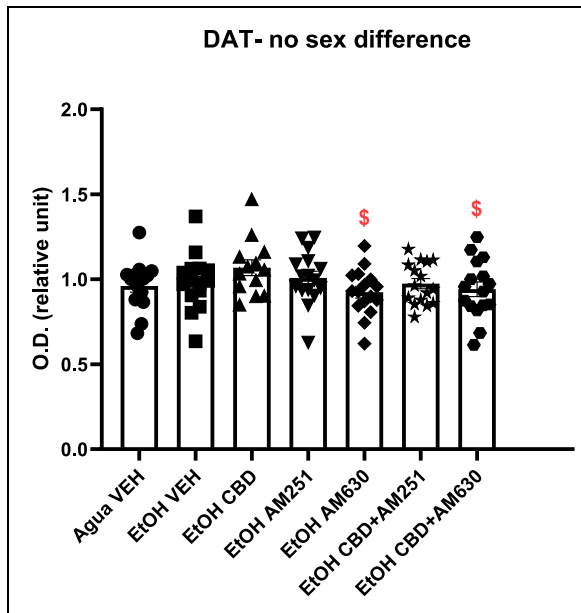


Figure 29: Optical density of DAT/Adaptin in males and females

SPSS Two-way ANOVA;

Tukey post-hoc

Comparisons:

\$ $p < 0.05$ vs EtOH CBD

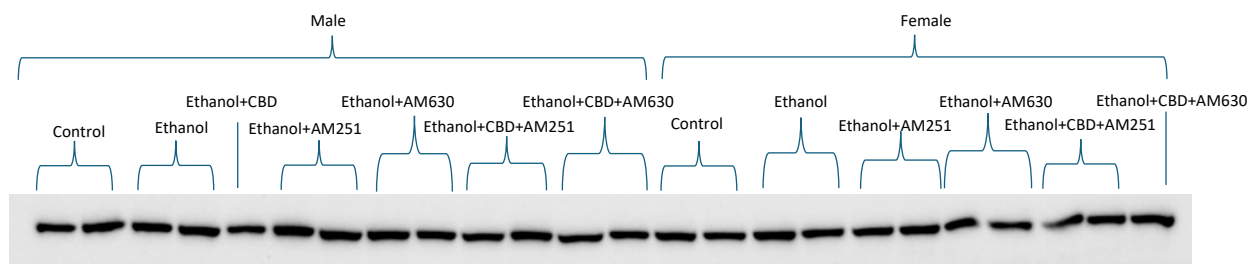


Figure 30: Representative immunoblot showing DAT

Overall, DAT did not differ across Treatment conditions in either sex under EtOH exposure, while a Sex main effect was present in the omnibus model. DAT is frequently upregulated in FASD and relates to dopaminergic signaling and affective dysregulation; here, however, neither the alcohol exposure, nor the cannabinoid-targeted manipulations did produce robust DAT changes.

3.1.6 Phosphorylated PKA (pPKA)

The three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) showed no main effects of Alcohol, Treatment, or Sex. No interactions were detected. In line with the analysis plan, we then conducted the predefined two-factor analyses by Sex within the EtOH arm.

Within EtOH, Treatment was not significant in males (Figure 31) and likewise not significant in females (Figure 32). The pooled (no Sex, Figure 33) two-way ANOVA (Alcohol $\times$ Treatment) also showed no Treatment effect and no Alcohol effect. The combination of CBD and AM630 lowered pPKA compared to EtOH Veh significantly ($p = 0.050$) in males.

Table 11: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	8	1 $\pm$ 0.178
Ethanol	Male	7	1.132 $\pm$ 0.103
Ethanol + CBD	Male	7	0.976 $\pm$ 0.186
Ethanol + AM251	Male	8	1.013 $\pm$ 0.327
Ethanol + AM630	Male	8	1.084 $\pm$ 0.204
Ethanol + CBD + AM251	Male	8	0.953 $\pm$ 0.257
Ethanol + CBD + AM630	Male	8	0.872 $\pm$ 0.362
Control	Female	7	0.951 $\pm$ 0.181
Ethanol	Female	7	0.862 $\pm$ 0.190
Ethanol + CBD	Female	6	1.048 $\pm$ 0.239
Ethanol + AM251	Female	8	0.958 $\pm$ 0.170
Ethanol + AM630	Female	8	0.941 $\pm$ 0.233
Ethanol + CBD + AM251	Female	8	0.942 $\pm$ 0.262
Ethanol + CBD + AM630	Female	8	0.981 $\pm$ 0.327

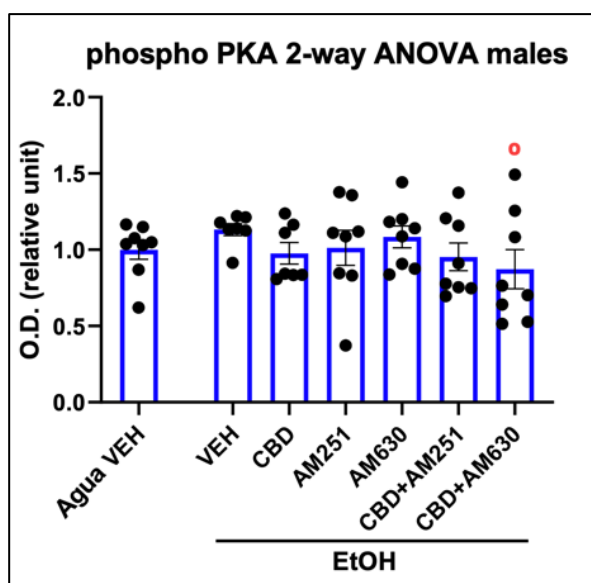


Figure 31: Optical density of pPKA/PKA total in males
SPSS Two-way ANOVA:
Tukey post-hoc
Comparisons in the same sex:
* $p < 0.05$ vs EtOH VEH

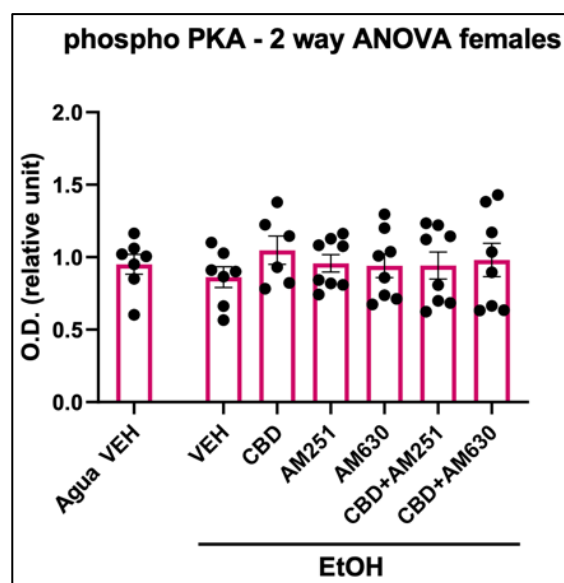


Figure 32: Optical density of pPKA/PKA total in females
SPSS Two-way ANOVA:
Tukey post-hoc

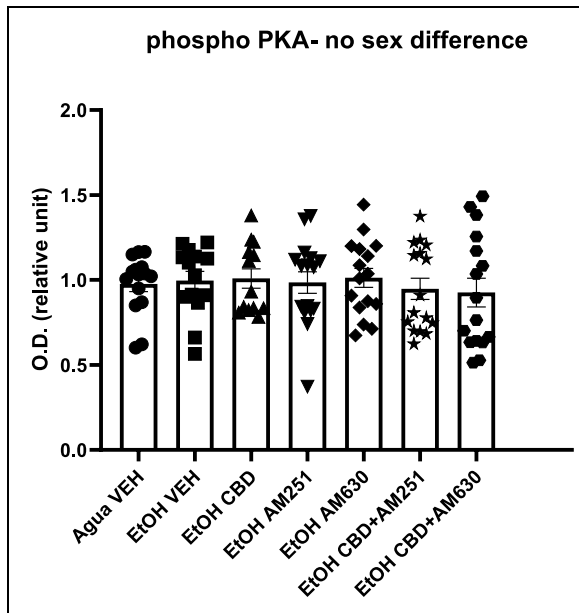


Figure 33: Optical density of pPKA/PKA total in males and females

SPSS three-way ANOVA:

Tukey post-hoc

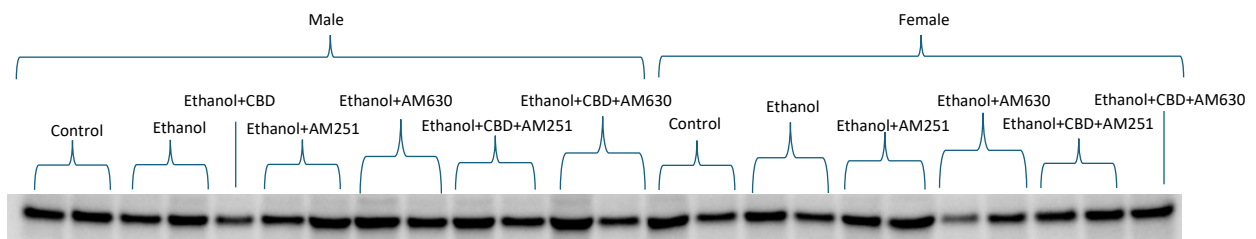


Figure 34: Representative immunoblot showing pPKA

Overall, hippocampal pPKA did not differ across Treatment conditions in either sex under EtOH exposure. CB1/CB2 signaling regulates adenylyl cyclases and therefore cAMP/PKA, and CBD's CB1 negative allosteric modulation and CB2 antagonism can shift cAMP upward⁴⁶ and thereby potentially increasing pPKA. Here, however, the cannabinoid-targeted manipulations did not produce detectable pPKA changes within the planned analysis framework.

3.1.7 pAMPK α

Adhering to the prespecified factorial framework, the three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) showed no main effects of Sex, Alcohol, or Treatment.

Within EtOH, Treatment was not significant in males (Figure 35) and likewise not significant in females (Figure 36). There was a significant ($p = 0.008$) spike in the AM251 group compared to the EtOH Veh group and compared to EtOH CBD ($p = 0.036$). The pooled (no Sex, Figure 37) two-way ANOVA (Alcohol $\times$ Treatment) also showed no Treatment effect and no Alcohol effect.

Table 12: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	8	1 $\pm$ 0.475
Ethanol	Male	8	1.101 $\pm$ 0.484
Ethanol + CBD	Male	7	1.096 $\pm$ 0.357
Ethanol + AM251	Male	8	1.098 $\pm$ 0.383
Ethanol + AM630	Male	8	1.342 $\pm$ 1.085
Ethanol + CBD + AM251	Male	8	0.843 $\pm$ 0.316
Ethanol + CBD + AM630	Male	8	0.962 $\pm$ 0.489
Control	Female	7	0.754 $\pm$ 0.456
Ethanol	Female	7	0.676 $\pm$ 0.280
Ethanol + CBD	Female	6	0.783 $\pm$ 0.177
Ethanol + AM251	Female	8	1.276 $\pm$ 0.530
Ethanol + AM630	Female	8	0.863 $\pm$ 0.363
Ethanol + CBD + AM251	Female	8	0.965 $\pm$ 0.560
Ethanol + CBD + AM630	Female	8	0.882 $\pm$ 0.369

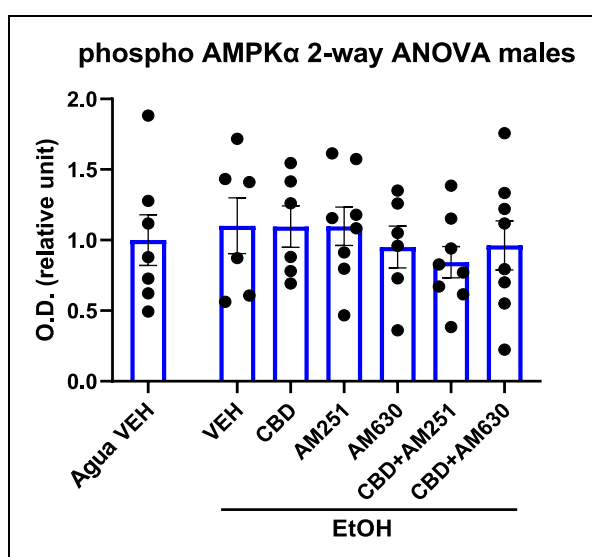


Figure 35: Optical density of pAMPK α /AMPK α total in males
SPSS Two-way ANOVA:
Tukey post-hoc

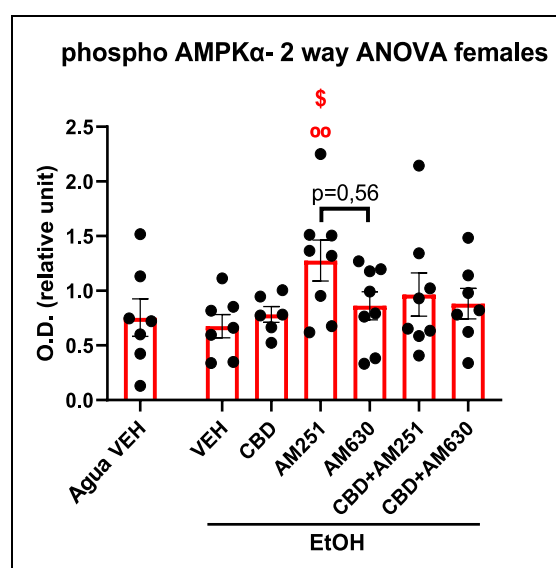


Figure 36: Optical density of pAMPK α /AMPK α total in females
SPSS Two-way ANOVA;
Tukey post-hoc
\$ p <0.05 vs EtOH CBD
 ^{oo} p <0.01 vs EtOH Veh

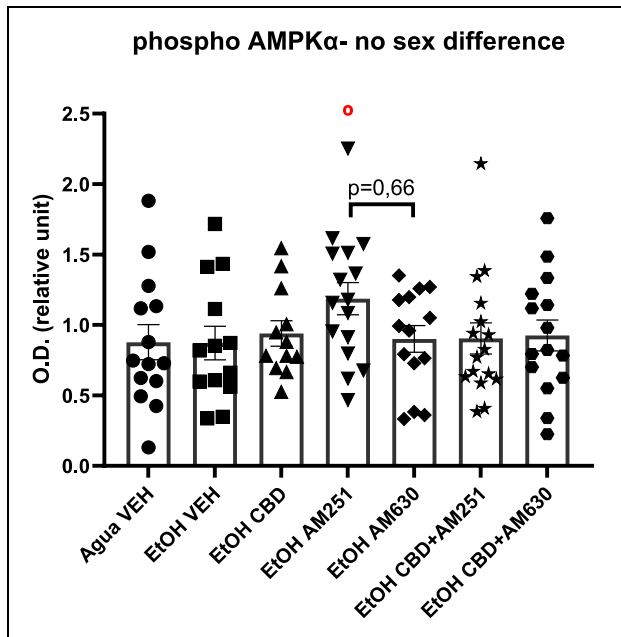


Figure 37: Optical density of pAMPKα/AMPKα total in males and females
SPSS Two-way ANOVA;
Tukey post-hoc
* $p < 0.05$ vs EtOH VEH

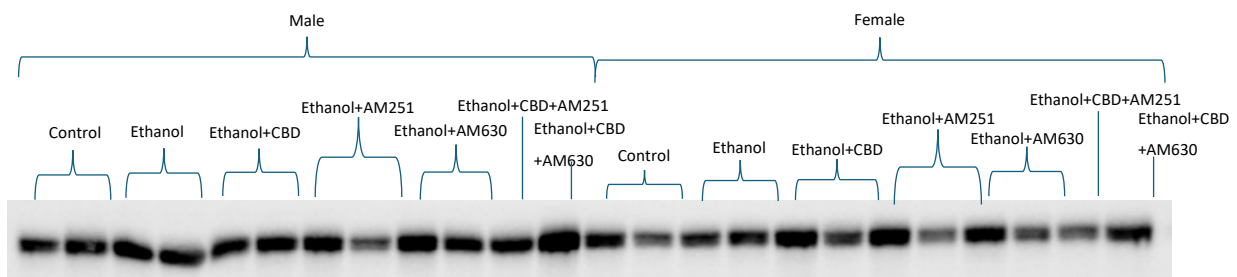


Figure 38: Representative immunoblot showing pAMPKα

Overall, hippocampal pAMPKα did not differ across Treatment conditions in either sex under EtOH exposure. AMPK is a metabolic stress sensor activated by ATP deficiency under ethanol; here, however, the cannabinoid-targeted manipulations did not produce detectable changes in pAMPKα within the planned analysis framework.

3.2 Immunohistochemistry

3.2.1 Iba1

3.2.1.1 Iba1 in dentate gyrus

Using the prespecified factorial approach, the three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) identified a significant main effect of Sex ($F(1, 133) = 8.667, p = 0.004$), Treatment ($F(5, 133) = 16.490, p = 0.000$), Alcohol ($F(1, 133) = 7.718, p = 0.006$) and Sex $\times$ Alcohol ($F(5, 133) = 0.001$).

Within EtOH, males (Figure 39) showed a significant Treatment effect ($F(5, 68) = 19.970, p = 0.000$). In females (Figure 40), Treatment ($F(5, 65) = 2.456, p = 0.042$) and Alcohol ($F(1, 65) = 4.320, p = 0.042$) were significant.

The pooled (no Sex, Figure 41) two-way ANOVA (Alcohol $\times$ Treatment) also showed significant Treatment effect ($F(5, 140) = 13.244, p = 0.000$) and Alcohol effect ($F(5, 140) = 4.703, p = 0.032$).

Post-hoc tests (EtOH, males) indicated significant lower Iba1 for CBD ($p = 0.022$), AM251 ($p = 0.000$), AM630 ($p = 0.000$), CBD+AM251 ($p = 0.000$) and CBD+AM630 ($p = 0.000$) compared with EtOH Veh. In females Iba1 was significant lower for AM251 ($p = 0.014$), AM630 ($p = 0.007$) and CBD + AM630 ($p = 0.009$) compared with EtOH Veh. Iba1 was heightened in the EtOH Veh group compared to Agua Veh ($p = 0.042$).

Table 13: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	12	0.0456 $\pm$ 0.0195
Ethanol	Male	11	0.0537 $\pm$ 0.0145
Ethanol + CBD	Male	12	0.0391 $\pm$ 0.0108
Ethanol + AM251	Male	10	0.0311 $\pm$ 0.0055
Ethanol + AM630	Male	12	0.0166 $\pm$ 0.0102
Ethanol + CBD + AM251	Male	10	0.0254 $\pm$ 0.0102
Ethanol + CBD + AM630	Male	11	0.0256 $\pm$ 0.0089
Control	Female	11	0.0261 $\pm$ 0.0125
Ethanol	Female	12	0.0339 $\pm$ 0.0094
Ethanol + CBD	Female	7	0.0304 $\pm$ 0.0086
Ethanol + AM251	Female	11	0.0277 $\pm$ 0.0141
Ethanol + AM630	Female	12	0.0259 $\pm$ 0.0101
Ethanol + CBD + AM251	Female	11	0.0274 $\pm$ 0.0086
Ethanol + CBD + AM630	Female	11	0.0237 $\pm$ 0.0093

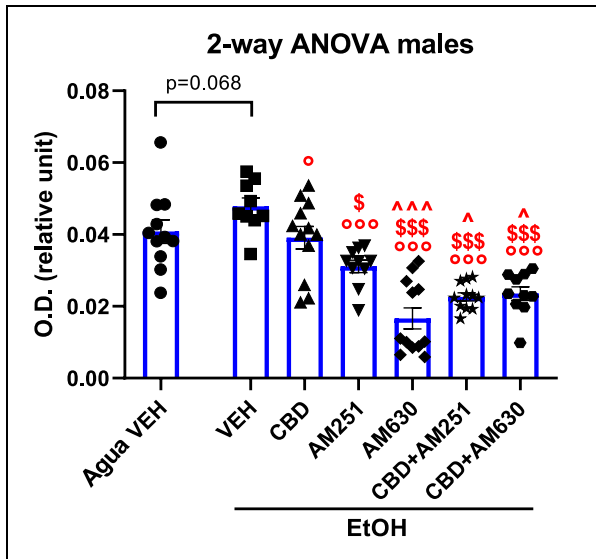


Figure 39: Optical density of Iba1 in dentate gyrus in males
SPSS Two-way ANOVA:
Significant effect of treatment ($p<0.001$)
Tukey post-hoc
Comparisons in the same sex:
* $p<0.05$, ** $p<0.001$ vs EtOH VEH
\$ $p<0.05$, \$\$\$ $p<0.001$ vs EtOH CBD
^ $p<0.05$, ^^ $p<0.001$ vs EtOH AM251

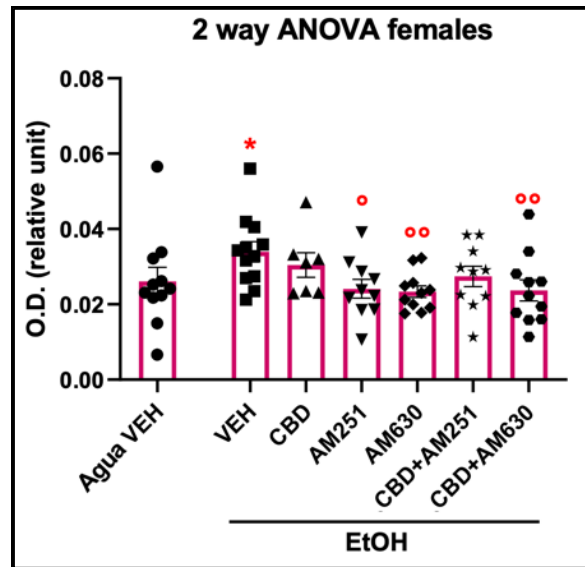


Figure 40: Optical density of Iba1 in dentate gyrus in females
SPSS Two-way ANOVA:
Significant effect of alcohol and treatment ($p<0.05$)
Tukey post-hoc
Comparisons in the same sex:
* $p<0.05$ vs AGUA Veh
° $p<0.05$, °° $p<0.01$ vs EtOH VEH

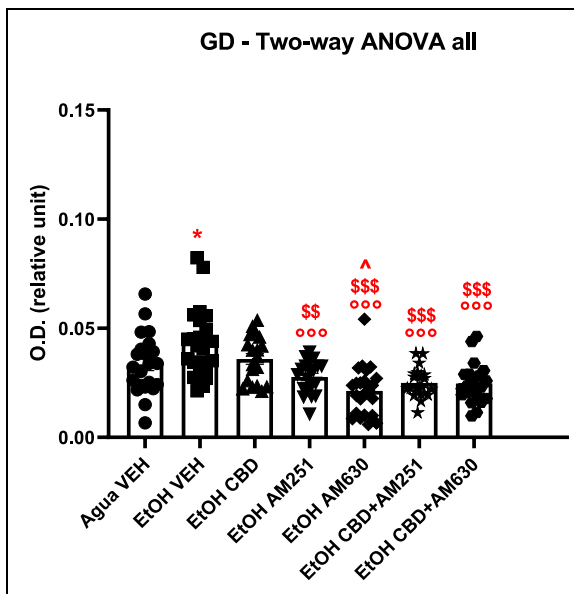


Figure 41: Optical density of Iba1 in dentate gyrus in males and females
SPSS Two-way ANOVA:
Significant effect of alcohol ($p<0.05$) and treatment ($p<0.05$)
Tukey post-hoc
Comparisons:
* $p<0.05$ vs AGUA VEH
°° $p<0.001$ vs EtOH VEH
\$\$\$ $p<0.01$, \$\$\$ $p<0.001$ vs EtOH CBD
^ $p<0.05$ vs EtOH AM251

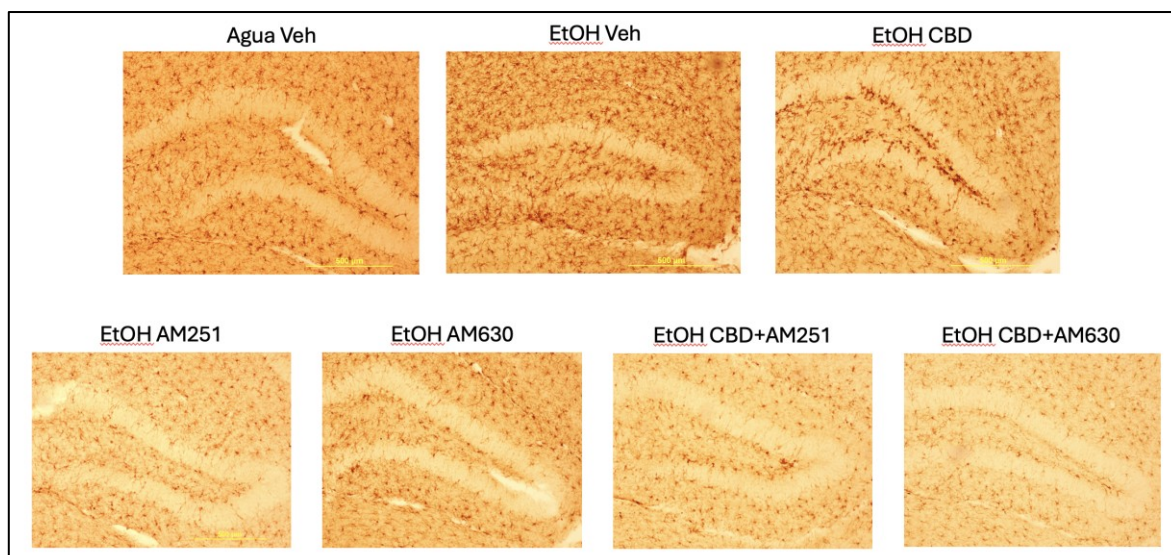


Figure 42: Iba1 marked glia cells in dentate gyrus (males, 10x enlargement)

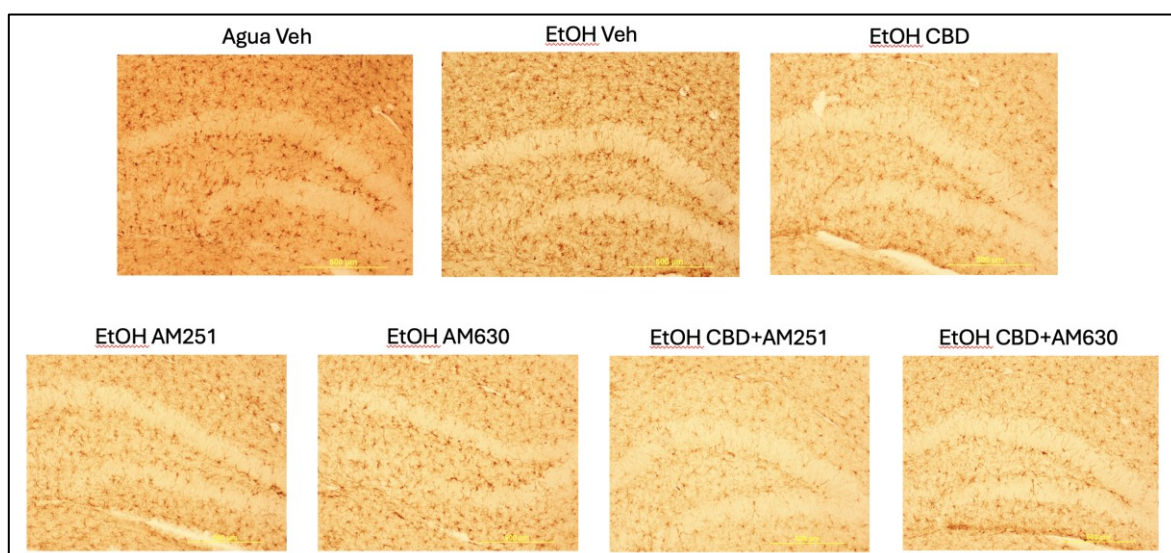


Figure 43: Iba1 marked glia cells in dentate gyrus (females, 10x enlargement)

3.2.1.2 Iba1 in CA3

The three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) identified a significant main effect of Treatment ($F(5, 145) = 9.159, p = 0.000$) and Sex $\times$ Treatment ($F(5, 145) = 2.578, p = 0.001$).

Within EtOH, males (Figure 44) showed a significant Treatment effect ($F(5, 78) = 7.699, p = 0.000$). In females (Figure 45), Treatment ($F(5, 67) = 3.904, p = 0.004$) was significant.

The pooled (no Sex, Figure 46) two-way ANOVA (Alcohol $\times$ Treatment) also showed significant Treatment effect ($F(5, 152) = 8.918, p = 0.000$).

Post-hoc tests (EtOH, males) indicated significant lower Iba1 for AM630 ($p = 0.000$), CBD+AM251 ($p = 0.000$) and CBD+AM630 ($p = 0.004$) compared with EtOH Veh. In females Iba1 was significant lower for CBD ($p = 0.022$), AM630 ($p = 0.022$) and CBD + AM630 ($p = 0.000$) compared with EtOH Veh.

Table 14: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	11	0.0249 $\pm$ 0.0091
Ethanol	Male	12	0.0240 $\pm$ 0.010
Ethanol + CBD	Male	15	0.0207 $\pm$ 0.0061
Ethanol + AM251	Male	12	0.0212 $\pm$ 0.0061
Ethanol + AM630	Male	12	0.0085 $\pm$ 0.0056
Ethanol + CBD + AM251	Male	12	0.0133 $\pm$ 0.0044
Ethanol + CBD + AM630	Male	11	0.0153 $\pm$ 0.0071
Control	Female	11	0.0207 $\pm$ 0.0082
Ethanol	Female	11	0.0207 $\pm$ 0.0063
Ethanol + CBD	Female	8	0.0139 $\pm$ 0.0048
Ethanol + AM251	Female	11	0.0189 $\pm$ 0.0080
Ethanol + AM630	Female	12	0.0146 $\pm$ 0.0049
Ethanol + CBD + AM251	Female	10	0.0202 $\pm$ 0.0158
Ethanol + CBD + AM630	Female	12	0.0105 $\pm$ 0.0038

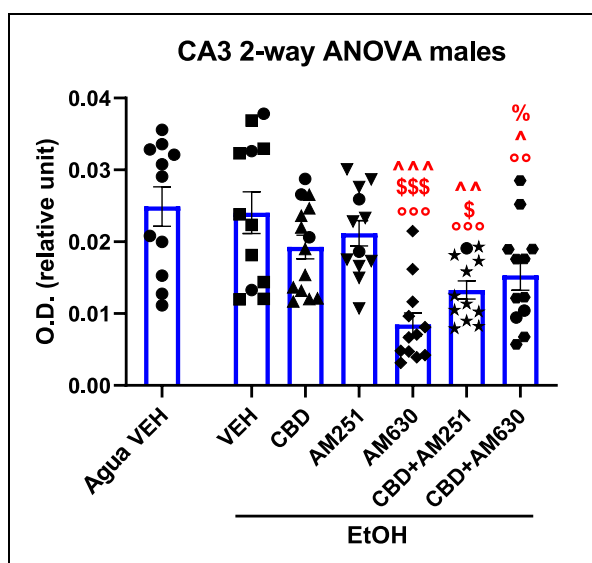


Figure 44: Optical density of Iba1 in CA3 in males
SPSS Two-way ANOVA:
Significant effect of treatment ($p < 0.001$)
Tukey post-hoc
Comparisons in the same sex:
 $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ vs EtOH VEH
 $^{\$}p < 0.05$, $^{\$ \$ \$}p < 0.001$ vs EtOH CBD
 $^{\wedge}p < 0.05$, $^{\wedge \wedge}p < 0.01$, $^{\wedge \wedge \wedge}p < 0.001$ vs EtOH AM251
 $^{\%}p < 0.05$ vs EtOH AM630

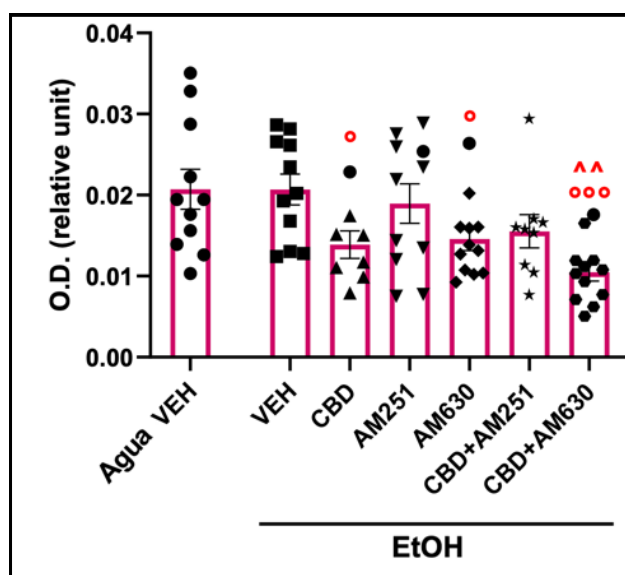


Figure 45: Optical density of Iba1 in CA3 in females
SPSS Two-way ANOVA:
Significant effect of treatment ($p < 0.01$)
Tukey post-hoc
Comparisons in the same sex:
 $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$ vs EtOH VEH, $^{\circ\circ\circ}p < 0.001$ vs EtOH VEH
 $^{\wedge \wedge}p < 0.01$ vs EtOH AM251

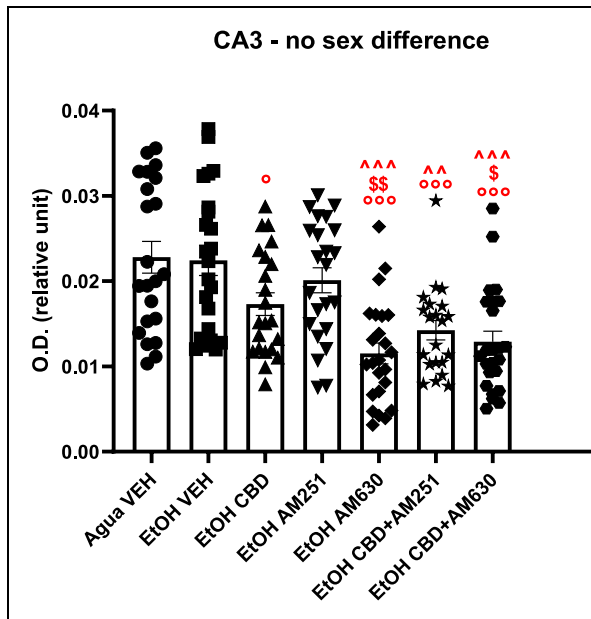


Figure 46: Optical density of Iba1 in CA3 in males and females

SPSS Two-way ANOVA:

Significant effect of treatment ($p < 0.001$)

Tukey post-hoc

Comparisons:

* $p < 0.05$ vs AGUA VEH

*** $p < 0.001$ vs EtOH VEH

\$ $p < 0.05$, \$\$ $p < 0.01$ vs EtOH CBD

^ $p < 0.05$ vs EtOH AM251

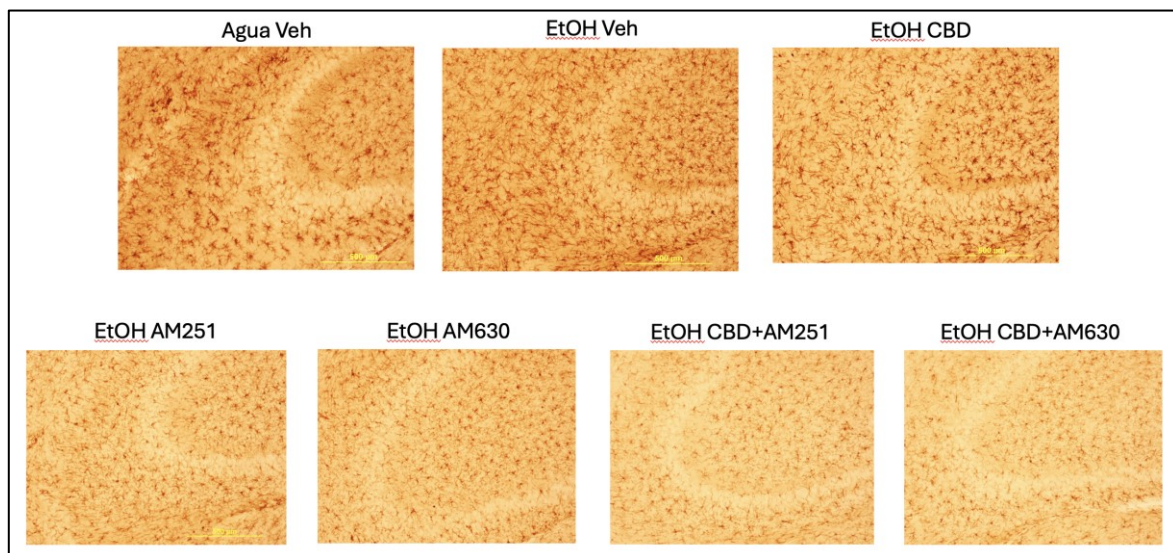


Figure 47: Iba1 marked glia cells in CA3 (males, 10x enlargement)

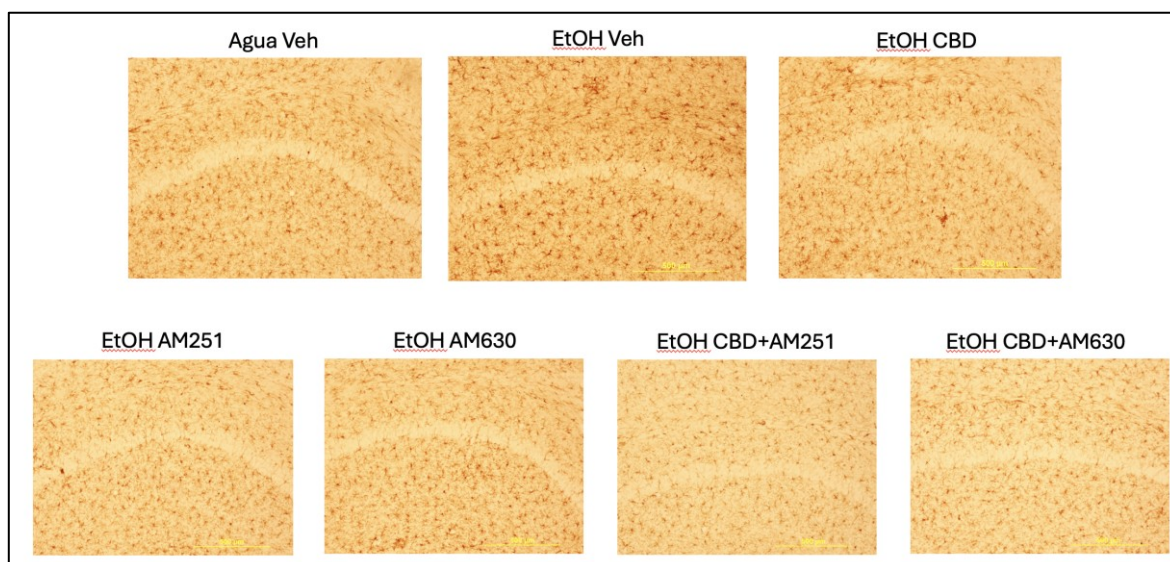


Figure 48: Iba1 marked glia cells in CA3 (female, 10x enlargement)

3.2.1.3 Iba1 in CA1

The three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) identified a significant main effect of Treatment ($F(5, 147) = 20.679, p = 0.000$), Sex ($F(1, 147) = 29.964, p = 0.000$), Sex $\times$ Alcohol ($F(1, 147) = 5.040, p = 0.026$) and Sex $\times$ Treatment ($F(5, 147) = 5.590, p = 0.000$).

Within EtOH, males (Figure 49) showed a significant Treatment effect ($F(5, 77) = 16.428, p = 0.000$). In females (Figure 50), Treatment ($F(5, 70) = 7.899, p = 0.004$) and Alcohol ($F(1, 70) = 14.377, p = 0.000$) was significant.

The pooled (no Sex, Figure 51) two-way ANOVA also showed significant Treatment effect ($F(5, 154) = 15.676, p = 0.000$). Post-hoc tests (EtOH, males) indicated significant lower Iba1 for AM251 ($p = 0.016$), AM630 ($p = 0.000$), CBD+AM251 ($p = 0.003$) and CBD+AM630 ($p = 0.000$) compared with EtOH Veh. In females Iba1 was significant lower for CBD ($p = 0.000$), AM251 ($p = 0.000$), AM630 ($p = 0.000$), CBD + AM251 ($p = 0.000$) and CBD + AM630 ($p = 0.000$) compared with EtOH Veh. Iba1 was heightened in the EtOH Veh group compared to Agua Veh ($p = 0.000$).

Table 15: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	12	0.0208 $\pm$ 0.0043
Ethanol	Male	11	0.0205 $\pm$ 0.0031
Ethanol + CBD	Male	15	0.0185 $\pm$ 0.0023
Ethanol + AM251	Male	12	0.0182 $\pm$ 0.0041
Ethanol + AM630	Male	12	0.0088 $\pm$ 0.0055
Ethanol + CBD + AM251	Male	12	0.0159 $\pm$ 0.0030
Ethanol + CBD + AM630	Male	12	0.0130 $\pm$ 0.0035

Control	Female	12	0.0140 ± 0.0018
Ethanol	Female	11	0.0180 ± 0.0027
Ethanol + CBD	Female	8	0.0137 ± 0.0024
Ethanol + AM251	Female	11	0.0140 ± 0.0020
Ethanol + AM630	Female	12	0.0124 ± 0.0031
Ethanol + CBD + AM251	Female	10	0.0125 ± 0.0023
Ethanol + CBD + AM630	Female	12	0.0131 ± 0.0022

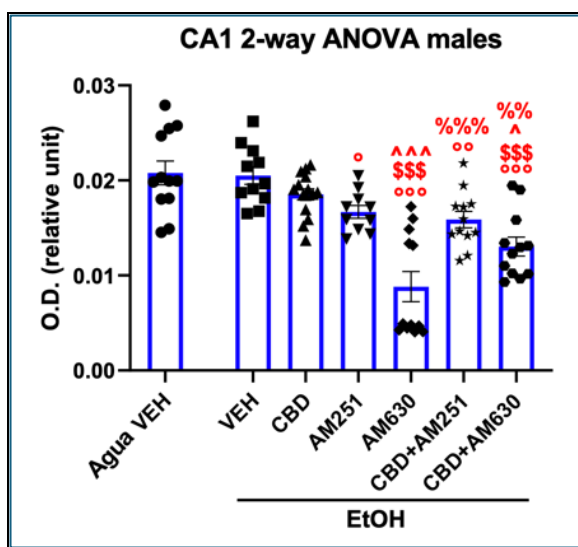


Figure 49: Optical density of Iba1 in CA1 in males
SPSS Two-way ANOVA:
Significant effect of treatment ($p < 0.001$)
Tukey post-hoc Comparisons in the same sex:
 $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ vs EtOH VEH
 $\$p < 0.05$, $\$ \$ \$p < 0.001$ vs EtOH CBD
 $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs EtOH AM251
 $\%p < 0.01$, $\% \% \%p < 0.001$ vs EtOH AM630

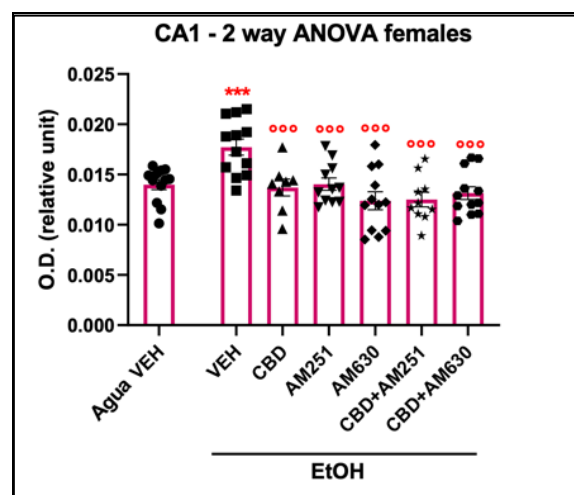


Figure 50: Optical density of CA1 in females
SPSS Two-way ANOVA:
Tukey post-hoc
Significant effect of alcohol and treatment ($p < 0.01$)
Comparisons in the same sex:
 $***p < 0.001$ vs Agua VEH
 $^{\circ\circ\circ}p < 0.001$ vs EtOH VEH

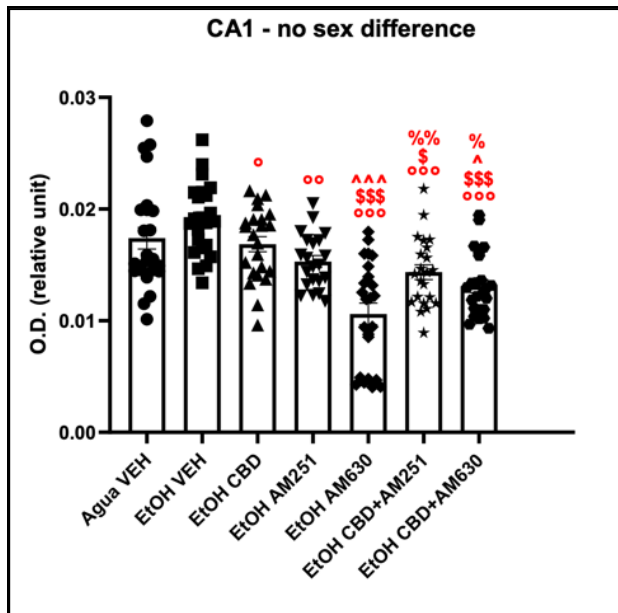


Figure 51: Optical density of Iba1 in CA1 in males and females
 SPSS Two-way ANOVA:
 significant effect of treatment ($p < 0.001$). Tukey post-hoc
 Comparisons:
 $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs EtOH VEH
 $^{\$}p < 0.05$, $^{\$ \$ \$}p < 0.001$ vs EtOH CBD
 $^{\wedge}p < 0.05$, $^{\wedge \wedge \wedge}p < 0.001$ vs EtOH AM251
 $^{\%}p < 0.05$, $^{\% \%}p < 0.01$ vs EtOH AM630

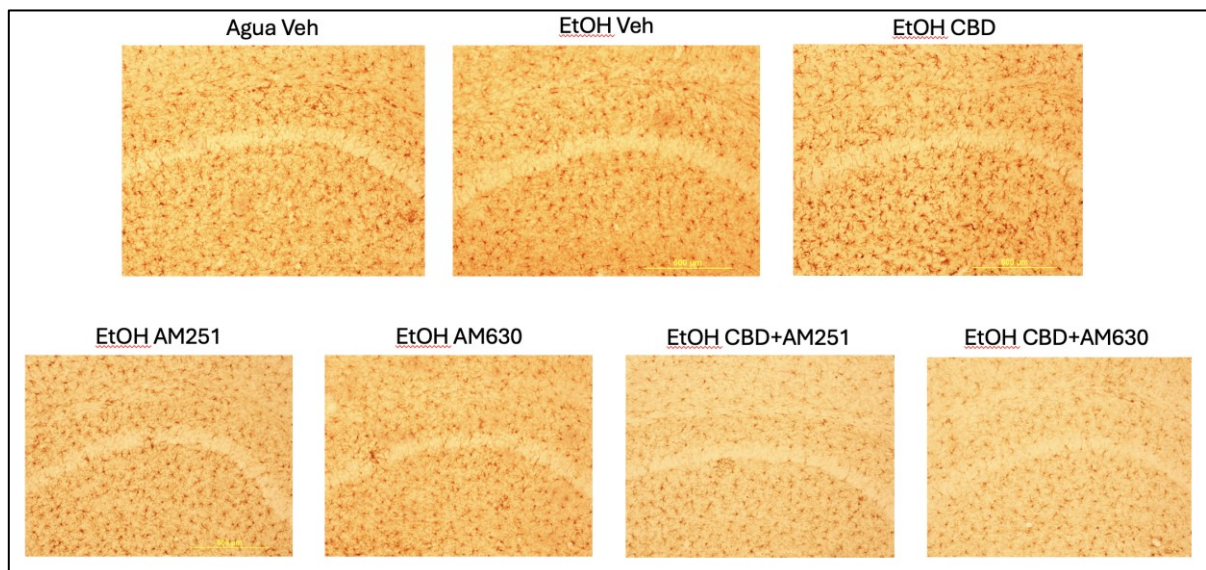


Figure 52: Iba1 marked glia cells in CA1 (males, 10x enlargement)

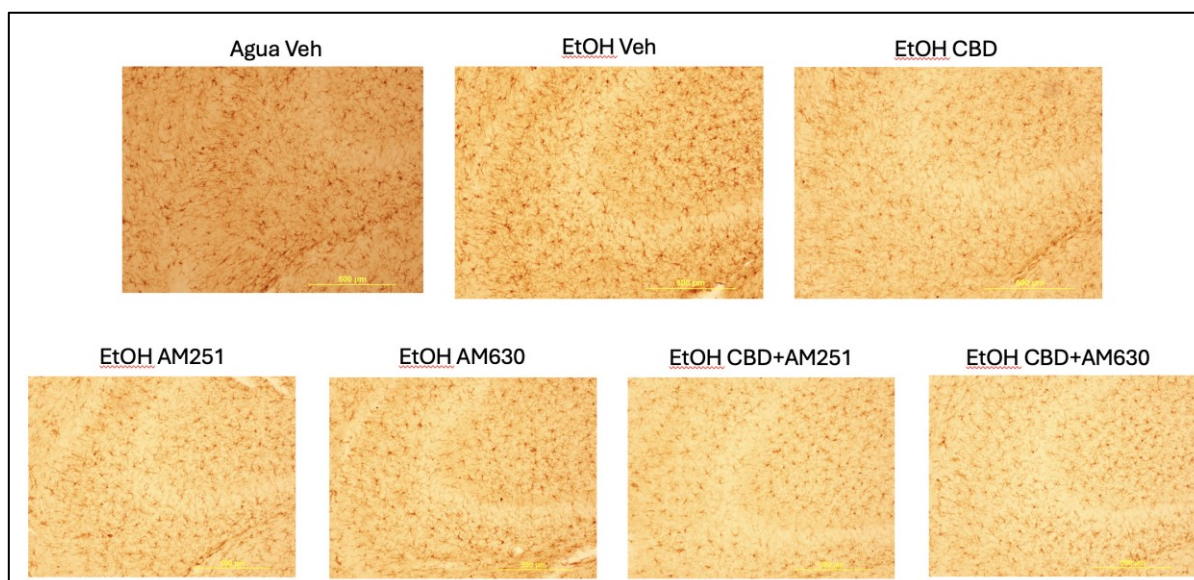


Figure 53: Iba1 marked glia cells in CA1 (females, 10x enlargement)

3.2.1.4 Iba1 in total hippocampus

The three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) identified a significant main effect of Treatment ($F(5, 149) = 25.150, p = 0.000$), Sex ($F(1, 149) = 21.331, p = 0.000$), Alcohol ($F(1, 149) = 4.887, p = 0.029$) and Sex $\times$ Treatment ($F(5, 149) = 6.752, p = 0.000$).

Within EtOH, males (Figure 54) showed a significant Treatment effect ($F(5, 80) = 22.959, p = 0.000$). In females (Figure 55), Treatment ($F(5, 69) = 6.656, p = 0.000$) and Alcohol ($F(1, 69) = 6.451, p = 0.013$) was significant.

The pooled (no Sex, Figure 56) two-way ANOVA (Alcohol $\times$ Treatment) also showed significant Treatment effect ($F(5, 156) = 19.075, p = 0.000$).

Post-hoc tests (EtOH, males) indicated significant lower Iba1 for CBD ($p = 0.009$), AM251 ($p = 0.001$), AM630 ($p = 0.000$), CBD + AM251 ($p = 0.000$) and CBD + AM630 ($p = 0.000$) compared with EtOH Veh. In females Iba1 was significant lower for CBD ($p = 0.002$), AM251 ($p = 0.010$), AM630 ($p = 0.000$), CBD + AM251 ($p = 0.000$) and CBD + AM630 ($p = 0.000$) compared with EtOH Veh. Iba1 was heightened in the EtOH Veh group compared to Agua Veh ($p = 0.013$).

Table 16: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	12	0.0278 $\pm$ 0.0052
Ethanol	Male	12	0.0295 $\pm$ 0.0045
Ethanol + CBD	Male	15	0.0245 $\pm$ 0.0047
Ethanol + AM251	Male	12	0.0223 $\pm$ 0.0038
Ethanol + AM630	Male	12	0.0106 $\pm$ 0.0064
Ethanol + CBD + AM251	Male	12	0.0172 $\pm$ 0.0043
Ethanol + CBD + AM630	Male	12	0.0164 $\pm$ 0.0034

Control	Female	12	0.0189 ± 0.0034
Ethanol	Female	12	0.0229 ± 0.0041
Ethanol + CBD	Female	8	0.0173 ± 0.0028
Ethanol + AM251	Female	12	0.0186 ± 0.0051
Ethanol + AM630	Female	12	0.0165 ± 0.0046
Ethanol + CBD + AM251	Female	12	0.0180 ± 0.0059
Ethanol + CBD + AM630	Female	12	0.0144 ± 0.0029

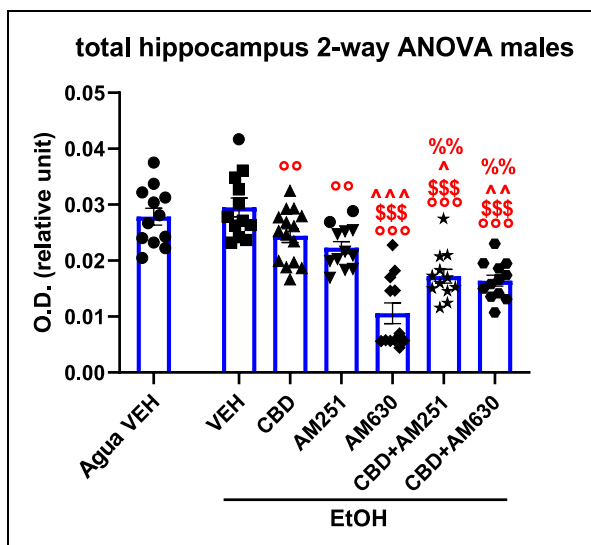


Figure 54: Optical density of Iba1 in total hippocampus in males

SPSS Two-way ANOVA:

Significant effect of treatment ($p < 0.001$)

Tukey post-hoc

Comparisons in the same sex:

$^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ vs EtOH VEH

$$$$p < 0.001$ vs EtOH CBD

$^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs EtOH AM251

$%%p < 0.01$ vs EtOH AM630

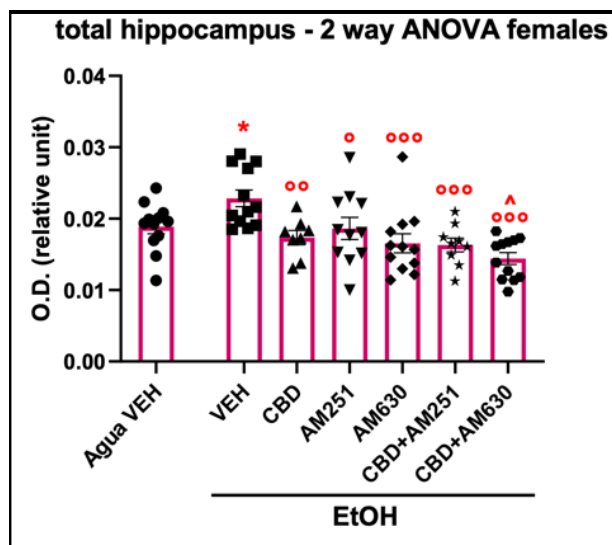


Figure 55: Optical density of Iba1 in total hippocampus in females

SPSS Two-way ANOVA:

Significant effect of alcohol ($p < 0.05$) and treatment ($p < 0.01$)

Comparisons in the same sex:

$*p < 0.05$ vs Agua VEH

$^{\circ}p < 0.05$, $^{\circ\circ}p < 0.001$ vs EtOH VEH

$^{\wedge}p < 0.05$ vs EtOH AM251

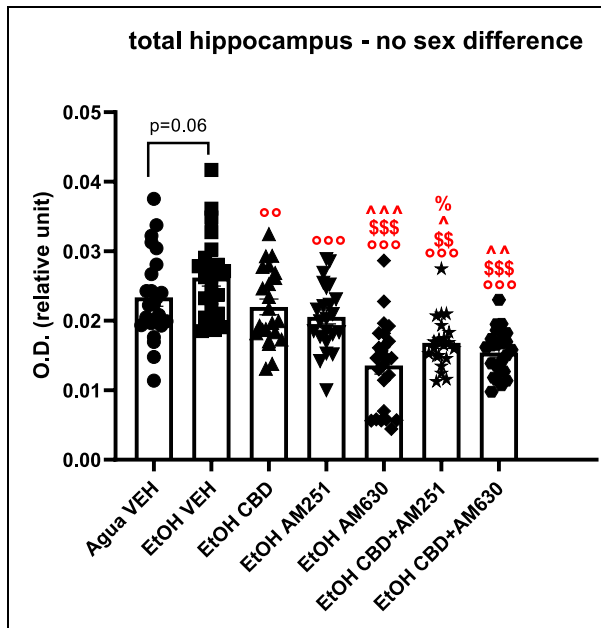


Figure 56: Optical density of Iba1 in total hippocampus in males and females

SPSS Two-way ANOVA:

Significant effect of treatment ($p < 0.001$)

Tukey post-hoc

Comparisons:

$^{**}p < 0.01$, $^{***}p < 0.001$ vs EtOH VEH

$^{$$$}p < 0.01$, $^{$$$$}p < 0.001$ vs EtOH CBD

$^{^}p < 0.05$, $^{^^}p < 0.01$, $^{^^^}p < 0.001$ vs EtOH AM251

$^{%}p < 0.05$ vs EtOH AM630

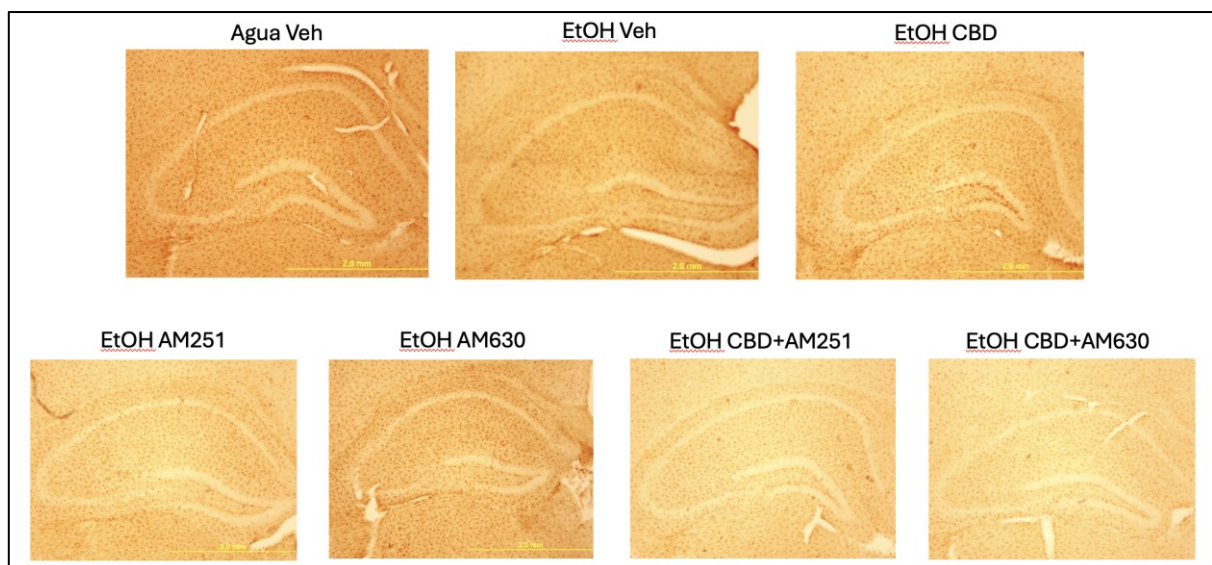


Figure 57: Iba1 marked glia cells in total hippocampus (males, 4x enlargement)

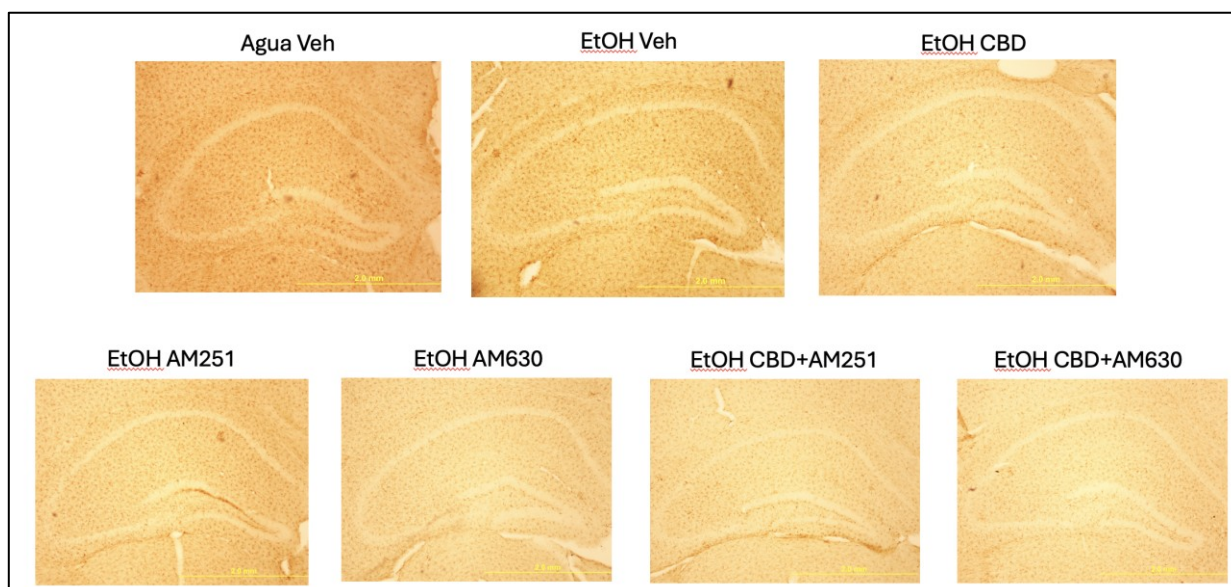


Figure 58: Iba1 marked glia cells in total hippocampus (females, 4x enlargement)

3.2.1.5 Discussion: Iba1

The experiment demonstrated that cannabidiol (CBD) significantly reduced Iba1 expression in the hippocampus, an established marker of neuroinflammation. AM251 and AM630 were also effective, with AM630 showing a particularly strong effect in males. This finding highlights the importance of CB2R signaling in neuroinflammatory processes in the male hippocampus. Consistent with its modulatory properties, CBD partially attenuated the effect of AM630.

In females, Iba1 levels were significantly elevated in the EtOH Veh group in the dentate gyrus and CA1 region, indicating neuroinflammation induced by prenatal ethanol exposure. This effect was not observed in males.

3.2.2 GFAP

3.2.2.1 GFAP in dentate gyrus

The three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) identified a significant main effect of Treatment ($F(5, 142) = 6.882, p = 0.000$).

Within EtOH, males (Figure 59) showed a significant Treatment effect ($F(5, 80) = 6.410, p = 0.000$). In females (Figure 60), Treatment ($F(5, 76) = 2.826, p = 0.022$) was significant.

The pooled (no Sex, Figure 61) two-way ANOVA (Alcohol $\times$ Treatment) also showed significant Treatment effect ($F(5, 149) = 7.563, p = 0.000$).

Post-hoc tests (males) indicated significant higher GFAP for CBD ($p = 0.001$) compared with EtOH Veh. In females GFAP was significant lower for AM251 ($p = 0.004$), AM630 ($p = 0.013$) and CBD + AM251 ($p = 0.029$) compared with EtOH Veh.

Table 17: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ SEM
Control	Male	11	0.0180 $\pm$ 0.0074
Ethanol	Male	12	0.0160 $\pm$ 0.0096
Ethanol + CBD	Male	16	0.0204 $\pm$ 0.0065
Ethanol + AM251	Male	12	0.0145 $\pm$ 0.0059
Ethanol + AM630	Male	12	0.0121 $\pm$ 0.0016
Ethanol + CBD + AM251	Male	12	0.0141 $\pm$ 0.0020
Ethanol + CBD + AM630	Male	12	0.0129 $\pm$ 0.0034
Control	Female	12	0.0159 $\pm$ 0.0064
Ethanol	Female	11	0.0165 $\pm$ 0.0064
Ethanol + CBD	Female	11	0.0157 $\pm$ 0.0051
Ethanol + AM251	Female	10	0.0115 $\pm$ 0.0015
Ethanol + AM630	Female	11	0.0121 $\pm$ 0.0018
Ethanol + CBD + AM251	Female	12	0.0129 $\pm$ 0.0039
Ethanol + CBD + AM630	Female	11	0.0133 $\pm$ 0.0021

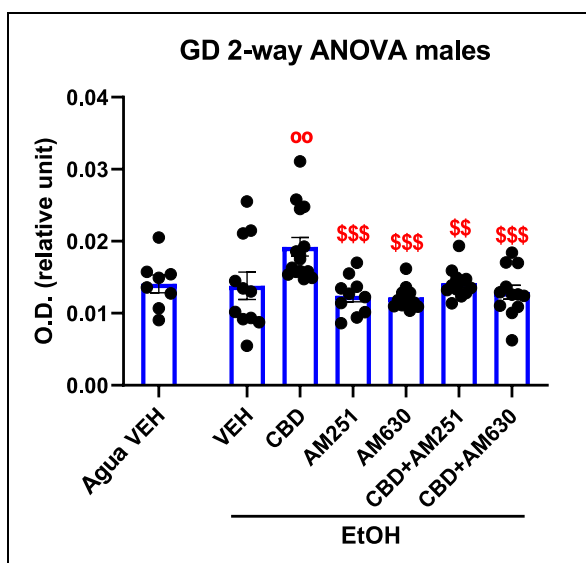


Figure 59: Optical density of GFAP in dentate gyrus in males
SPSS Two-way ANOVA:
Tukey post-hoc
Significant effect of treatment ($p < 0.001$)
Comparisons in the same sex:
°° $p < 0.01$ vs EtOH VEH
\$\$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs EtOH CBD

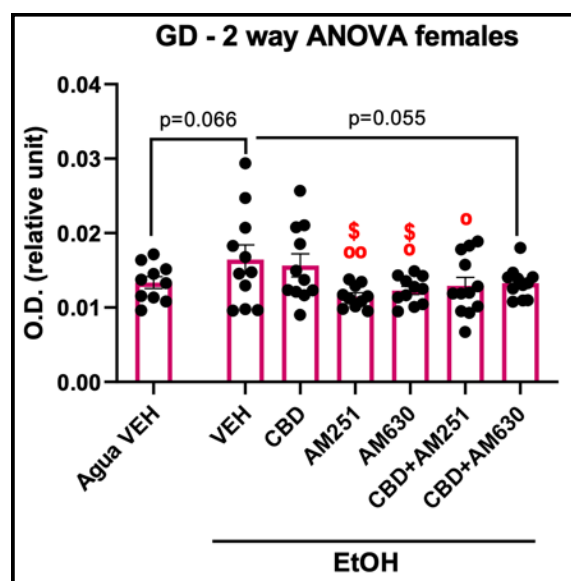


Figure 60: Optical density of GFAP in dentate gyrus in females
SPSS Two-way ANOVA:
Tukey post-hoc
Significant effect of treatment ($p < 0.05$)
Comparisons in the same sex:
° $p < 0.05$, °° $p < 0.01$ vs EtOH VEH
\$ $p < 0.05$ vs EtOH CBD

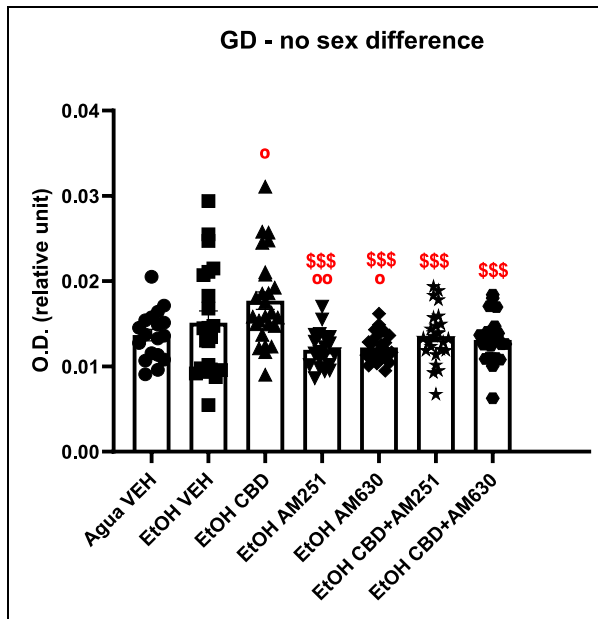


Figure 61: Optical density of GFAP in dentate gyrus in males and females

SPSS Two-way ANOVA:

Tukey post-hoc

Significant effect of treatment ($p < 0.001$)

Comparisons:

° $p < 0.05$, °° $p < 0.01$ vs EtOH VEH

\$\$\$ $p < 0.001$ vs EtOH CBD

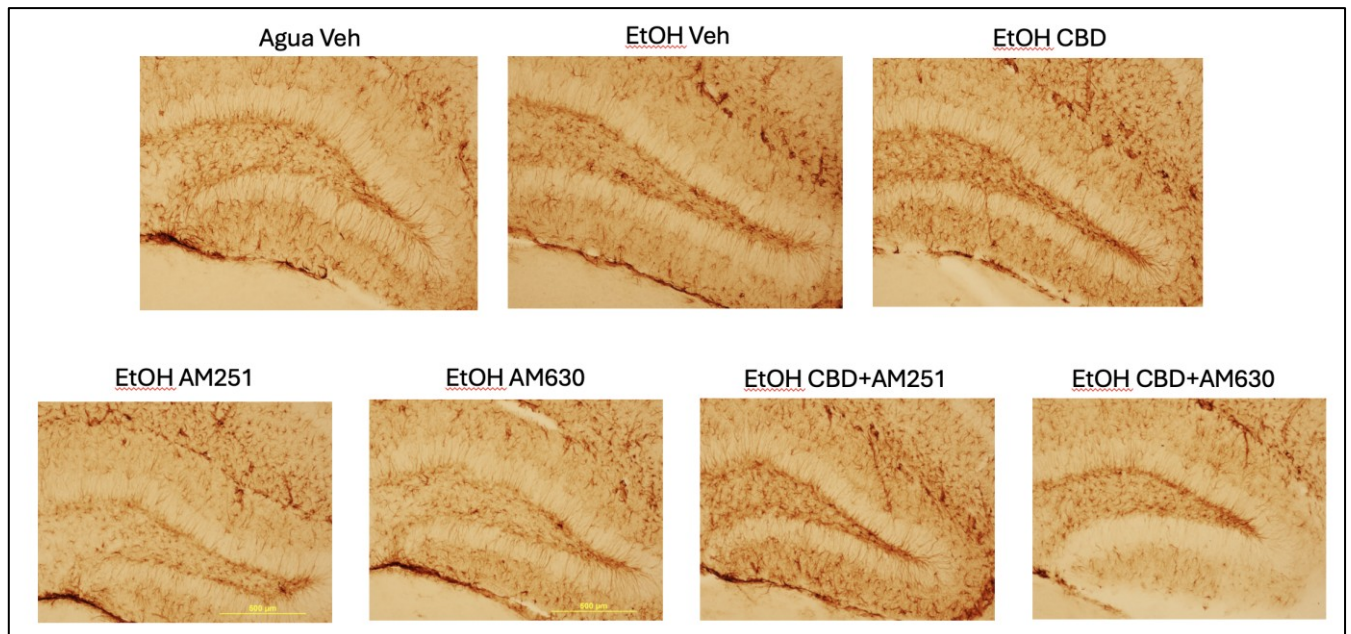


Figure 62: GFAP marked glia cells in dentate gyrus (males, 10x enlargement)

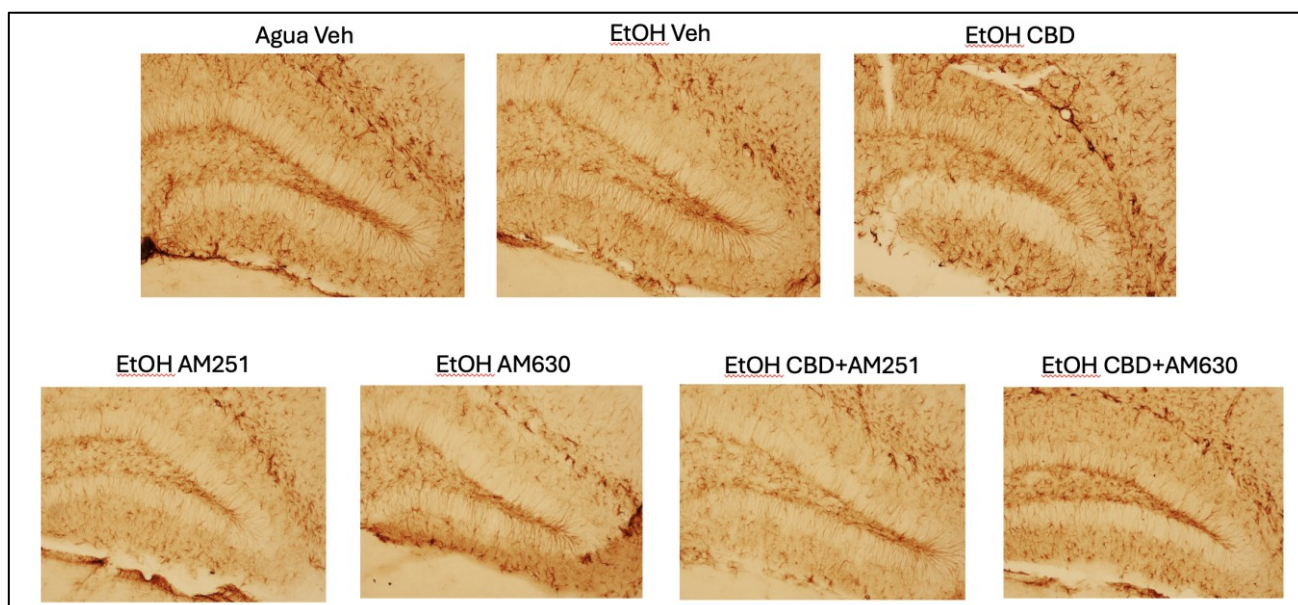


Figure 63: GFAP marked glia cells in dentate gyrus (females, 10x enlargement)

3.2.2.2 GFAP in CA3

There were no significant effects detected (three-way and two-way ANOVA).

Table 18: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ Standard Deviation
Control	Male	11	0.0061 $\pm$ 0.0036
Ethanol	Male	12	0.0058 $\pm$ 0.0027
Ethanol + CBD	Male	16	0.0074 $\pm$ 0.0031
Ethanol + AM251	Male	12	0.0051 $\pm$ 0.0031
Ethanol + AM630	Male	12	0.0061 $\pm$ 0.0014
Ethanol + CBD + AM251	Male	12	0.0056 $\pm$ 0.0019
Ethanol + CBD + AM630	Male	12	0.0057 $\pm$ 0.0019
Control	Female	12	0.0056 $\pm$ 0.0032
Ethanol	Female	10	0.0047 $\pm$ 0.0015
Ethanol + CBD	Female	12	0.0052 $\pm$ 0.0024
Ethanol + AM251	Female	10	0.0051 $\pm$ 0.0021
Ethanol + AM630	Female	11	0.0041 $\pm$ 0.0017
Ethanol + CBD + AM251	Female	12	0.0047 $\pm$ 0.0018
Ethanol + CBD + AM630	Female	11	0.0059 $\pm$ 0.0018

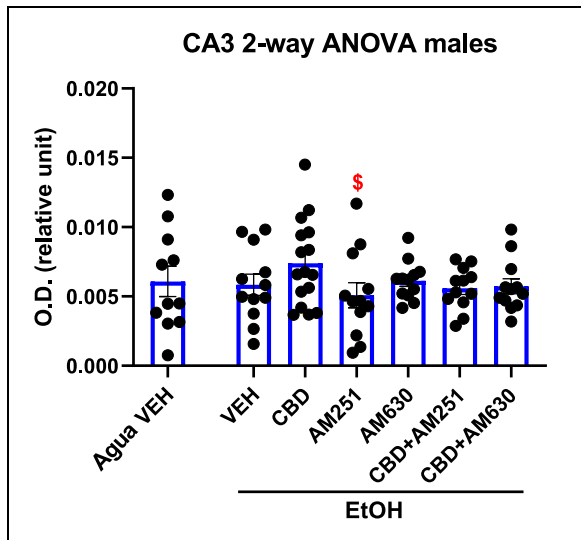


Figure 64: Optical density of GFAP in CA3 in males
SPSS Two-way ANOVA:
Tukey post-hoc
No significant effect
Comparisons in the same sex:
\$ $p < 0.05$ vs EtOH CBD

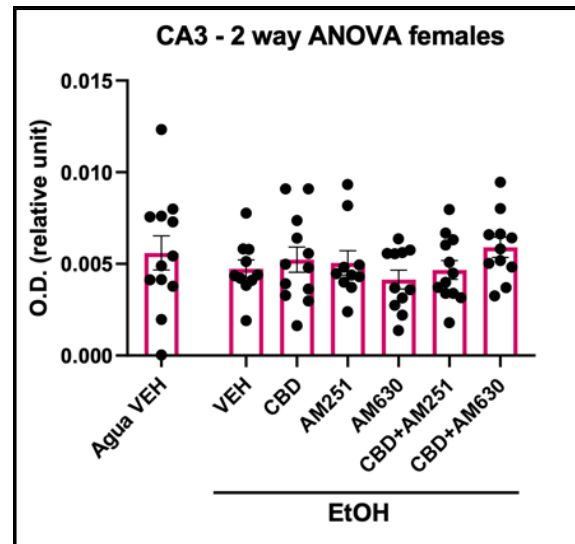


Figure 65: Optical density of GFAP in CA3 in females
SPSS Two-way ANOVA:
Tukey post-hoc
No significant effect.

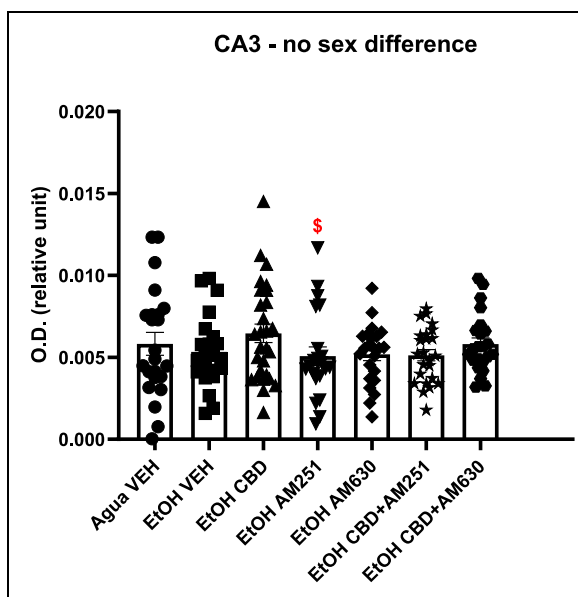


Figure 66: Optical density of GFAP in CA3 in males and females
SPSS Two-way ANOVA:
Tukey post-hoc
No significant effect

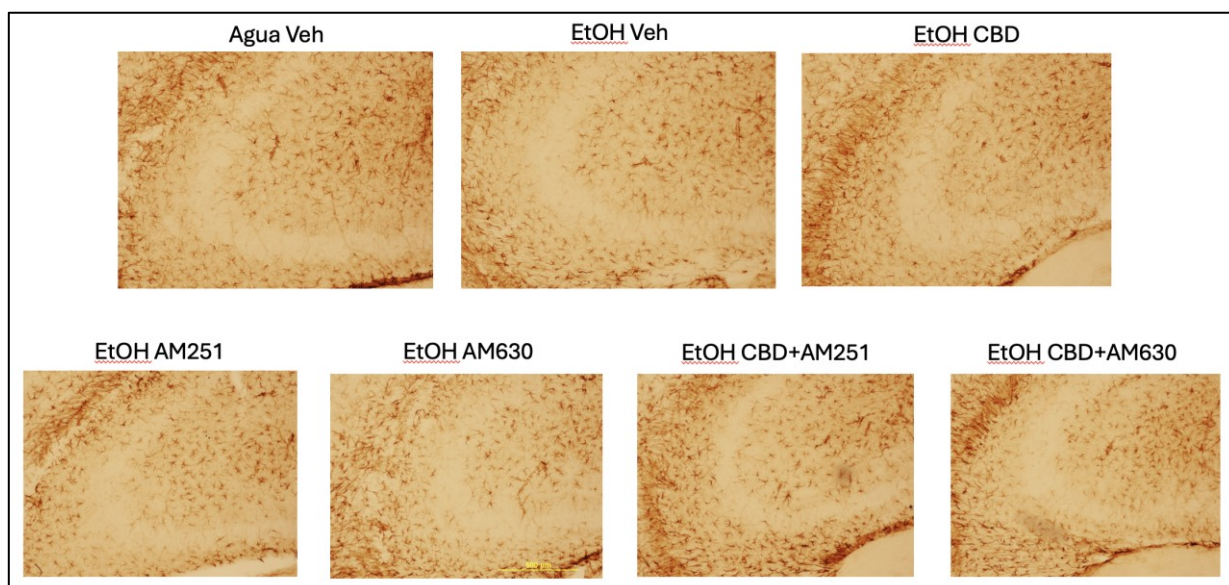


Figure 67: GFAP marked glia cells in CA3 (males, 10x enlargement)

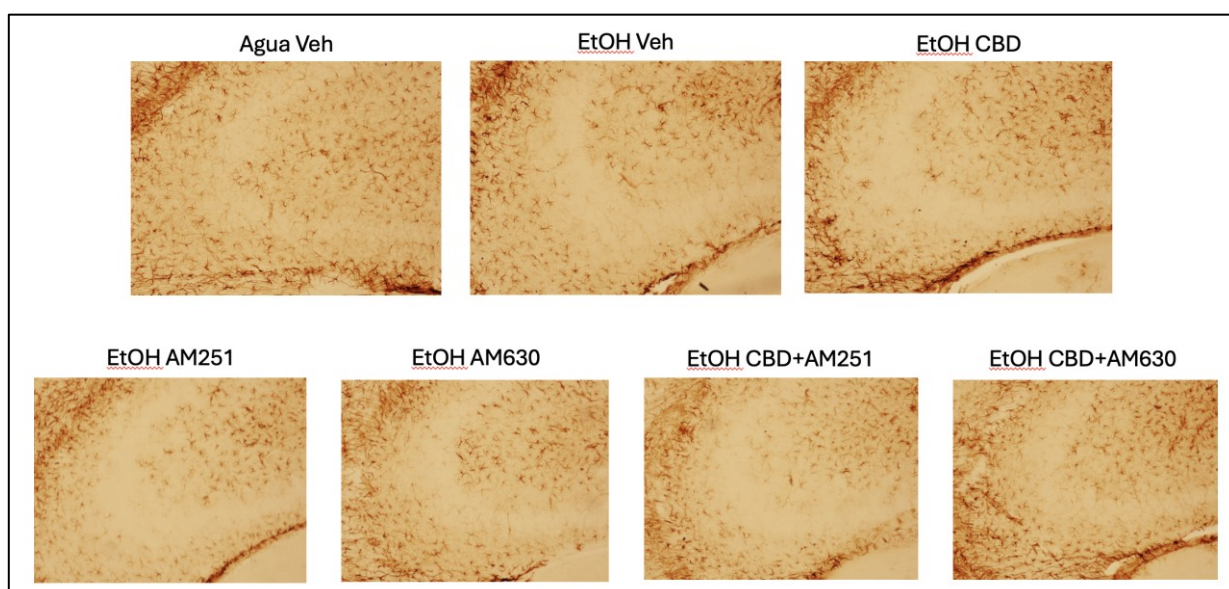


Figure 68: GFAP marked glia cells in CA3 (females, 10x enlargement)

3.2.2.3 GFAP in CA1

The three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) identified a significant main effect of Treatment ($F(5, 147) = 10.663, p = 0.000$), Sex ($F(1, 147) = 10.342, p = 0.002$), Alcohol ($F(1, 147) = 7.532, p = 0.007$) and Sex $\times$ Treatment ($F(5, 147) = 6.477, p = 0.000$).

Within EtOH, males (Figure 69) showed a significant Treatment effect ($F(5, 80) = 22.959, p = 0.000$). In females (Figure 70), Treatment ($F(5, 69) = 6.656, p = 0.000$) was significant.

The pooled (no Sex, Figure 71) two-way ANOVA (Alcohol $\times$ Treatment) also showed a significant Treatment effect ($F(5, 154) = 8.069, p = 0.000$) and Alcohol effect ($F(1, 156) = 5.618, p = 0.019$).

Post-hoc tests (males) indicated significant lower GFAP for CBD + AM251 ($p = 0.002$) and CBD + AM630 ($p = 0.001$) compared with EtOH Veh. In females GFAP was significant lower for CBD ($p = 0.000$), AM251 ($p = 0.001$) compared with EtOH Veh.

= 0.000), AM630 ($p = 0.007$), CBD + AM251 ($p = 0.000$) and CBD + AM630 ($p = 0.000$) compared with EtOH Veh.

Table 19: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ Standard Deviation
Control	Male	11	0.0129 ± 0.0021
Ethanol	Male	11	0.0151 ± 0.0038
Ethanol + CBD	Male	14	0.017 ± 0.0032
Ethanol + AM251	Male	12	0.0130 ± 0.0025
Ethanol + AM630	Male	12	0.0133 ± 0.0021
Ethanol + CBD + AM251	Male	12	0.0116 ± 0.0031
Ethanol + CBD + AM630	Male	12	0.0130 ± 0.0045
Control	Female	12	0.0135 ± 0.0020
Ethanol	Female	11	0.0154 ± 0.0044
Ethanol + CBD	Female	12	0.0109 ± 0.0017
Ethanol + AM251	Female	11	0.0109 ± 0.0018
Ethanol + AM630	Female	10	0.0091 ± 0.0020
Ethanol + CBD + AM251	Female	11	0.0097 ± 0.0025
Ethanol + CBD + AM630	Female	12	0.0126 ± 0.0015

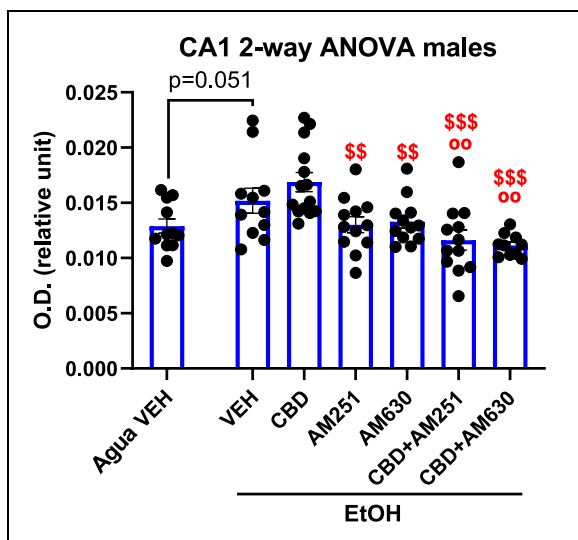


Figure 69: Optical density of GFAP in CA1 in males
SPSS Two-way ANOVA:
Tukey post-hoc
Significant effect of treatment ($p < 0.001$)
Comparisons in the same sex:
 $^{**}p < 0.01$ vs EtOH VEH
 $^{***}p < 0.01$, $^{$$$}p < 0.001$ vs EtOH CBD

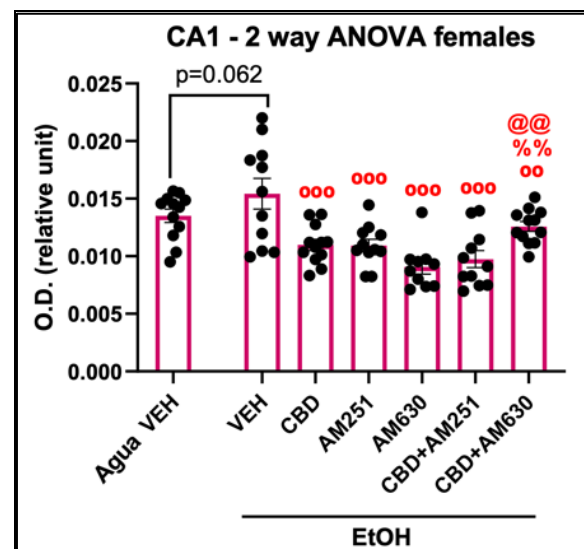


Figure 70: Optical density of GFAP in CA1 in females
SPSS Two-way ANOVA:
Tukey post-hoc
Significant effect of treatment ($p < 0.001$).
 $^{***}p < 0.001$ vs EtOH VEH
 $^{%%}p < 0.01$ vs EtOH AM630
 $^{@@}p < 0.01$ vs EtOH CBD+AM251

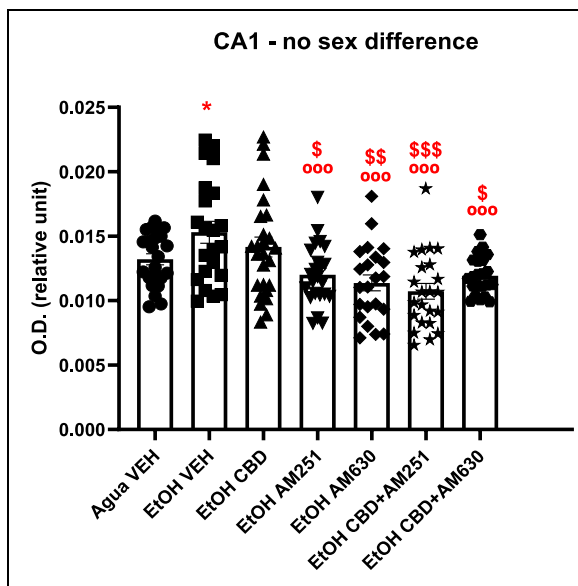


Figure 71: Optical density of GFAP in CA1 in males and females

SPSS Two-way ANOVA:

Significant effect of treatment ($p < 0.001$)

Tukey post-hoc

Comparisons:

$^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ vs EtOH VEH

$$$$p < 0.01$, $$$$$p < 0.001$ vs EtOH CBD

$^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs EtOH AM251

$^{\%}p < 0.05$ vs EtOH AM630

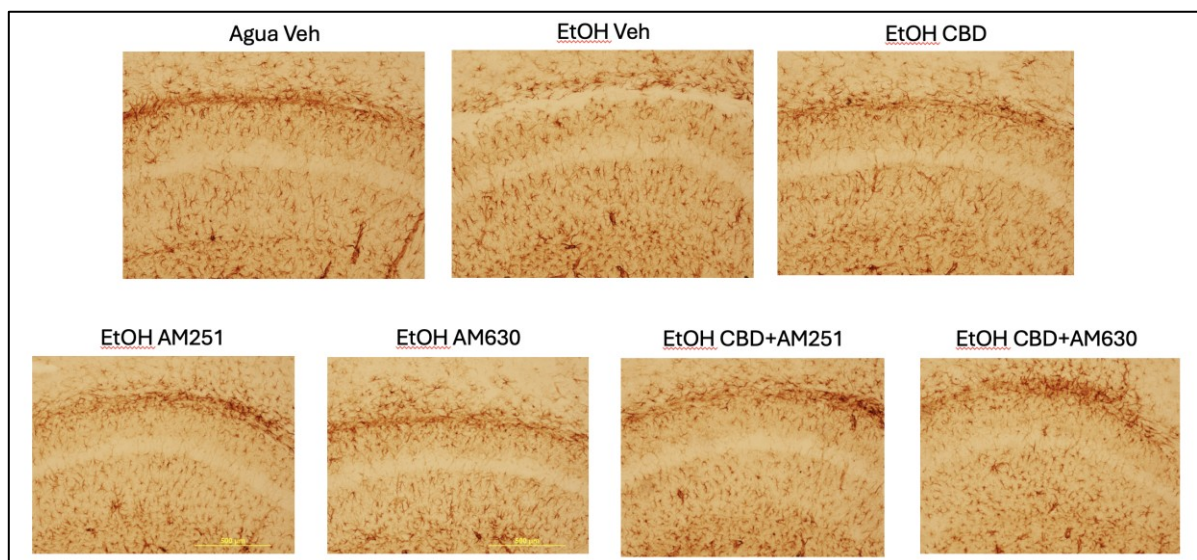


Figure 72: GFAP marked glia cells in CA1 (males, 4x enlargement)

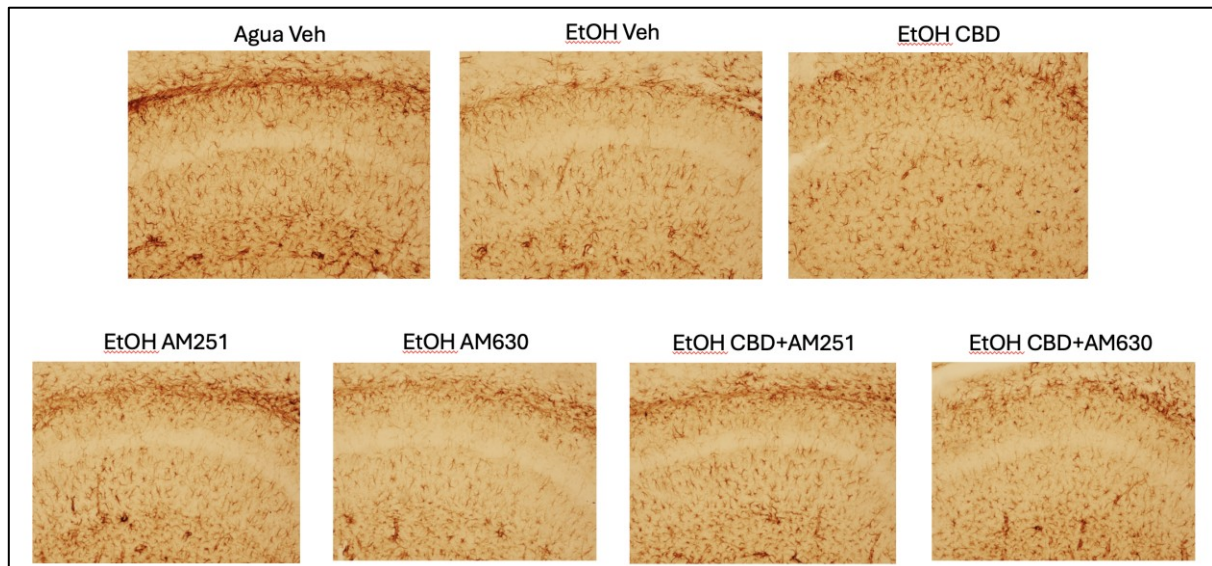


Figure 73: GFAP marked glia cells in CA1 (females, 10x enlargement)

3.2.2.4 GFAP in total hippocampus

The three-way ANOVA (Sex $\times$ Alcohol $\times$ Treatment) identified a significant main effect of Treatment ($F(5, 151) = 5.259$, $p = 0.000$), of Sex ($F(5, 151) = 9.972$, $p = 0.002$) and of Sex $\times$ Treatment ($F(5, 151) = 3.330$, $p = 0.007$).

Within EtOH, males (Figure 74) showed a significant Treatment effect ($F(5, 79) = 5.316$, $p = 0.000$). In females (Figure 75), Treatment ($F(5, 72) = 3.483$, $p = 0.007$) was significant.

The pooled (no Sex, Figure 76) two-way ANOVA (Alcohol $\times$ Treatment) also showed significant Treatment effect ($F(5, 158) = 5.326$, $p = 0.000$).

Post-hoc tests (males) indicated significant higher GFAP for CBD ($p = 0.013$) compared with EtOH Veh. In females GFAP was significant lower for AM251 ($p = 0.015$), AM630 ($p = 0.001$) and CBD + AM251 ($p = 0.016$) compared with EtOH Veh.

Table 20: Values of mean and SEM of each experimental group, sex and sample size.

Group	Sex	n	Mean $\pm$ Standard Deviation
Control	Male	11	0.0113 $\pm$ 0.0033
Ethanol	Male	12	0.0110 $\pm$ 0.0035
Ethanol + CBD	Male	16	0.0133 $\pm$ 0.0023
Ethanol + AM251	Male	12	0.0099 $\pm$ 0.0023
Ethanol + AM630	Male	12	0.0100 $\pm$ 0.0013
Ethanol + CBD + AM251	Male	12	0.0099 $\pm$ 0.0013
Ethanol + CBD + AM630	Male	12	0.0100 $\pm$ 0.0025
Control	Female	12	0.0107 $\pm$ 0.0021
Ethanol	Female	11	0.0125 $\pm$ 0.0051
Ethanol + CBD	Female	12	0.0095 $\pm$ 0.0020

Ethanol + AM251	Female	10	0.0087 ± 0.0012
Ethanol + CBD + AM251	Female	12	0.0088 ± 0.0020
Ethanol + CBD + AM630	Female	12	0.0101 ± 0.0012

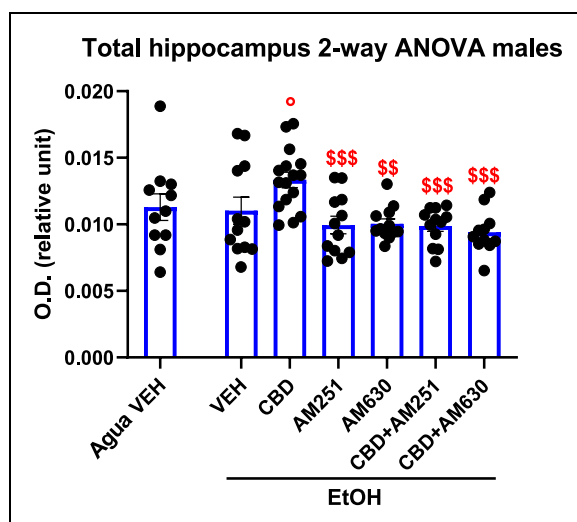


Figure 74: Optical density of Iba1 in total hippocampus in males

SPSS Two-way ANOVA:

Tukey post-hoc

Significant effect of treatment ($p < 0.001$)

Comparisons in the same sex:

$^{\circ}p < 0.05$ vs EtOH VEH

$$$$p < 0.01$, $$$$$p < 0.001$ vs EtOH CBD

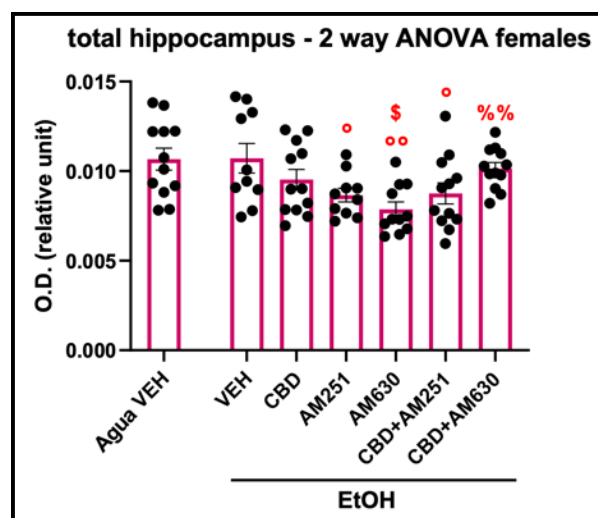


Figure 75: Optical density of Iba1 in total hippocampus in females

SPSS Two-way ANOVA:

Significant effect of alcohol ($p < 0.05$) and treatment ($p < 0.01$)

Comparisons in the same sex:

$*p < 0.05$ vs Agua VEH

$^{\circ}p < 0.05$, $^{\circ\circ}p < 0.001$ vs EtOH VEH

$^{\wedge}p < 0.05$ vs EtOH AM251

$%%p < 0.01$ vs EtOH AM630

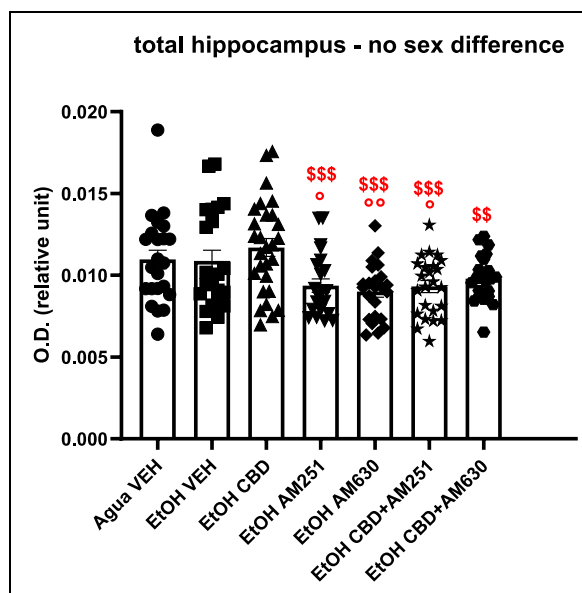


Figure 76: Optical density of Iba1 in total hippocampus in males and females

SPSS Two-way ANOVA:

Tukey post-hoc

Significant effect of treatment ($p < 0.001$)

Comparisons:

$^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$ vs EtOH VEH

$$$$p < 0.01$, $$$$$p < 0.001$ vs EtOH CBD

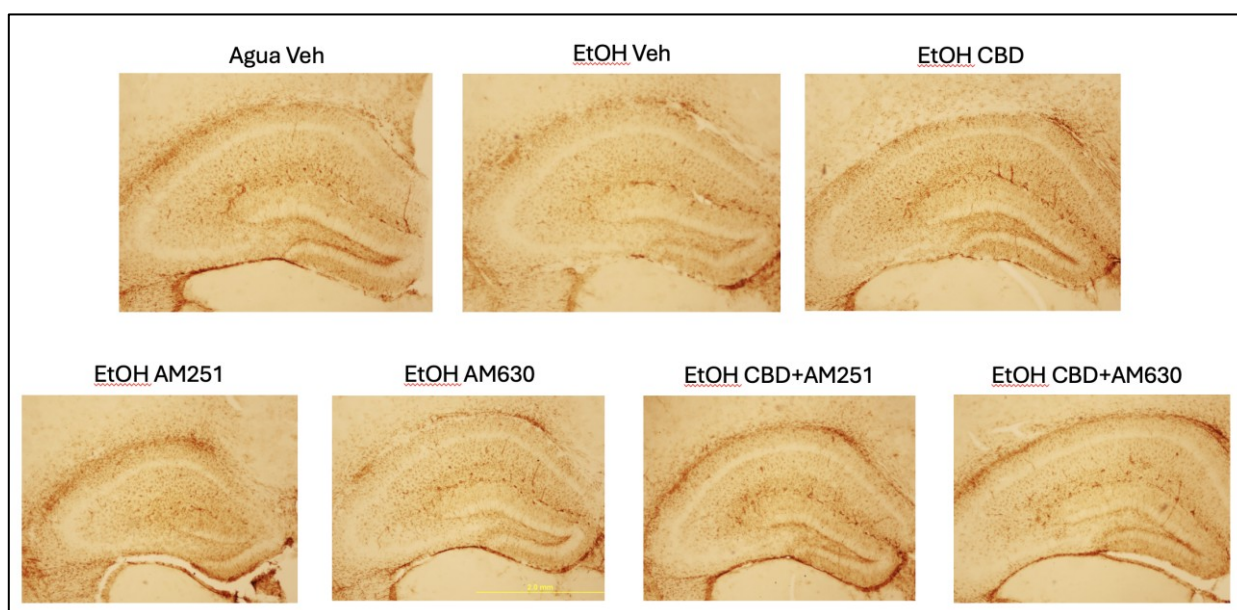


Figure 77: GFAP marked glia cells in total hippocampus (males, 4x enlargement)

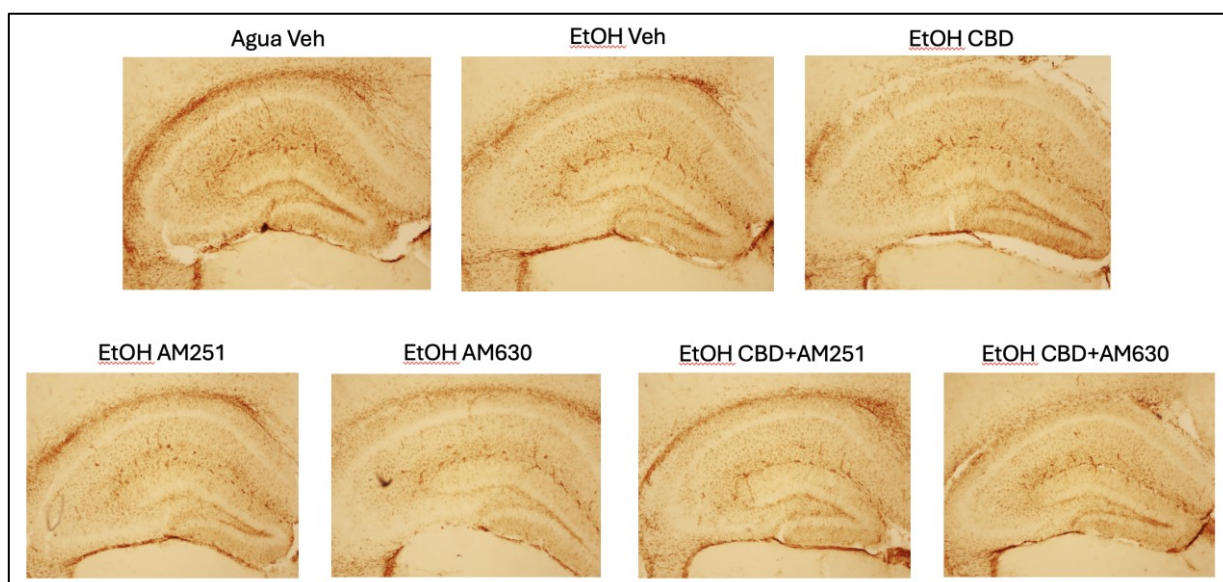


Figure 78: GFAP marked glia cells in total hippocampus (females, 4x enlargement)

3.2.2.5 Discussion: GFAP

The bidirectional nature of GFAP, as a marker of both disrupted neuronal migration and neuroinflammation, combined with the absence of changes in any EtOH Veh group, complicates interpretation. In males, CBD increased GFAP in the dentate gyrus, which could indicate improved neuronal migration, although alternative explanations (e.g., astrogliosis) cannot be excluded. In females, AM251, AM630, and AM251 combined with CBD reduced GFAP in the dentate gyrus, potentially reflecting lower neuroinflammation. Moreover, all treatments decreased GFAP in the female CA3 region, again consistent with reduced

neuroinflammation. The significant main effect of treatment indicates that the endocannabinoid system modulates GFAP expression, although the relevance of this finding for FASD requires further investigation. The bidirectional profile of GFAP in our dataset limits its specificity for distinguishing reactive astrogliosis from altered astrogenesis. To reduce interpretative ambiguity, future work should incorporate complementary astrocytic markers and co-localization analyses alongside the current readouts.

3.3 Discussion in the Context of the Literature

This study shows that pharmacological manipulation of the endocannabinoid system (ECS) with CBD, AM251, and AM630 yields region- and sex-dependent effects on neuroinflammatory status and intracellular signaling cascades disrupted by prenatal alcohol exposure (PAE). Our observations align with prior work indicating that PAE provokes chronic neuroimmune activation and signaling imbalances in the hippocampus, contributing to the cognitive and affective symptoms associated with FASD. Consistent with the literature, PAE elevates proinflammatory mediators and enhances microglial reactivity within hippocampal networks. These are core processes in ethanol-driven neurotoxicity.⁴³

Neuroinflammation: Iba1 and GFAP

In line with previous reports, both CBD and the CB2 inverse agonist AM630 diminished microglial activation, as indexed by Iba1, across several hippocampal subfields. CBD has been shown to dampen PAE-elicited neuroinflammation by normalizing hippocampal TNF- α and IL-6 levels⁴³, and the pronounced efficacy of AM630 in males reinforces the central role of CB2 receptors in calibrating microglial responsivity and neuroimmune tone. Although CB2 activation is generally linked to anti-inflammatory signaling, context-dependent outcomes—wherein CB2 blockade or inverse agonism can induce a microglial shift towards a M2-like phenotype.⁷⁴ The sex-selective effects observed here likely reflect sexually dimorphic trajectories of microglial maturation and ECS component expression during development.

Astroglial responses, assessed by GFAP, were more variable. An increase in dentate gyrus GFAP in males following CBD contrasts with reductions observed in females under AM251 and AM630. Such divergence is compatible with evidence that astrocyte reactivity to cannabinoids depends on sex and developmental stage, and with the notion that ethanol exposure itself induces persistent astroglial activation. The broadly observed reductions in CA1 GFAP across conditions in both sexes are congruent with CBD's reported capacity to lower astrocytic activation in FASD-like contexts.⁴³

Intracellular signaling: mTOR, AKT, AMPK, PKA

A male-specific elevation of phosphorylated mTOR under AM630 implies functional crosstalk between CB2 signaling and mTOR-dependent pathways governing neurodevelopment and synaptic plasticity. Given that PAE disturbs mTOR signaling with consequences for neuronal growth and metabolic homeostasis,

these results indicate that CB2-directed modulation can partially restore mTOR phosphorylation without altering total mTOR protein abundance.

In females, AM251 and CBD+AM251 increased pAKT, consistent with the influence of CB1 receptor modulation on AKT, a pathway fundamental to neuronal survival and synaptic efficacy. AKT dysregulation is a recognized contributor to PAE-related hippocampal dysfunction, and CBD has been shown to normalize apoptotic and inflammatory signals downstream of AKT in FASD models.⁴³

By contrast, phosphorylation of AMPK and PKA was largely resistant to the interventions. Despite CBD's capacity to impact cAMP/PKA signaling via negative allosteric modulation of CB1 and antagonism at CB2 (see thesis introduction), the absence of measurable effects here may reflect constraints related to developmental stage, dosing parameters, or regional specificity.

Dopaminergic markers: DARPP-32 and DAT

DARPP-32 exhibited treatment sensitivity, with notable increases following CBD in females. This pattern aligns with evidence that CBD can modulate dopaminergic transmission and improve behavioral outcomes in FASD models.⁷⁵ In contrast, DAT levels were unchanged, which is consistent with reports that dopamine transporter expression is comparatively unresponsive to cannabinoid-based interventions in PAE paradigms.

Overall interpretation

Taken together, these findings show the potential of ECS-focused strategies for attenuating PAE-induced neuroinflammation and signaling dysregulation. CBD's ability to suppress microglial activation and adjust key intracellular pathways accords with prior demonstrations of its efficacy in ameliorating cognitive and neuroimmune disturbances in FASD-like models.⁷⁵ The robust actions of AM630 further position CB2 receptors as compelling targets for neuroimmune modulation, while CB1-linked effects on AKT point to complementary neuroprotective mechanisms.

To refine therapeutic applications for FASD, future work should delineate long-term behavioral efficacy, characterize dose–response functions, and define critical developmental windows for ECS-targeted interventions.

4. Conclusion

Microglial activation (Iba1)

- Pharmacological modulation of the endocannabinoid system attenuated neuroinflammatory alterations induced by prenatal alcohol exposure (PAE) in the hippocampus.
- Iba1 immunoreactivity decreased across total hippocampus and in DG, CA3, and CA1 following CBD, AM251, and especially AM630.
- Sex- and region-specific effects: females showed ethanol-related elevations of Iba1 in DG and CA1, highlighting the therapeutic relevance of cannabinoid-targeted reductions.

Astrocytic responses (GFAP)

- Sex- and region-dependent, bidirectional effects.
- In males, CBD increased GFAP in DG.
- In females, GFAP decreased in DG and CA1 under inverse agonist and combination conditions; no significant changes in CA3.
- These patterns underscore GFAP's dual interpretability as a marker of neuroinflammation and potentially impaired neuronal migration.

Intracellular signaling

- mTOR pathway: phosphorylation state (pmTOR) was treatment-sensitive; total mTOR was largely unchanged, with ethanol selectively reducing total mTOR in males.
- Dopamine-related signaling: DARPP-32 increased under CBD in ethanol-exposed females, partially attenuated by CBD+AM251, suggesting receptor-dependent modulation.
- Energetic signaling: pAMPK showed no consistent treatment-dependent differences.

Overall interpretation

- Endocannabinoid-directed interventions mitigate microglia-associated neuroinflammation and modulate intracellular signaling in a sex-specific manner following PAE.
- Findings support the therapeutic potential of CBD-based strategies (including combinations with receptor antagonists or inverse agonists) in the context of FASD.

Limitations

- TNF- α quantification was not feasible due to insufficient signal intensity.
- In several assays, alcohol exposure did not yield significant changes in target proteins, limiting the interpretability of treatment-induced effects.

Future directions

- Link molecular changes more tightly to functional outcomes (behavior and cognition).
- Incorporate complementary astrocytic markers and co-localization analyses to improve the interpretability of GFAP.

- Optimize dosing and timing of prenatal alcohol exposure to elicit robust changes in relevant molecular markers.

1. Popova, S., Lange, S., Probst, C., Gmel, G. & Rehm, J. Estimation of national, regional, and global prevalence of alcohol use during pregnancy and fetal alcohol syndrome: a systematic review and meta-analysis. *The Lancet Global Health* **5**, e290–e299 (2017).
2. Cook, J. L. *et al.* Fetal alcohol spectrum disorder: a guideline for diagnosis across the lifespan. *CMAJ* **188**, 191–197 (2016).
3. Gupta, K. K., Gupta, V. K. & Shirasaka, T. An Update on Fetal Alcohol Syndrome—Pathogenesis, Risks, and Treatment. *Alcoholism: Clinical and Experimental Research* **40**, 1594–1602 (2016).
4. Lange, S. *et al.* Global Prevalence of Fetal Alcohol Spectrum Disorder Among Children and Youth: A Systematic Review and Meta-analysis. *JAMA Pediatr* **171**, 948–956 (2017).
5. Morin, C. *et al.* Qualitative study of the lived experience of methylphenidate prescribed for children with a fetal alcohol spectrum disorder. *Eur Child Adolesc Psychiatry* **33**, 4219–4235 (2024).
6. Ordenewitz, L. K. *et al.* Evidence-based interventions for children and adolescents with fetal alcohol spectrum disorders – A systematic review. *European Journal of Paediatric Neurology* **33**, 50–60 (2021).
7. Burton, S. *et al.* The stigma of alcohol use during pregnancy: Exploring the impact of alcohol strength and social context on public perceptions. *PLoS One* **21**, e0339954 (2026).
8. Kane, C. J. M. & Drew, P. D. Neuroinflammatory contribution of microglia and astrocytes in fetal alcohol spectrum disorders. *Journal of Neuroscience Research* **99**, 1973–1985 (2021).
9. Hillmer, A. T., Nadim, H., Devine, L., Jatlow, P. & O’Malley, S. S. Acute alcohol consumption alters the peripheral cytokines IL-8 and TNF- α . *Alcohol* **85**, 95–99 (2020).
10. Bodnar, T. S., Hill, L. A. & Weinberg, J. Evidence for an immune signature of prenatal alcohol exposure in female rats. *Brain, Behavior, and Immunity* **58**, 130–141 (2016).
11. Noor, S. & Milligan, E. D. Lifelong Impacts of Moderate Prenatal Alcohol Exposure on Neuroimmune Function. *Front. Immunol.* **9**, (2018).
12. Kane, C. J. M. *et al.* Ethanol modulation of cerebellar neuroinflammation in a postnatal mouse model of fetal alcohol spectrum disorders. *Journal of Neuroscience Research* **99**, 1986–2007 (2021).
13. Zhang, W., Xiao, D., Mao, Q. & Xia, H. Role of neuroinflammation in neurodegeneration development. *Signal Transduct Target Ther* **8**, 267 (2023).

14. Padilla-Valdez, M. M., Díaz-Iñiguez, M. I., Ortuño-Sahagún, D. & Rojas-Mayorquín, A. E. Neuroinflammation in fetal alcohol spectrum disorders and related novel therapeutic approaches. *Biochim Biophys Acta Mol Basis Dis* **1870**, 166854 (2024).
15. Mandolesi, G. *et al.* Interleukin-1 β Alters Glutamate Transmission at Purkinje Cell Synapses in a Mouse Model of Multiple Sclerosis. *J. Neurosci.* **33**, 12105–12121 (2013).
16. Rossi, S. *et al.* Interleukin-1 β causes excitotoxic neurodegeneration and multiple sclerosis disease progression by activating the apoptotic protein p53. *Mol Neurodegeneration* **9**, 56 (2014).
17. Brown, G. C. & Neher, J. J. Microglial phagocytosis of live neurons. *Nat Rev Neurosci* **15**, 209–216 (2014).
18. Saito, M., Chakraborty, G., Hui, M., Masiello, K. & Saito, M. Ethanol-Induced Neurodegeneration and Glial Activation in the Developing Brain. *Brain Sciences* **6**, 31 (2016).
19. Smith, J. A., Das, A., Ray, S. K. & Banik, N. L. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Research Bulletin* **87**, 10–20 (2012).
20. Liddelow, S. A. *et al.* Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **541**, 481–487 (2017).
21. Hattori, Y. The behavior and functions of embryonic microglia. *Anat Sci Int* **97**, 1–14 (2022).
22. Gomes, T. M., Dias da Silva, D., Carmo, H., Carvalho, F. & Silva, J. P. Epigenetics and the endocannabinoid system signaling: An intricate interplay modulating neurodevelopment. *Pharmacol Res* **162**, 105237 (2020).
23. Salim, S. Oxidative Stress and the Central Nervous System. *The Journal of Pharmacology and Experimental Therapeutics* **360**, 201–205 (2017).
24. Huang, M.-C., Chen, C.-H., Peng, F.-C., Tang, S.-H. & Chen, C.-C. Alterations in Oxidative Stress Status During Early Alcohol Withdrawal in Alcoholic Patients. *Journal of the Formosan Medical Association* **108**, 560–569 (2009).
25. Komada, M. *et al.* Mechanisms underlying neuro-inflammation and neurodevelopmental toxicity in the mouse neocortex following prenatal exposure to ethanol. *Sci Rep* **7**, 4934 (2017).

26. Shichiri, M. The role of lipid peroxidation in neurological disorders. *J Clin Biochem Nutr* **54**, 151–160 (2014).
27. Di Marzo, V., Bifulco, M. & De Petrocellis, L. The endocannabinoid system and its therapeutic exploitation. *Nat Rev Drug Discov* **3**, 771–784 (2004).
28. Costanzi, M., Battaglia, M., Populin, R., Cestari, V. & Castellano, C. Anandamide and memory in CD1 mice: effects of immobilization stress and of prior experience. *Neurobiol Learn Mem* **79**, 204–211 (2003).
29. Nogueras-Ortiz, C. & Yudowski, G. A. The Multiple Waves of Cannabinoid 1 Receptor Signaling. *Mol Pharmacol* **90**, 620–626 (2016).
30. Rhee, M. H., Bayewitch, M., Avidor-Reiss, T., Levy, R. & Vogel, Z. Cannabinoid receptor activation differentially regulates the various adenylyl cyclase isozymes. *J Neurochem* **71**, 1525–1534 (1998).
31. Felder, C. C. *et al.* Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol* **48**, 443–450 (1995).
32. Brusco, A., Tagliaferro, P. A., Saez, T. & Onaivi, E. S. Ultrastructural localization of neuronal brain CB2 cannabinoid receptors. *Ann N Y Acad Sci* **1139**, 450–457 (2008).
33. Stempel, A. V. *et al.* Cannabinoid Type 2 Receptors Mediate a Cell Type-Specific Plasticity in the Hippocampus. *Neuron* **90**, 795–809 (2016).
34. Castillo, P. E., Younts, T. J., Chávez, A. E. & Hashimoto, Y. Endocannabinoid signaling and synaptic function. *Neuron* **76**, 70–81 (2012).
35. Simard, M. *et al.* Biosynthesis and metabolism of endocannabinoids and their congeners from the monoacylglycerol and N-acyl-ethanolamine families. *Biochem Pharmacol* **205**, 115261 (2022).
36. Hillard, C. J. Biochemistry and pharmacology of the endocannabinoids arachidonyl ethanolamide and 2-arachidonylglycerol. *Prostaglandins Other Lipid Mediat* **61**, 3–18 (2000).
37. Gonsiorek, W. *et al.* Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide. *Mol Pharmacol* **57**, 1045–1050 (2000).

38. Egertová, M., Cravatt, B. F. & Elphick, M. R. Comparative analysis of fatty acid amide hydrolase and cb(1) cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. *Neuroscience* **119**, 481–496 (2003).
39. Rodrigues, R. J., Marques, J. M. & Köfalvi, A. Cannabis, Endocannabinoids and Brain Development: From Embryogenesis to Adolescence. *Cells* **13**, 1875 (2024).
40. Basavarajappa, B. S. Endocannabinoid System and Alcohol Abuse Disorders. *Adv Exp Med Biol* **1162**, 89–127 (2019).
41. Reid, H. M. O., Elford, H., Till, M. A. C. & Christie, B. R. Animal Models of Prenatal Alcohol Exposure. in *Molecular Mechanisms and Lifelong Consequences of Prenatal Exposure to Psychoactive Substances* (eds Bukiya, A. N. & Ali, D. W.) 1–25 (Springer Nature Switzerland, Cham, 2026). doi:10.1007/978-3-032-12741-9_1.
42. Gasparyan, A. *et al.* Cannabidiol repairs behavioral and brain disturbances in a model of fetal alcohol spectrum disorder. *Pharmacol Res* **188**, 106655 (2023).
43. García-Baos, A., Puig-Reyne, X., García-Algar, Ó. & Valverde, O. Cannabidiol attenuates cognitive deficits and neuroinflammation induced by early alcohol exposure in a mice model. *Biomed Pharmacother* **141**, 111813 (2021).
44. Laprairie, R. B., Bagher, A. M., Kelly, M. E. M. & Denovan-Wright, E. M. Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *Br J Pharmacol* **172**, 4790–4805 (2015).
45. Thomas, A. *et al.* Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. *Br J Pharmacol* **150**, 613–623 (2007).
46. Tham, M. *et al.* Allosteric and orthosteric pharmacology of cannabidiol and cannabidiol-dimethylheptyl at the type 1 and type 2 cannabinoid receptors. *Br J Pharmacol* **176**, 1455–1469 (2019).
47. Wu, H.-Y., Tang, X.-Q., Liu, H., Mao, X.-F. & Wang, Y.-X. Both classic Gs-cAMP/PKA/CREB and alternative Gs-cAMP/PKA/p38 β /CREB signal pathways mediate exenatide-stimulated expression of M2 microglial markers. *J Neuroimmunol* **316**, 17–22 (2018).

48. Woo, M.-S., Jung, S.-H., Hyun, J.-W. & Kim, H.-S. Differential regulation of inducible nitric oxide synthase and cytokine gene expression by forskolin and dibutyl-cAMP in lipopolysaccharide-stimulated murine BV2 microglial cells. *Neurosci Lett* **356**, 187–190 (2004).
49. Ruffell, D. *et al.* A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci U S A* **106**, 17475–17480 (2009).
50. Caldwell, R. W., Rodriguez, P. C., Toque, H. A., Narayanan, S. P. & Caldwell, R. B. Arginase: A Multifaceted Enzyme Important in Health and Disease. *Physiol Rev* **98**, 641–665 (2018).
51. Feinberg, H. *et al.* Structural analysis of carbohydrate binding by the macrophage mannose receptor CD206. *J Biol Chem* **296**, 100368 (2021).
52. Sawada, M., Suzumura, A., Hosoya, H., Marunouchi, T. & Nagatsu, T. Interleukin-10 inhibits both production of cytokines and expression of cytokine receptors in microglia. *J Neurochem* **72**, 1466–1471 (1999).
53. He, Y. *et al.* IL-4 Switches Microglia/macrophage M1/M2 Polarization and Alleviates Neurological Damage by Modulating the JAK1/STAT6 Pathway Following ICH. *Neuroscience* **437**, 161–171 (2020).
54. Rodrigues, F. da S. *et al.* Cannabidiol prevents LPS-induced inflammation by inhibiting the NLRP3 inflammasome and iNOS activity in BV2 microglia cells via CB2 receptors and PPAR γ . *Neurochem Int* **177**, 105769 (2024).
55. Rajan, T. S. *et al.* Anti-inflammatory and antioxidant effects of a combination of cannabidiol and moringin in LPS-stimulated macrophages. *Fitoterapia* **112**, 104–115 (2016).
56. Stampanoni Bassi, M. *et al.* Transient Receptor Potential Vanilloid 1 Modulates Central Inflammation in Multiple Sclerosis. *Front Neurol* **10**, 30 (2019).
57. Leweke, F. M. *et al.* Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. *Transl Psychiatry* **2**, e94 (2012).
58. Navarrete, F. *et al.* Cannabidiol modulates brain molecular alterations, gut microbiota dysbiosis and alcohol self-administration in a mouse model of fetal alcohol spectrum disorder. *Biomedicine & Pharmacotherapy* **193**, 118791 (2025).

59. Zheng, C. *et al.* The second intracellular loop of the human cannabinoid CB2 receptor governs G protein coupling in coordination with the carboxyl terminal domain. *PLoS One* **8**, e63262 (2013).
60. Correa, F. *et al.* Anandamide enhances IL-10 production in activated microglia by targeting CB(2) receptors: roles of ERK1/2, JNK, and NF-kappaB. *Glia* **58**, 135–147 (2010).
61. Lee, J. *et al.* Chronic Binge Alcohol Exposure During Pregnancy Alters mTOR System in Rat Fetal Hippocampus. *Alcohol Clin Exp Res* **44**, 1329–1336 (2020).
62. Lopatynska-Mazurek, M., Komsta, L., Gibula-Tarlowska, E. & Kotlinska, J. H. Aversive Learning Deficits and Depressive-Like Behaviors Are Accompanied by an Increase in Oxidative Stress in a Rat Model of Fetal Alcohol Spectrum Disorders: The Protective Effect of Rapamycin. *Int J Mol Sci* **22**, 7083 (2021).
63. Hahn-Windgassen, A. *et al.* Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. *J Biol Chem* **280**, 32081–32089 (2005).
64. Susick, L. L., Chrumka, A. C., Hool, S. M. & Conti, A. C. Dysregulation of TrkB phosphorylation and proBDNF protein in adenylyl cyclase 1 and 8 knockout mice in a model of fetal alcohol spectrum disorder. *Alcohol* **51**, 25–35 (2016).
65. Nuutinen, S., Kiianmaa, K. & Panula, P. DARPP-32 and Akt regulation in ethanol-preferring AA and ethanol-avoiding ANA rats. *Neurosci Lett* **503**, 31–36 (2011).
66. Nishi, A. & Shuto, T. Potential for targeting dopamine/DARPP-32 signaling in neuropsychiatric and neurodegenerative disorders. *Expert Opin Ther Targets* **21**, 259–272 (2017).
67. Darbinian, N. *et al.* Biomarkers of Affective Dysregulation Associated with In Utero Exposure to EtOH. *Cells* **13**, 2 (2023).
68. Ehrhart, F. *et al.* Review and gap analysis: molecular pathways leading to fetal alcohol spectrum disorders. *Mol Psychiatry* **24**, 10–17 (2019).
69. Muraleedharan, R. & Dasgupta, B. AMPK in the brain: its roles in glucose and neural metabolism. *FEBS J* **289**, 2247–2262 (2022).
70. Boschen, K. E., Ruggiero, M. J. & Klintsova, A. Y. Neonatal binge alcohol exposure increases microglial activation in the developing rat hippocampus. *Neuroscience* **324**, 355–366 (2016).

71. Li, D. *et al.* Neurochemical regulation of the expression and function of glial fibrillary acidic protein in astrocytes. *Glia* **68**, 878–897 (2020).
72. Vallés, S. *et al.* Glial fibrillary acidic protein expression in rat brain and in radial glia culture is delayed by prenatal ethanol exposure. *J Neurochem* **67**, 2425–2433 (1996).
73. Takahashi, Y. *et al.* Aberrant neurogenesis and late onset suppression of synaptic plasticity as well as sustained neuroinflammation in the hippocampal dentate gyrus after developmental exposure to ethanol in rats. *Toxicology* **462**, 152958 (2021).
74. García-Baos, A., Alegre-Zurano, L., Cantacorps, L., Martín-Sánchez, A. & Valverde, O. Role of cannabinoids in alcohol-induced neuroinflammation. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **104**, 110054 (2021).
75. Gasparyan, A. *et al.* Cannabidiol repairs behavioral and brain disturbances in a model of fetal alcohol spectrum disorder. *Pharmacological Research* **188**, 106655 (2023).