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Extracellular vesicles from mesenchymal stem cells reverse neuroinflammation and cognitive and motor impairment in rats with mild liver damage

Doctoral Thesis

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CERTIFICAN:

Que la memoria de la Tesis Doctoral realizada por Dª Gergana Mincheva Ganeva, titulada: "Extracellular vesicles from mesenchymal stem cells reverse neuroinflammation and cognitive and motor impairment in rats with mild liver damage", ha sido realizada bajo su dirección y reúne todos los requisitos necesarios para su juicio y calificación.

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Cirrhotic patients may develop minimal hepatic encephalopathy (MHE), with mild cognitive impairment, psychomotor slowing and motor incoordination, that reduce their life quality and span. Mild cognitive impairment in patients with no-alcoholic fatty liver disease (NAFLD) has been also recently reported. Human mesenchymal stem cells derived extracellular vesicles (MSC-EVs) have been proposed as therapy for different diseases including liver injury and neurodegenerative diseases, due to their immunomodulatory, anti-inflammatory and regenerative properties. The administration of carbon tetrachloride (CCl_4) to rats is a model of progressive liver damage and hepatic encephalopathy (HE). The aim of this study was to assess if MSC-EVs reverse cognitive and motor impairment in rats with mild liver damage induced by CCl_4 and MHE, as a model of NAFLD, and to assess the underlying mechanisms. In addition, we assessed if TGF-β, main component of MSC-EVs and with anti-inflammatory properties, has a role in the effects of MSC-EVs.

Mild liver damage was induced by intraperitoneal injection of CCl_4 during four weeks. MSC-EVs were isolated from human adipose tissue-derived stem cells and also from these cells after silencing TGF-β expression to assess the role of TGF-β. MSC-EVs were injected intravenously the second and the third weeks of CCl_4 treatment. Cognitive and motor functions were assessed. Glutamatergic and GABAergic neurotransmission and neuroinflammation in the cerebellum and the hippocampus were analysed by immunohistochemistry and Western blot. Peripheral inflammation, liver damage and gut microbiota were also analyzed.

MSC-EVs improve spatial learning and memory and working memory in rats with mild liver damage reducing neuroinflammation and normalizing some alterations in glutamatergic and GABAergic neurotransmission in the hippocampus. MSC-EVs improve motor coordination and altered locomotor gait by reducing neuroinflammation and the activation of pathways induced by TNFα that lead to GABAergic neurotransmission alterations. MSC-EVs also reduce peripheral inflammation, liver steatosis and fibrosis, and liver inflammation and counteract some consequences of gut dysbiosis. These effects are different depending on if the EVs proceed from unmodified MSCs or from MSCs with silenced TGF-β, indicating that this modification and the presence of TGF-β, that should also affect the rest of his cargo, is determinant to the effects of MSCs-EVs and the involved mechanisms.

MSC-EVs could be a promising therapy for MHE, and also for other diseases involving similar pathological mechanisms, such as diseases associated with sustained peripheral inflammation and neuroinflammation.

INDEX

ABBREVIATIONS

ABC	Avidin Biotin Complex
ALT/GPT	Alanine aminotransferase
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	Ammonium persulfate
ASC	Adipose tissue derived mesenchymal stem cells
ASH	Alcoholic steatohepatitis
AST/GOT	Aspartate aminotransferase
ATP	adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine
BDL	Bile duct ligation
BDNF	Brain derived neurotrophic factor
BS3	Bis(sulfosuccinimidyl)suberate
BSA	Bovin serum albumin
CA	Cornu Ammonis
CC	CCl_4-induced liver damage-PBS group
CCE	CCl_4-with C-Evs group
CCE-T	CCl_4-with T-EVs group
CCL2	Chemokine -ligand 2; also known as monocyte chemoattractant protein 1, MCP1
CCL20	chemokine (C-C motif) ligand 20 or macrophage inflammatory protein-3 MIP3A
CCl_4	Carbon Tetrachloride
CCL5	Chemokine (C-C motif) ligand 5, or RANTES
CCR2	CCL2 receptor
CD	cluster differentiation
CE	Control-treated with C-EVs group
CE-T	Control treated with T-EVs group
C-EVs	EVS from control ASC
clr	Centered log-ratio
CNS	Central nervous system
COX2	cyclooxygenase-2
CV	Control-PBS group
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL10	C-X-C motif chemokine ligand 10
CXCL12	C-X-C motif chemokine 12, also known as stromal cell-derived factor 1
DAB	Diaminobenzidine
DG	Dentate gyrus
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dsDNA	Double strand deoxyribonucleic acid
ECM	Extracellular matrix proteins
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)
EVs	extracellular vesicles
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GABA	γ-Aminobutyric acid
$GABA_A$	GABA receptor type A
GAD	Glutamate decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GAT-1	GABA transporter 1
GAT-3	GABA transporter 3
GDNF	Glial cell–derived neurotrophic factor
GLMM	Generalized linear mixed models
GFAP	Glial fibrillary acid protein
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter 1
GluA1	AMPA receptor subunit 1
GluA2	AMPA receptor subunit 2
GluN1	NMDA receptor subunit 1
GluN2A	NMDA receptor subunit 2A
GluN2B	NMDA receptor subunit 2B
GTR	Generalized time-reversible
H&E	Hematoxylin and eosin
HE	Hepatic encephalopathy
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1
HPA	Hypothalamic–pituitary–adrenal (pathway)
HRP	Horse-raddish peroxidase
HSC	Hepatic stellate cells
HSP70	70 kilodalton heat shock protein
Iba1	Ionized calcium-binding adapter molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
IGF-I	Insulin-like growth factor I
IL	interleukin
IL-1R1	Interleukin 1 receptor, type I
IL-1Ra	Antagonist of IL-1 receptor
KC	Kupffer cells
KCC2	Kotassium chloride cotransporter 2
$(LD)_{50}$	Lethal dose
LMM	Linear mixed models
LPS	Lipopolysaccharide (bacterial)
LTP	Long-term potentiation
MEA	Multielectrode array
mGluRs	Metabotropic glutamate receptors
MHE	Minimal hepatic encephalopathy
miRNA	Micro ribonucleic acid
MMPs	Matrix metalloproteinases
mRNA	messenger ribonucleic acid
MSC	Mesenchymal stem cells or mesenchymal stromal cells
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NBT	4-Nitro blue tetrazolium chloride
NGS	Normal goat serum
NH3	Ammonia
NH_4^+	Ammonium ion
NMDA	N-methyl-D-aspartate
NO	nitric oxide

NOL	Novel object location
OTU	Operational Taxonomic Unit
P2X4	P2X purinoceptor 4
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCS	Portacaval shunts
PD	Phylogenetic distance
PDGF	Platelet derived growth factor
PGE2	Prostaglandin E2
PHES	Psychometric Hepatic Encephalopathy Score
PRRs	pattern-recognition receptors
PSS	Portosystemic shunts
PTA	Phosphate-buffered-saline-triton-albumin
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
S1PR2	Sphingosine-1-phosphate receptor 2
SCFA	short chain fatty acids
SDs	Standard deviations
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
STAT3	Signal transducer and activator of transcription 3
TBS-T	Tris-buffered saline-Tween
TEMED	Tetramethylethylenediamine
T-EVs	EVs from ASC with silenced TGF-β gene
Tfh	T follicular helper
TGF-β	Transforming growth factor beta
TGF-β1	Transforming growth factor beta 1
Th	T helper
TIMP1	TIMP metallopeptidase inhibitor 1
TJ	Tight junctions
TJP-1/ZO-1	Tight junction protein 1
TLR	toll-like receptor
TNFR1	TNF-α receptor
TNF-α	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRIS	Tris(hydroxymethyl)aminomethane
TrkB	Tropomyosin receptor kinase B, BDNF receptor
VEGF-A	Vascular endothelial growth factor-A
YM-1	Chitinase-like protein 3
α-SMA	α-smooth muscle actin

INTRODUCTION

1. Liver failure

1.1. Liver: structure and function

The liver is a large organ present only in vertebrates that participates in many important biological processes. In humans, it represents approximately 2% to 3% of average body weight and can be found in the right upper quadrant of the abdomen, below the diaphragm (Abdel-Misih & Bloomston, 2010). The liver is related to many physiological processes such as detoxification of toxic substances for the organism, metabolism of macronutrients, storage of glucose as glycogen, lipid and cholesterol homeostasis, protein and amino acid metabolism, blood volume regulation, immune system support, endocrine control of growth signalling pathways, and the breakdown of compounds, including many current drugs (Trefts et al., 2017). It is also an accessory digestive organ, it produces bile, a liquid that helps in the breakdown of fat. After production, bile is stored in the gallbladder (cholecyst, located under the liver) and when needed, is moved to the small intestine to complete digestion.

The liver is a reddish-brown, wedge-shaped organ that can present unequal-sized lobes. It receives blood from the hepatic artery and the portal vein. The portal vein contains blood, rich in nutrients, coming from the gastrointestinal tract, while the hepatic artery contains highly oxygenated blood. Both enter the liver through the hilum, a transverse fissure in its lower surface. The blood is mixed in the hepatic sinusoids and is drained into the systemic circulation via the hepatic venous system that converges into the inferior cava vein (Abdel-Misih & Bloomston, 2010). Because of this specific circulation, all blood from the digestive tract passes through the liver. The hepatic sinusoids lead to the lobules, the functional units of the liver.

The hepatocytes are the liver's main parenchymal cells and they are arranged to form the hepatic lobules. The portal triads can be found in between the lobules. They consist mainly of a bile ductule and ramifications of the portal vein and the hepatic artery. When the blood arrives at the centre of the lobule, it drains from the liver towards the vena cava. During this short path, hepatocytes can exchange nutrients and residues with the blood (Kmieć, 2001). This microscopic structure of the liver is generally preserved in all mammals and is essential for liver function (Kruepunga et al., 2019).

Around 60 to 80 % of the cells constituting the liver are hepatocytes. The remaining population of nonparenchymal cells includes different cell types such as endothelial cells, Kupffer cells (KC), lymphocytes, biliary cells, and hepatic stellate cells (HSC). KC, which can be found mainly in the periportal area, are the resident macrophages in the liver. From there, they clear endotoxins from the blood and phagocytose debris and microorganisms. They can be in contact with hepatocytes and phagocytose them in case of apoptosis. KC are a key component of the innate immune system (Racanelli & Rehermann, 2006). In case of infection, they start an effective first response, they produce cytokines such as PDGF (platelet derived growth factor), TGF-β1 (Transforming growth factor beta 1) and CCL2 (chemokine -ligand 2; also known as monocyte chemoattractant protein 1, MCP1) that are important for modulating the differentiation and proliferation of other cells, for instance, HSC. This type of cell is localized in the subendothelial space of Disse. In a normal healthy liver, HSC present a non-proliferative phenotype. Nevertheless, after liver injury and due to the following cytokine production, HSC become activated transdifferentiating to a proliferative, inflammatory and chemotactic phenotype that is also characterized by enhanced production of extracellular matrix proteins (ECM) that will start the process of fibrosis (Robinson et al., 2016; Koyama & Brenner, 2017).

1.2. Chronic liver disease

The liver starts to accumulate an excessive amount of triacylglycerides in intracytoplasmic vesicles as a common response to injuries or diseases. This condition is known as steatosis and it can be related to the abuse of certain substances such as alcohol or illegal drugs, but also to other factors such as obesity, diabetes type 2, or some medications. In many of these cases, when the disease is not related to alcohol abuse, it is known as non-alcoholic fatty liver disease (NAFLD).

Steatosis is the result of defective fatty acid metabolism and it is usually diagnosed when lipid content reaches 5 to 10 % of liver weight. Hepatic steatosis can be observed as the accumulation of macrovesicular or microvesicular intracytoplasmic droplets in hepatocytes. Steatosis in early and mild stages is reversible and allows a relatively normal life. Nevertheless, steatosis can worsen significantly. Overaccumulation of lipids in hepatocytes can disrupt cell constituents and lead to apoptosis or bursting. With the death of hepatocytes, triacylglycerides and toxic fatty acids are released in the liver, damaging it further, and producing inflammation and thus

turning into steatohepatitis (Wang, David Q-H et al., 2013). Depending on the presence or absence of alcohol consumption related to liver disease, alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH) can be differentiated. The inflammatory response is directly related to the lipidic release that is followed by peroxidation and reactive oxygen species production. On one side, in response to oxidative stress, KC produce cytokines that activate HSC, involved in fibrogenesis. On the other side, reactive oxygen species, lipid peroxidation, and some hepatocyte constituents produce immunological responses that also lead to inflammation (Spengler & Loomba, 2015). Moreover, steatohepatitis increases the risk to advance toward the next stages of liver damage, fibrosis, cirrhosis, and hepatic carcinoma (Schuppan & Kim, 2013) (Figure 1).

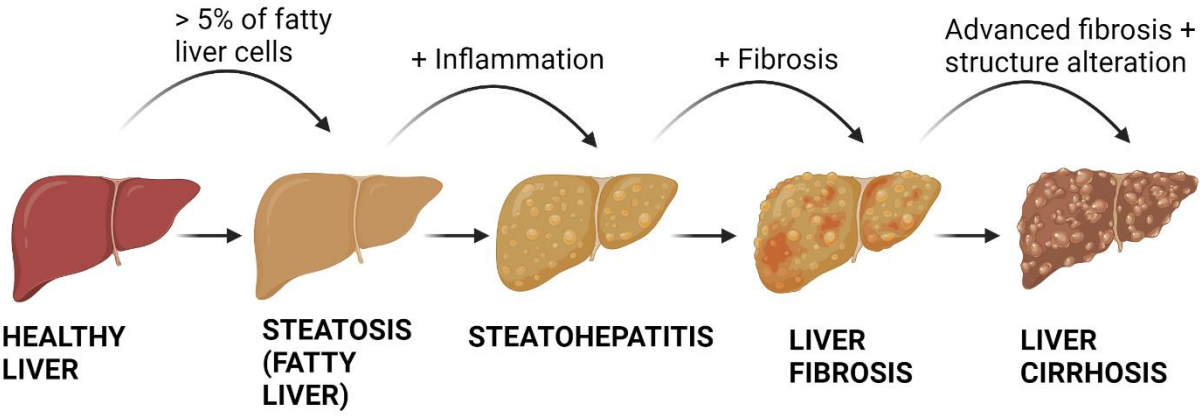

Figure 1. *Stages of liver disease. Created with Biorender.com*

Liver fibrosis consists of an accumulation of ECM proteins, such as collagen, in the liver (Tacke & Weiskirchen, 2012). This is common in most types of chronic liver diseases. Activated HSC play the main role in liver fibrosis as major collagen-producing cells after liver damage. Consequently, the accumulation of ECM proteins alters liver architecture and leads to the formation of a fibrous scar. In addition, liver injury and cell apoptosis stimulate hepatic regeneration which contributes to the distortion of the architecture and the formation of hepatic nodules. This environment leads to cirrhosis which can be defined as a process characterized by aggressive fibrogenesis and the subsequent development of abnormal nodule structure. Some of the morphological features of cirrhosis are diffuse fibrosis, nodules, altered lobular architecture and the creation of intrahepatic vascular shunts between afferent and efferent vessels of the liver (Pinzani et al., 2011). Cirrhosis produces hepatic cell dysfunction and complications in the blood flow in the liver which leads to hepatic insufficiency and portal

hypertension (Bataller & Brenner, 2005). Indeed, portal hypertension is the main mechanism leading to the death of cirrhotic patients (Pinzani et al., 2011).

In 2017, globally 1.5 billion people suffered a chronic liver disease. The median cirrhosis prevalence was 833 out of 100,000 in European countries in 2017 (Moon et al., 2020). In industrialized countries, some common causes of cirrhosis are chronic hepatitis C, alcohol abuse and obesity (associated with NASH), either as a unique cause or in combination (Pinzani et al., 2011). Alcohol is estimated to be related to 20% to 36% of cirrhosis cases. Moreover, the increasing number of children with obesity is concerning due to its relation with NAFLD and cirrhosis. It is estimated that 34.2% of obese children suffer from NAFLD (Moon et al., 2020).

2. Hepatic encephalopathy

Liver failure causes alterations in many functions such as the detoxification of toxic compounds from the blood. These compounds, such as ammonia, start accumulating and may reach the brain and affect its function. Alterations in cerebral function due to liver failure cause a complex neuropsychiatric syndrome known as hepatic encephalopathy (HE). Chronic hyperammonemia, increased levels of ammonia in blood, is one of the main causes of HE. HE includes alterations in motor function as bradykinesia and asterixis, dysfunction of cognitive abilities, impairment of circadian rhythms, and changes of personality and consciousness that in advanced stages can lead to coma or even death.

HE can be classified into three types or levels (A, B, and C) depending on the type of liver failure (Ferenci et al., 2002). Type A HE is related to acute liver failure, patients show tissue necrosis and a rapid advance of symptoms having as a consequence a high mortality rate. Type B is associated with chronic liver disease produced by portosystemic shunts (PSS) but without any intrinsic hepatocellular disease. A PSS is an abnormal connection between the systemic circulation and the portal vascular system. In these cases, the blood coming from the digestive system that should pass by the portal vein into the liver is shunted to the systemic circulation and thus it is not properly detoxified. PSS related to type B HE can be produced spontaneously or due to genetic factors. Type C is a chronic HE associated to cirrhosis and portal hypertension

and it is the most common type of HE. Chronic HE (as type B and C HE) usually develops progressively.

HE can also be classified depending on the duration and characteristics of the symptoms. Episodic HE is characterized by the appearance of neuropsychiatric alterations in a short period. Episodes can vary in severity and can proceed with or without precipitating factors such as the use of some medications, infections, dehydration, homeostatic imbalances, etc. If two episodes occur within one year, the term recurrent HE can be used. HE can also become persistent showing evident and continuous neurological alterations. Persistent HE can be mild or severe depending on the level of autonomy of the patient (Weissenborn, 2019).

2.1.Minimal hepatic encephalopathy

Patients with chronic liver damage may not show evident neurological alterations in the first stages of chronic HE, but they can show some mild impairments at cognitive and motor levels as alterations in movement speed, bimanual coordination, attention, concentration, and visuospatial perception. The presence of those alterations in cirrhotic patients is defined as minimal hepatic encephalopathy (MHE), considered a subclinical type of HE (nowadays also denominated covert HE). The neurological impairments related to MHE are usually not severe, but enough to impede or complicate daily life activities, for instance, driving. MHE also increases the risk of domestic and work accidents, reduces the life quality and lifespan of patients and increases the possibility to suffer clinical HE (Felipo, 2013).

Although MHE symptoms are not obvious in a standard neurological exam, they can be observed when evaluating patients with the proper psychometric tests. For the diagnosis of MHE, there is consensus to use the Psychometric Hepatic Encephalopathy Score (PHES). This test includes five psychometric scored tasks in which the presence of MHE in cirrhotic patients can be identified. It is considered that a patient has MHE when, after normalization, the PHES score is lower than -4. PHES evaluates cognitive functions as visual perception, visuo-spatial orientation, and visuo-constructive abilities, motor speed and accuracy, working memory and attention (Weissenborn, K. et al., 2001).

Approximately 30% of patients with cirrhosis are also affected by MHE (Bajaj et al., 2009), which means around 2.2 million people in the European Union and a similar number in the

USA with MHE, making it a major global health concern, yet many patients are undiagnosed (Minguez et al., 2006; Sharma et al., 2008; Mina et al., 2014). Firstly, MHE is not properly diagnosed due to the need for standardization of the test by age, education and employment of the patients thereby they are more time and budget consuming. Secondly, there is a lack of testing for MHE through patients. In the USA, in 2007, a survey showed that although most clinicians believed that MHE is a major problem, only approximately half of them assessed if their patients suffered from this disease (Bajaj et al., 2007; Ridola et al., 2018).

2.2.Motor function impairment

Motor symptoms are important in HE. Some type of motor impairments such as tremor, ataxia, slowing of fingers movements, altered handwriting and bimanual incoordination can be observed in cirrhotic patients without any evident signs of HE nor MHE (Butz et al., 2010; Felipo et al., 2012b; Dellatore et al., 2020). Motor symptoms progress from mild to more severe, such as asterixis (flapping tremor), hyporeflexia, hyperreflexia, clonus and rigidity, which is a sign of more severe HE (Dellatore et al., 2020) Thus, there is a close relationship between motor alterations and HE severity (Butz et al., 2010; Dellatore et al., 2020)

The cerebellum, located underneath the cerebral hemispheres, plays a main role in motor control, motor coordination and learning, and equilibrium among others. The impairment of motor coordination is related to dysfunction in the cerebellum (Hanchar et al., 2005). Motor incoordination and other movement related symptoms are also common in other diseases such as cerebellar ataxia or multiple sclerosis and are related to cerebellar alterations (Tornes et al., 2014; Marsden, 2018). The early motor impairments and brain magnetic resonance studies show that the cerebellum is one of the earliest brain areas to be affected in cirrhotic patients (Felipo et al., 2014). The cerebellum of patients with steatohepatitis already shows some alterations including neuronal loss (Balzano et al., 2018).

2.3.Cognitive impairment

Personality changes as apathy, disinhibition and irritability are the first type of symptoms reported by patient's close family or friends. These psychological symptoms may turn into cognitive impairments if they are not treated. These impairments include confusion, disorientation, memory impairment, shortened attention span and difficulties in the speech

(Dellatore et al., 2020). Some milder alterations are usually also present as difficulties for sleeping, socialization, working or doing domestic chores (Groeneweg et al., 1998; Prasad et al., 2007). Attentional functions as vigilance, orienting and higher executive function are also altered in MHE (Weissenborn et al., 2005) Traditionally, the grade of HE has been classified depending on the severity of these alterations from grade 0 (only cirrhosis without measurable cognitive symptoms) to grade IV (highest severity, coma) (Dharel & Bajaj, 2015).

Functions as learning, memory and cognition are directly associated to the hippocampus. The hippocampus is part of the limbic system and it is located deep in the medial temporal lobe. This region has a very characteristic structure formed by two main areas: the hippocampus proper (CA derived from Cornu Ammonis) and the dentate gyrus (DG). In the hippocampus proper, regions CA1, CA2, CA3 and CA4 can be differentiated. The classic anatomical connectivity between the subdivisions of the hippocampus is known as a "trisynaptic loop". The main cortical input comes from the entorhinal cortex through the perforant path to the DG. The DG projects the mossy fibers to CA3, and this projects to CA1 via the Schaffer Collateral pathway. Finally, the loop is completed by CA1 projecting back to the entorhinal cortex (Andersen, 2007; Knierim, 2015).

The hippocampus has a key role in memory and learning. Concretely, it is essential for spatial related memory and the ability to remember specific events (Lisman et al., 2017). In Alzheimer's disease, alterations in dendritic complexity and spine density of hippocampal neurons appear in the early phase of the disease which are related to cognitive decline. Patients with other diseases such as Parkinson's disease, multiple sclerosis and Huntington's disease show symptoms due to alterations in hippocampal function (Weerasinghe-Mudiyanselage et al., 2022). Patients deceased with steatohepatitis and cirrhosis showed some alterations in hippocampus that could be responsible for the cognitive impairment (Leone et al., 2023).

3. Animal models in the study of HE and MHE

To better understand the mechanisms involved in the development of MHE and HE different animal models can be used, depending on the type of HE of interest. These animal models are useful to study the mechanism of the disease, at the different stages of the disease and for the preclinical assessment of possible treatments. Rats and mice are the most common animals

used in these studies due to the availability of information and materials and the easy and cost-effective housing.

HE is a difficult-to-model disease mainly due to the wide range of different aetiologies and the complexity of the syndrome. However, some models are well established both for acute and chronic HE (Butterworth et al., 2009; DeMorrow et al., 2021). Several ways to induce liver failure or liver circulation impairment are well stablished to models for types A, B and C HE (DeMorrow et al., 2021) (Figure 2).

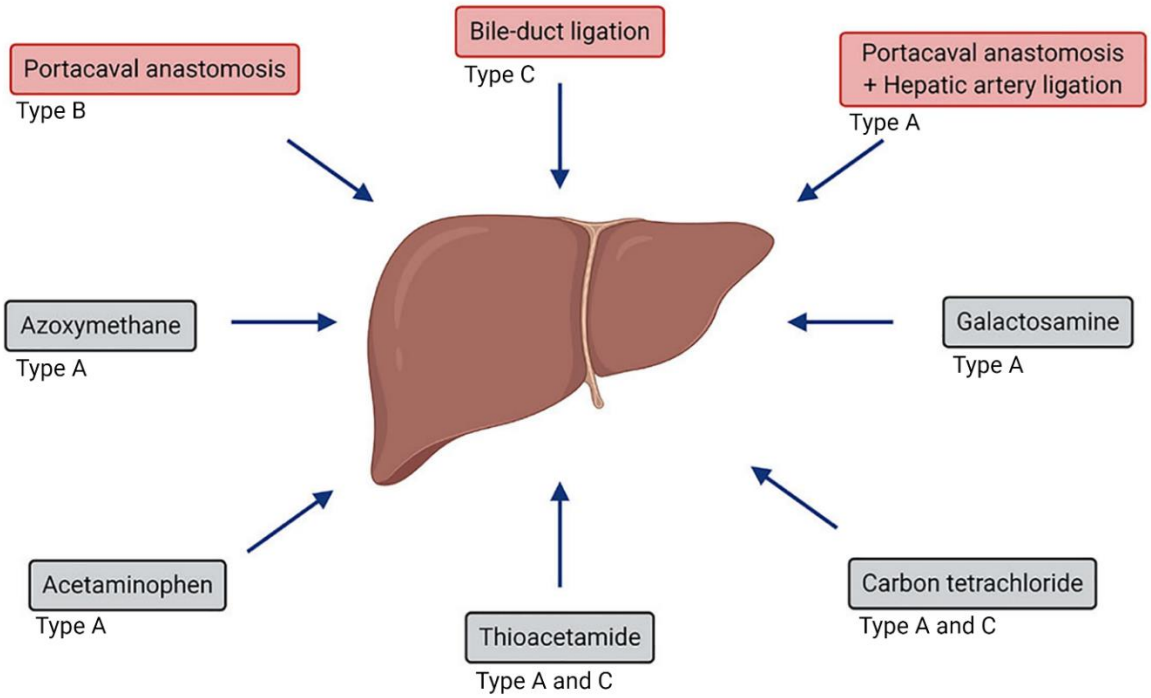

Figure 2. *Main HE models with liver failure/injury induction. Surgical methods are shown in red boxes while toxins appear in blue boxes. Under each method is indicated the uses depending on HE types (A, B or C). Adapted from DeMorrow et al 2021.*

Two common rat models to reproduce chronic liver disease and MHE are portacaval shunts (PCS) and bile duct ligation (BDL). Both models show neurological alterations that reproduce HE symptoms in patients. In PCS rats, the portal vein and the inferior vena cava are surgically connected being a type B HE animal model. PCS rats show hyperammonaemia, increased brain ammonia/glutamine relation, alterations in circadian rhythms and motor activity and coordination, memory and learning ability. BDL model consists of ligation (2-3 points) and resection of the common bile duct. This is the most widely used model for type C HE. BDL rats develop biliary liver cirrhosis after around five weeks of surgery and show symptoms

related to hepatic failure including jaundice, changes in liver architecture and increased plasma levels of bilirubin and liver enzymes (AST/GOT, aspartate aminotransferase; and ALT/GPT, alanine aminotransferase). These rats also develop dysfunction of the immune system, hyperammonemia and motor and cognitive deficits such as hypokinesia, and reduced memory and learning (Butterworth et al., 2009; Felipo, 2013; DeMorrow et al., 2021). However, BDL rats only survive 6 to 8 weeks post-ligation (DeMorrow et al., 2021).

There are also models to study the effects of hyperammonaemia *per se*, without any type of liver failure. An easy and inexpensive method for this model is the feeding of the rats with a diet rich in ammonium acetate (Felipo, V. et al., 1988; Azorin et al., 1989) . Hyperammonemic rats show neuroinflammation, altered neurotransmission and disturbances in learning, memory and motor functions (Hermenegildo et al., 1998; Cauli et al., 2009c; Rodrigo et al., 2010).

Some animal models of chronic liver disease are based on induction of liver damage by chronic administration of hepatotoxins, such as thioacetamide or carbon tetrachloride (CCl_4) (DeMorrow et al., 2021).

3.1.Model of chronic liver damage induced by carbon tetrachloride

CCl_4 is a hepatic toxin widely used for the induction of liver fibrosis and cirrhosis in laboratory animals. Specifically, it is a clear, colourless, volatile and very stable chlorinated hydrocarbon.

CCl_4 presents toxicity on different organs and tissues, but it affects mainly the liver where it has strong hepatotoxic effects, inducing acute liver failure by high dose acute administration and also hepatic cirrhosis and carcinogenesis when it is administrated chronically at lower doses. CCl_4 is first metabolized via cytochrome P450 producing free radicals which will lead to cellular damage (Manibusan et al., 2007).

Regarding HE, CCl_4 can be used in two different manners, either for modelling acute liver failure, when a high dose is administered or to reproduce a chronic liver disease using lower doses (Butterworth et al., 2009). The lethal dose $(LD)_{50}$ after acute oral uptake in rats is in the range of 4.7–14.7 mL/kg body weight but a lower dose of 1.0 ml/kg body weight of 10% CCl_4, administrated chronically (three times per week), is enough to model liver damage reproducing

all the stages of chronic liver disease with increasing severity of liver injury in time (Lee et al., 2005; Scholten et al., 2015; Balzano et al., 2021; Leone et al., 2022).

Early after mild liver damage induction by CCl_4, after four weeks of administration, rats show steatosis and mild steatohepatitis but not hyperammonemia, which appears after six weeks of chronic CCl_4 administration. This can be considered a NAFLD and NASH model. These rats already show motor and cognitive impairment (Balzano et al., 2021; Leone et al., 2022). Patients with NAFLD already show mild cognitive impairment (Colognesi et al., 2020a; Gimenez-Garzo et al., 2021).

4. Pathophysiology of HE: Synergy between hyperammonaemia and inflammation

The accepted hypothesis is that neurological alterations observed in HE are mainly produced by the synergistic action of two main factors: hyperammonaemia and inflammation. The interplay between both factors induces neuroinflammation which leads to alterations in neurotransmission and these to the symptoms of HE (Figure 3) (Shawcross et al., 2004; Shawcross et al., 2007; Montoliu et al., 2009; Felipo, et al., 2012a; Felipo, 2013).

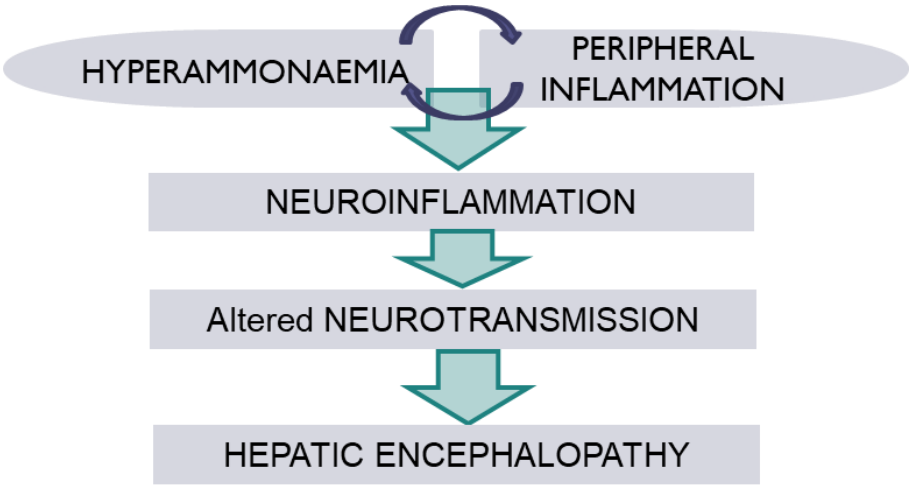

Figure 3. *Hyperammonaemia and peripheral inflammation act synergistically and induce neuroinflammation and alter neurotransmission which leads to the symptoms of HE.*

4.1.Hyperammonemia / ammonia toxicity

Normally, large quantities of ammonia enter the body from protein digestion in the gastrointestinal system. Besides that, ammonia can be produced by all types of cells derived from nitrogenous metabolism and the degradation of amino acids, nucleotides and other nitrogen compounds. Ammonia participates in many essential biological reactions, for example, it has a role in the maintenance of homeostasis of nitrogen and the synthesis of glutamine and glutamate. In mammals, ammonia formation depends on glutamate dehydrogenase, glutaminase and purine nucleotide cycle enzymes, although it can be also produced by urease producing bacteria of the intestinal flora (Rai et al., 2015)

Ammonium (NH_4^+) is the ion of ammonia (NH_3). In an aqueous solution, they form an equilibrium (Equation 1) that is displaced towards the formation of NH_4^+ at physiological pH, indeed more than 98% of ammonia is present as NH_4^+ at physiological conditions. NH_3 in gas form can freely cross cellular membranes while NH_4^+ can only cross them through ion channels or on membrane transporters (Felipo & Butterworth, 2002).

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \qquad Eq.\ 1$$

In humans and other ureotelic animals, the liver is the main detoxifier of ammonia, through the urea cycle, after which urea is excreted in the urine. The liver reduces ammonia in the blood to maintain its concentration low (50-100 μM). In the brain, which lacks the urea cycle, ammonia is detoxified by glutamine synthetase. Glutamine synthetase is an enzyme specific to astrocytes and oligodendrocytes. It participates in the glutamate-glutamine cycle between astrocytes and neurons, the mechanism by which glutamate is recycled (Marcaggi & Coles, 2001). The enzyme incorporates ammonia into glutamate and consumes adenosine triphosphate (ATP) to produce glutamine. Glutamine is a non-toxic form to transport ammonia to the liver where it can be eliminated through the urea cycle (Cabrera-Pastor et al., 2018)

After liver failure, detoxification of ammonia is not properly carried out and thus ammonia tends to accumulate. In addition, altered microbiota, gut dysbiosis and increase of the permeability of intestinal barrier associated to liver injury also leads to increase of ammonia production and permeation in the blood circulation. Hyperammonaemia refers to increased levels of ammonia in the blood. Hyperammonemia was considered for a long period of time

the main factor responsible of HE. In fact, the severity of the disease correlates with ammonia concentration in blood (Clemmesen et al., 1999). Moreover, at the moment all effective HE treatments are based in reduction of ammonia levels (Rose, 2012).

Because of the role of ammonia in the glutamate-glutamine cycle, hyperammonaemia affects glutamatergic neurotransmission (Felipo, 2013; Cabrera-Pastor et al., 2018). Hyperammonaemia also affects other neurotransmitters as dopamine, norepinephrine, serotonin and histamine. Moreover, high ammonia levels increase the blood-brain barrier (BBB) permeability (Oja et al., 2017). Animal models of chronic hyperammonaemia, without liver damage, reproduce some alterations observed in cirrhotic patients with HE and show neuroinflammation and altered glutamatergic and γ-Aminobutyric acid (GABA) neurotransmission (Cauli et al., 2009a; Rodrigo et al., 2010).

4.2. Role of inflammation

In the last decades, inflammation has been also related to the appearance of HE. In some chronic diseases such as obesity, diabetes, and rheumatoid arthritis, the presence of chronic inflammation leads to cognitive and motor impairment. In patients with liver cirrhosis there is a correlation between inflammation and the effects of ammonia in the neurological function. Shawcross et al. (2004) showed that induced hyperammonemia results in significant worsening of the neuropsychological scores of patients only if systemic inflammation was also present. Liver cirrhosis induces peripheral inflammation that usually exacerbates the neuropsychological alterations induced by hyperammonaemia. Moreover, in patients with diseases involving alteration of ammonia levels and inflammation, cognitive impairment may appear even without liver disease, if hyperammonaemia and inflammation are high enough (Felipo, et al., 2012a). In fact, the concentration of some inflammatory mediators such as IL-6 and IL-18 could be a possible marker for MHE in cirrhotic patients (Montoliu et al., 2009). Peripheral inflammation is mostly transduced to the brain in chronic pathological situations causing neuroinflammation (Sun et al., 2022).

Neuroinflammation is reduced and cognitive and motor functions are improved in PCS, BDL and hyperammonemic rats treated with ibuprofen, a non-steroidal anti-inflammatory drug (Cauli et al., 2007; Cauli et al., 2009c; Rodrigo et al., 2010). In HE, patients and animal models show increased tumor necrosis factor alpha (TNF-α) levels and the treatment with anti-TNF-α

in rats with HE reduces peripheral inflammation and neuroinflammation and improves motor coordination and cognitive alterations (Dadsetan et al., 2016a; Dadsetan et al., 2016b). Other anti-inflammatory treatments also show improvement of motor and cognitive alterations. Treatment with sulforaphane, that enhances antioxidant and anti-inflammatory responses, improves cognitive and motor alterations observed in hyperammonemic rats (Hernandez-Rabaza et al., 2016a; Hernandez-Rabaza et al., 2016b).

Balzano et al. (2020) reported that in the model of chronic hyperammonemia, without liver failure, rats also show peripheral inflammation. Hyperammonaemia induces peripheral inflammation in rats in a rapid and reversible manner which is observed with increased proinflammatory factors (Prostaglandin E_2 known as PGE2, TNF-α and IL-6) and later, reduced anti-inflammatory factors (IL-10). This can be prevented peripherally with an anti-TNFα treatment (Infliximab), which also improves neuroinflammation and cognitive impairment (Balzano et al., 2020a).

Therefore, although hyperammonaemia is traditionally understood as a key driving factor in HE, peripheral inflammation by itself may contribute to the alterations observed in the disease (Azhari & Swain, 2018; Cabrera-Pastor et al., 2019).

5. Peripheral inflammation in HE

Inflammation is a coordinated process produced by infection or tissue damage. Its function is to solve the problem and return to homeostasis. This response should be quick and efficient, but sometimes sustained inflammatory response can cause more damage. After an infection or tissue injury, inflammation is first initiated by the innate immune system and then it can be exacerbated by the adaptative immune response. In the case of tissue damage, cell death can produce the liberation of multiple molecules that can be recognized by the innate immune system as the toll-like receptor (TLR) family members. Then, the inflammatory cascade would start and cytokines and inflammatory mediators would be produced in order to recruit other immune cells. The DNA–binding protein high-mobility group box 1 (HMGB1) has been related to TLR activation in some inflammatory settings (Barton, 2008).

Cytokines are small proteins that act as short-distance cell signaling messengers and are important mediators of inflammation. Different protein families are considered cytokines including interleukins, chemokines, interferons, and tumor necrosis factors. Cytokines are secreted by many different cells, mainly immune cells (macrophages, B lymphocytes, T lymphocytes), endothelial cells, and fibroblasts among others. They exert their functions through cell surface receptors and have a key role in the immune system that include the maintenance of the balance between humoral and cell-based immune responses, and the maturation, growth, and responsiveness of specific cell populations (Marisa et al., 2018). Interleukins regulate leukocytes activation, differentiation and proliferation, antibody secretion, chemotaxis, and the release of other cytokines; they mediate communication between cells of the immune system. Chemokines are cytokines that have the ability to activate, attract, and direct different families of circulating leukocytes toward damaged sites.

Cytokines are important for inflammatory responses. TNF-α, the interleukins IL-6, IL-1β, IL-12 and IL-18, and interferon gamma (IFN-γ) are the main proinflammatory cytokines. Other cytokines are also relevant for inflammatory processes such as IL-17, IL-21, IL-23, CCL2 and chemokine (C-C motif) ligand 20 (CCL20; also known as macrophage inflammatory protein-3 , MIP3A). Cytokines may have also anti-inflammatory properties such as IL-4, IL-13 and IL-10, and TGF-β.

5.1.Hepatic inflammation

The liver plays a main role in lipid and glucose metabolism. Because of the constant metabolic activity and the regular exposure to gut-derived bacterial products it has a persistent but regulated inflammation. The inflammatory processes are upregulated when the elimination of toxic products, pathogens or malignant cells is needed, but the failure of this clearance may lead to chronic pathological inflammation. Nevertheless, this controlled inflammation is essential to maintain homeostasis (Robinson et al., 2016).

A common response to metabolic or toxic stress is hepatic steatosis which in some cases may progress to liver injury. Injured steatotic hepatocytes change their gene expression (increasing the expression of TGF-β, IL-1A, hedgehog ligands, C-X-C motif chemokine ligand 10 (CXCL10), twist and snail) inducing inflammation and fibrosis. Steatosis of hepatocytes is usually the result of the increase of lipotoxicity, such as the presence of cytotoxic saturated free

fatty acids palmitate and stearate. This type of fatty acids is related to activation of proapoptotic signaling, insulin resistance, steatohepatitis and extracellular vesicles (EVs) release. EVs release is a communication method for cells to transmit signals. In fact, some components of injured hepatocytes EVs cargo as TNF-related apoptosis-inducing ligand (TRAIL), CXCL10, and sphingosine-1-phosphate could be involved in the activation or chemotaxis of macrophages to liver of NASH patients (Ibrahim et al., 2016; Hirsova et al., 2016; Kakazu et al., 2016; Koyama & Brenner, 2017). Moreover, liver inflammation can be modified by gut-derived microbiota. Boursier et al (2016) associated NAFLD severity with gut dysbiosis. They identified the association between NASH and *Bacteroides* and fibrosis with *Ruminococcus* (Boursier et al., 2016).

KC have an important role in liver inflammation. They are localized in the lumen of liver sinusoids and they are resident macrophages. Their function is to recognize and remove pathogens and toxic molecules from the blood arriving through the portal circulation via pattern-recognition receptors (PRRs). In the presence of liver injury, KC express pro-inflammatory cytokines and signaling molecules such as TNFα, IL-1β, IL-6, IL-12, nitric oxide (NO), among others (Ansari et al., 2016; Abdullah & Knolle, 2017).

Fu et al (2008) showed that CCl_4 administration to rats for 8 weeks induced oxidative stress, HSC activation and inflammation with increased levels of cytokines IFN-γ, TNF-α and IL-6 in liver (Fu et al., 2008). Similar results were showed after seven weeks of CCl_4 injection with increased levels of TNF-α, IL-6 and cyclooxygenase-2 (COX2) (Hamid et al., 2017). In fact, CCl_4 administration in rats induced fat accumulation after only two weeks of treatment and induced lobular inflammation and collagen accumulation after four weeks of treatment. Rats treated with CCl_4 also show increased levels of TGF-β and TNF-α (produced by KC under oxidative stress), IL-15 (produced by infiltrated monocytes) and IL-4 in liver at four weeks of CCl_4 administration. In contrast, IL-10 was reduced in livers of CCl_4-injected rats (Balzano et al., 2021).

5.2. Inflammation in peripheral blood

Peripheral inflammation is manifested by alteration of levels of proinflammatory and anti-inflammatory cytokines. Some cytokines can be also used even for discrimination of the presence or absence of MHE. For instance, in cirrhotic patients, the serum levels of interleukins

IL-6 and IL-8 can be correlated with the presence of MHE (Montoliu et al., 2009). Mangas-Losada et al. (2017) proposed that there is a shift in peripheral inflammation in MHE patients, characterized by increased activation and differentiation of CD4+ (cluster differentiation) T lymphocytes to Th17 (T helper), T follicular helper (Tfh), and Th22 types. This shift is associated with increased levels of cytokines IL-17, CCL20, chemokine (C-X3-C motif) ligand 1(CX3CL1, also known as fractalkine), and IL-15, which facilitate infiltration of lymphocytes into the brain. Cytokines are key in promoting peripheral inflammation and neuroinflammation, leading to cognitive and motor alterations in patients with MHE and HE (Mangas-Losada et al., 2017).

The importance of peripheral inflammation for the induction of the cognitive and motor alterations of MHE has also been analysed in rats with PCS. PCS rats show increased plasma levels of IL-6 and PGE-2 (both proinflammatory) and reduced levels of IL-10 (anti-inflammatory). Moreover, the administration of peripheral drugs as Infliximab, an anti-TNFα antibody, in PCS rats, prevents peripheral inflammation (normalizes IL-6, PGE-2 and IL-10), neuroinflammation and the alterations in neurotransmission, restoring motor coordination and learning and memory impairments (Dadsetan et al., 2016a; Dadsetan et al., 2016b). As seen before, hyperammonaemia is able to induce peripheral inflammation in rats. This peripheral inflammation is observed in hyperammonemic rats as increased plasma levels of PGE2, IL-6, IL-10 and TNFα which are prevented by the peripheral administration of Infliximab (Balzano et al., 2020a). Moreover, CCl_4 administration to rats increased TNF-α, CCL20, CX3CL1, IL-17 and IL-15 in plasma after four weeks of treatment (Balzano et al., 2021).

5.3.Role of microbiota in the immune system alterations

Gastrointestinal microbiota includes approximately 100 trillion microorganisms (mainly bacteria) that reside in the human gastrointestinal tract (Rath & Dorrestein, 2012; Bull & Plummer, 2014). This complex microbiome produces thousands of metabolites that may influence human health (Bull & Plummer, 2014). The enterohepatic circulation between the gastrointestinal tract and the liver provides direct access of host metabolites to the microbiome and vice versa. Some studies in animals and humans have shown that gut microbiota has a key role in many aspects of human health, including immune system, metabolic regulation and behaviour (Jandhyala et al., 2015). In healthy adults, gut microbiota composition is determined by diet and drugs and is relatively stable and individual-specific (Goodrich et al., 2014; Valdes

et al., 2018). However, repeated disturbances in the microbiota due to antibiotics, infection, changes in diet, or non-infectious diseases may lead to alterations in host physiology.

Intestinal microbiota plays an important role in metabolism of nutrients. Some organisms as *Bacteroides, Roseburia, Bifidobacterium*, *Faecalibacterium* and *Enterobacteria* are able to fermentate residual carbohydrates and indigestible oligosaccharides producing short chain fatty acids (SCFA) such as butyrate, propionate and acetate that are used as sources of energy by the host (Jandhyala et al., 2015). SCFAs, specifically butyrate, are an important source for enterocytes and influence gastrointestinal barrier function (Willemsen et al., 2003; Brahe et al., 2013).

The microbiota promotes and modulates multiple aspects of the immune system. It is important for induction, training and function of the human immune system. On the other side, the immune system has evolved to maintain a symbiotic relationship with the microbiota. This relationship, in optimal conditions, would produce protective responses against pathogens and maintenance of tolerance to innocuous antigens. However, misdirected immune responses derived from alterations in the regulation of this symbiosis would lead to allergies, or autoimmune, metabolic and inflammatory diseases (Belkaid & Hand, 2014).

The gastrointestinal tract and the central nervous system (CNS) have a specific bidirectional communication known as “gut-brain axis”. Gastrointestinal microbiota also plays a role in this exchange of information being part of the “microbiota-gut-brain axis” (Bercik, 2011; Socala et al., 2021). The modulation of this axis by microbiota has led to new concepts of the pathophysiology of diseases (Dinan & Cryan, 2017). The microbiota-gut-brain axis communication network is complex and includes the hepatic and gallbladder metabolism, immune-modulatory responses, neuronal innervation (mainly vagal nerve), the enteric nervous system, enteroendocrine, and microbial metabolite signaling as well as the gut epithelium (Figure 4) (Cryan et al., 2019).

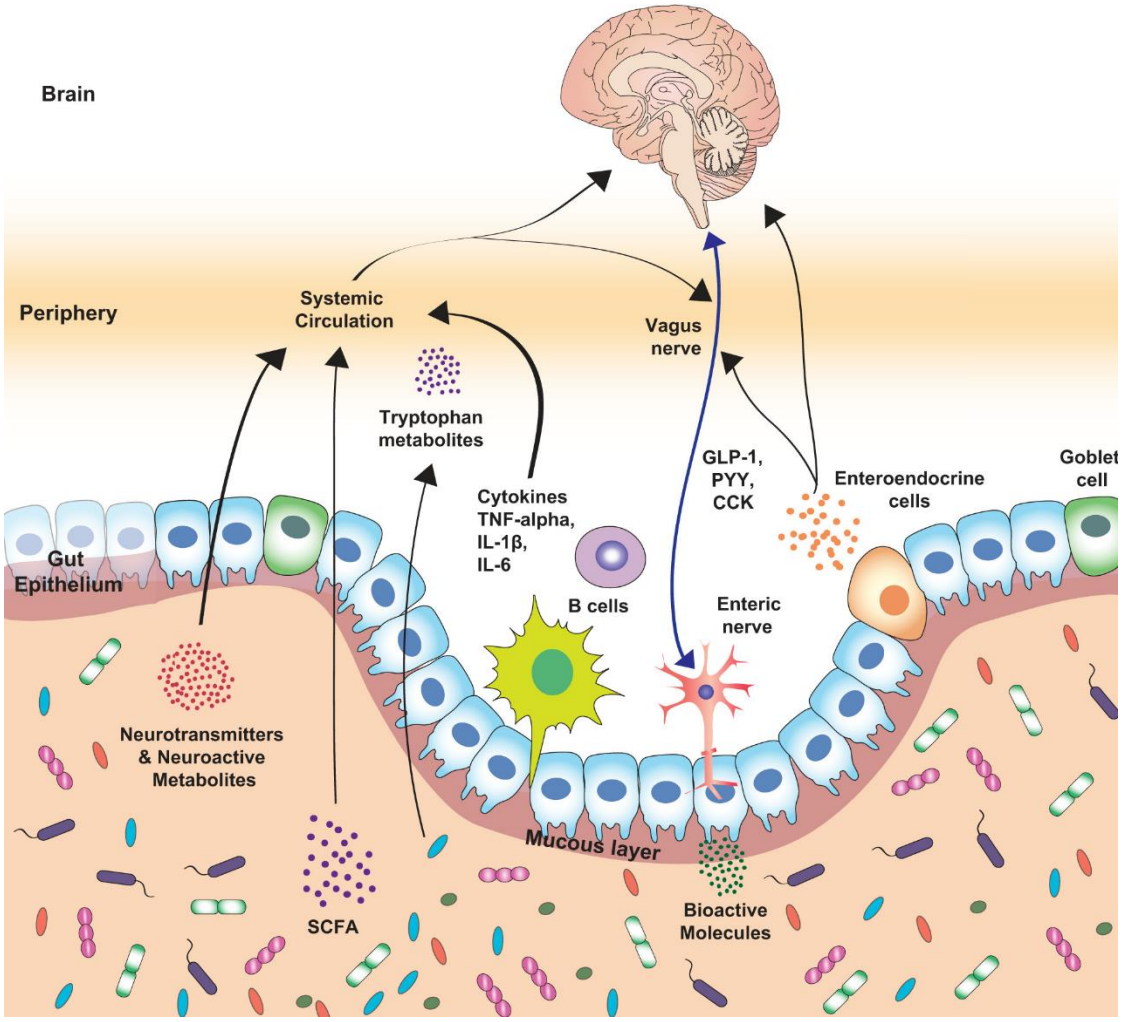

Figure 4. *Some pathways of communication in the microbiota-gut-brain axis including the nervous, enteroendocrine and immune systems, the hepatic and gallbladder metabolism, the epithelium and several involved metabolites. CCK, cholecystokinin; GLP-1, glucagon-like peptide-1; IL, interleukin; PYY, peptide YY; TNF, tumor necrosis factor; SCFA, short-chain fatty acid. From Cryan et al 2019.*

In chronic liver disease, increased intestinal permeability due to dysbiosis results in the translocation of bacteria and endotoxins, which may contribute to the development of HE (Bashiardes et al., 2016). Different molecules derived from bacteria can penetrate to the liver and activate pathways leading to inflammation or production of reactive oxygen species (Sarkola & Eriksson, 2001; Hartmann et al., 2012; Ilan, 2012; Paolella et al., 2014).

It has been shown that NASH patients have lower *Bacteroidetes* counts compared to obese people without NASH (Mehal, 2013). In patients with NAFLD, an increased abundance of *Escherichia, Anaerobacter,* and *Streptococcus* is observed, while *Ruminococcaceae, Alistipes,*

and *Prevotella* are decreased (Power et al., 2014; Jiang et al., 2015). Cirrhotic patients show enriched levels of *Firmicutes* in the duodenum, while the most abundant bacteria in controls are *Proteobacteria* (Bajaj, 2015). In addition, cirrhotic patients with HE show a decrease in the relative abundance of native benefitial taxa *Lachnospiraceae*, *Ruminococcaceae* and *Clostridiales XIV* (Bajaj et al., 2014).

5.4. Transmission of peripheral inflammation to the brain

Inflammation can reach the CNS by various means. Transmission of the peripheral inflammation to the brain is produced often by the disruption of the BBB.

The BBB impedes the transmission of peripheral signals to the CNS. The BBB is composed of capillary wall endothelial cells, pericytes, and astrocytes. An alteration in any of these components such as pericyte detachment, astrocytic loss or swollen end-feet or impaired transporter function will increase BBB permeability and produce neuroinflammation (Obermeier et al., 2013). Endothelial cells are joined by tight junctions (TJ) that maintain BBB impermeability. Some inflammatory circulating factors may disrupt TJ increasing BBB permeability (Takeshita et al., 2021; Zhao et al., 2022). In astrocytes, in response to IL-1β, vascular endothelial growth factor-A (VEGF-A) production is increased inducing the downregulation of TJ proteins claudin-5, TJ protein 1 (TJP-1/ZO-1) and occludin, disrupting TJ and BBB integrity (Linnerbauer et al., 2020). BBB permeabilization allows the entrance of inflammatory cytokines, ions and immune cells to the CNS (Sun et al., 2022).

Other pathways by which the peripheral inflammatory mediators can access the CNS, even without disruption of BBB, are passing through specific "leaky regions" in the circumventricular organs, through transporter protein channels, by activation and movement of immune cells such as monocytes and T-lymphocytes from the peripheral blood towards the CNS and infiltration into the brain tissue, release of inflammatory mediators by endothelial cells and perivascular macrophages in the cerebral vasculature, by the activation by peripheral cytokines of receptors in endothelial cells, and by EVs that can transduce inflammatory signaling to endothelial cells and also enter the CNS (Sun et al., 2022).

All those inflammatory processes would lead to additional cytokine production within the brain which together would trigger neuroinflammation. Therefore, persistent peripheral inflammation leads to neuroinflammation.

The microbiota-gut-brain axis also has a role in the relation between peripheral inflammation and brain function. Bidirectional communication of inflammatory signals through this axis is key for the physiological behaviours and for the pathology of diseases associated to inflammation. Inflammatory signaling exchanges occurs through three main pathways: the humoral systemic, the cellular immune and the neuronal pathways (Figure 5). In the systemic pathway (Figure 5 top) inflammatory factors released in the intestine can pass into the systemic circulation reaching the CNS and alter BBB integrity and brain function. Moreover, the activation of the hypothalamic–pituitary–adrenal (HPA) axis due to inflammation triggers systemic release of glucocorticoid, which modifies intestinal function. In the immune pathway (Figure 5 middle), dysbiosis activates enteric immune cells that can translocate to the CNS to promote neuroinflammation which can be also induced by metabolites produced by gut microbiota. This process can be initiated by stress responses in the CNS that can alter gut microbiota. Finally, in the neuronal pathway (Figure 5 bottom), the stimulation of afferent neurons such as the vagal nerve triggers neural circuits involved in HPA activation, sickness behaviour, and visceral pain perception. This circuits induce activation of glial cells and start activation of inhibitory pathways to suppress intestinal inflammation (Agirman et al., 2021).

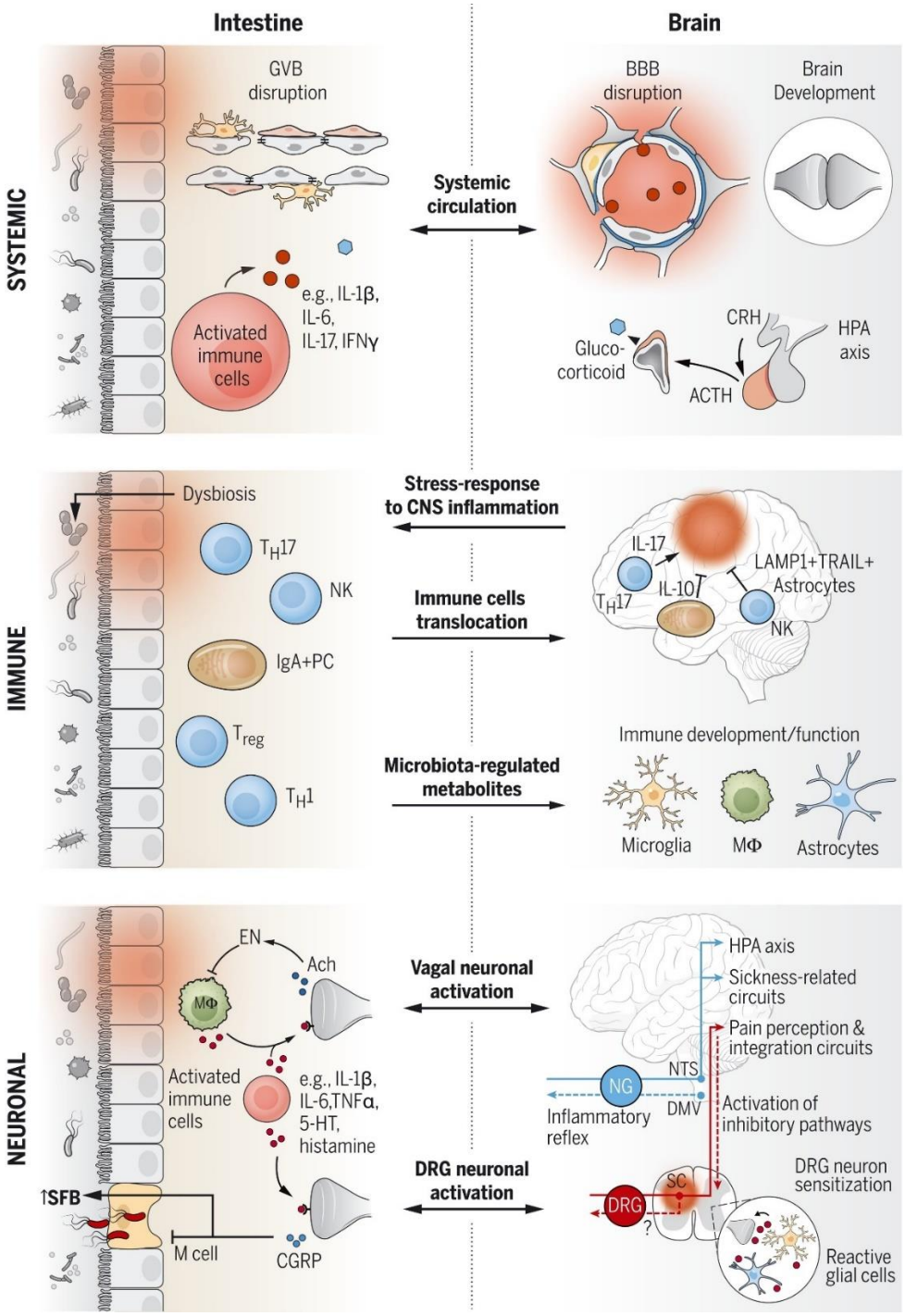

Figure 5. *Inflammatory related interactions in the gut-brain-axis in which the humoral systemic (top), immune cellular (middle) and neuronal (bottom) pathways are involved. Ach, acetylcholine; ACTH, adrenocorticotropic hormone; BBB, blood-brain barrier; CRH, corticotrophin-releasing hormone; DMV, dorsal motor nucleus of the vagus; DRG, dorsal root ganglia; EN, enteric neuron; GVB, gut-vascular barrier; HPA, hypothalamic–pituitary–adrenal; MΦ, macrophage; NG, nodose ganglion; NK, natural killer cell; NTS, nucleus tractus solitarius; PC, plasma cell; SC, spinal cord; SFB, segmented filamentous bacteria; TH, T helper; Treg, regulatory T cell; and VN, vagus nerve. From Agirman et al 2021*

5.4.1. Infiltration of immune cells into the brain

The peripheral immune system can induce neuroinflammation through peripheral cell infiltration into the CNS. Usually, T cells initiate immune responses by interacting with antigen-presenting cells (such as dendritic cells). However, cells from CNS cannot migrate to promote the immune response as happens with other organs. Thus, the immune cells are the ones migrating to the CNS to deal with the problem (Ransohoff & Brown, 2012).

Activated leukocytes have selectin ligands that recognize the selectins of the endothelial cells. This recognition produces clustering and a conformational change in leukocytes, resulting in high-affinity binding of integrins to their ligands and mediating the adhesion of leukocytes at the endothelium. After adhesion, leukocytes move across the endothelial surface until they reach inter-endothelial junctions. Then leukocytes emit protuberances via the inter-endothelial junction, looking for chemokines such as CXCL1 (chemokine (C-X-C motif) ligand 1), CCL2, CCL5 (Chemokine (C-C motif) ligand 5, or RANTES), and CXCL12 (C-X-C motif chemokine 12, also known as stromal cell-derived factor 1), which will function as guide signals for extravasation. Transmigration occurs in response to these chemokines. Extravasating cells first accumulate in the perivascular space between the endothelial basement membrane and the glia limitans, formed by astrocytic and microglial foot processes. Leukocyte transverse across the glia limitans involves the action of matrix metalloproteinases (MMPs) (Barreiro & Sanchez-Madrid, 2009; McEver, 2015).

Another route of entry is the meninges, a group of membranes that surround the brain and the spinal cord. The outer membrane is the dura which is attached to the skull and the vertebral column. The other two membranes are the arachnoid and the pia mater that are separated by the subarachnoid space. Inside the meninges the cerebrospinal fluid is circulating. In normal conditions, lymphocytes can access the CNS for examination purposes, but with neuroinflammatory conditions, their migration can increase and they can migrate to specific locations of the brain depending on the expression levels of adhesion molecules (Engelhardt & Ransohoff, 2012). Lymphocyte infiltration in the meninges plays an important role in the pathogenesis of multiple sclerosis (Pikor et al., 2016).Other important point of access for immune cells, is the choroid plexus that has a fenestrated structure that can be easily crossed by immune cells.

Infiltration of immune cells into the brain has been observed in patients with steatohepatitis, cirrhosis and HE in which has been observed the presence of T lymphocytes (Th17 and Tfh) in meninges of cerebellum (Balzano et al., 2018) and also in the hippocampus of patients with steatohepatitis infiltration of $CD4^+$ lymphocytes and macrophages was observed (Leone et al., 2023). Similarly, in the CCl_4 induced liver damage rat model, at an early stage with mild steatohepatitis, infiltration of $CD4^+CD28^-$ and Th17 CD4 lymphocytes and monocytes was observed in meninges of cerebellum (Balzano et al., 2021) and also in the hippocampus (Leone et al., 2022). Therefore, infiltration of immune cells into the brain has an important role in the induction and maintenance of neuroinflammation, that ultimately leads to impairment of brain function and neurological alterations in HE.

5.4.2. Extracellular vesicles

EVs are small membrane vesicles (~30-200 nm of diameter) secreted by a wide range of cells, from prokaryotes to higher eukaryotes. They have different functions in physiological and pathophysiological processes. They have a main role in intercellular communication due to their transfer information capacity by transfering their cargo, which include proteins, lipids, nucleic acids, sugar, etc (Yanez-Mo et al., 2015). They can modulate the immune response in several ways such as participating in the antigen presentation process and stimulating the liberation of pro or anti-inflammatory mediators (Bhatnagar & Schorey, 2007). They are also key in the communication between the periphery and the brain.

EVs from peripheral blood may transmit deleterious effects to the CNS in some pathological conditions. Ridder et al. (2014) injected EVs from the serum of transgenic mice (expressing Cre recombinase in hematopoietic lineage) into control mice. The injected EVs were capable of transferring functional RNAs (ribonucleic acid) to different neuronal types in the brain, in a process favoured by peripheral inflammation that induced significant changes in recipient cells (Ridder et al., 2014). Li et al. (2018) purified and fluorescently labelled EVs from the serum of mice with LPS-induced (lipopolysaccharides) endotoxemia. They were able to detect these EVs in the hippocampus of control mice 24 hours after injection. They also observed that injection of the EVs induced neuroinflammation in the brain of recipient mice (Li, J. J. et al., 2018).

In relation to HE, Izquierdo-Altarejos et al (2020) demonstrated that EVs play a key role in the transmission of peripheral alterations to the brain in hyperammonemic rats. The injection of plasma EVs from hyperammonemic rats to control rats induced neuroinflammation and motor incoordination to those (Izquierdo-Altarejos et al., 2020). Injection of plasma EVs from hyperammonemic rats also impaired learning and memory in normal rats and induced neuroinflammation in the hippocampus (Izquierdo-Altarejos et al., 2023).

Somehow, EVs are able to cross the BBB, but the mechanisms that allow them to do this are not fully known. However, some studies point out that these processes would be facilitated in pathological situations. Transcytosis (a type of transcellular transport) has been observed as a possible mechanism. Chen et al. (2016) showed that EVs were capable of crossing the endothelial cell monolayer under inflammation conditions (cells stimulated with TNF-α) but not under normal conditions in a transwell system that simulated the BBB. In this same work, it was seen that the vesicles were internalized by endothelial cells, suggesting that they followed the transcellular route (Chen, C. C. et al., 2016). Morad et al. (2019) have shown that tumor-derived EVs are capable of crossing the BBB in vivo through transcytosis (Morad et al., 2019).

5.4.3. Receptors of interleukins

Transmission of inflammatory signals to the brain can be also produced by the activation of receptors of peripheral cytokines such as TNF-α, IL-1β, IL-6 and IL-17 in the endothelial cells of the BBB. These cytokines present in the circulating blood may activate their receptors in the endothelial cells and trigger the release of inflammatory factors into the brain (Rummel et al., 2006). The injection of LPS in rats increases IL-6 in plasma that activates its receptors in the endothelial cells of the BBB leading to STAT3 (signal transducer and activator of transcription 3) activation which increases COX2 and PGE2 in the brain (Rummel et al., 2006). Upon the activation of cytokine receptors, endothelial cells are able to secrete signals into the parenchyma that induce neuroinflammation. Peripheral IL-1β binds to IL-1R1 (interleukin 1 receptor, type I) in the endothelial cells that may produce a microglial response (Krasnow et al., 2017). Endothelial cells can also respond to TNF-α that induces different inflammatory responses in the brain (O'Carroll et al., 2015).

6. Neuroinflammation in HE and MHE

The inflammatory response that occurs in the CNS due to infections, traumatic injuries, toxic metabolites, or autoimmunity is called neuroinflammation. Neuroinflammation is mediated by the production and release of cytokines, chemokines, reactive oxygen species (ROS) and secondary messengers by the CNS resident glia (microglia and astrocytes), peripheral immune cells and endothelial cells (DiSabato et al., 2016). Cytokines found in the CNS can come from local glial cells or neurons, from immune cells infiltrated into the CNS or from the periphery by active or passive transport through the BBB (Marisa et al., 2018).

Cytokine levels are altered in brain of animal models of MHE indicating presence of neuroinflammation. IL-6 is increased in cortex of PCS rats (Cauli et al., 2007). IL-1β is increased in cerebellum of hyperammonemic and BDL rats (Rodrigo et al., 2010). TNF-α is also increased in the cerebellum and the hippocampus of hyperammonemic and PCS rats (Hernandez-Rabaza et al., 2015; Cabrera-Pastor et al., 2016; Dadsetan et al., 2016a; Hernandez-Rabaza et al., 2016a; Dadsetan et al., 2016b). Rats with induced liver damage show increased levels of CCL2 in the hippocampus and the cerebellum. Moreover, IL-1β and TNF-α levels are also increased in neurons in the CA1 region of the hippocampus (Leone et al., 2022), while levels of TNF-α, TGF-β, IL-15, and IL-4 are increased and the levels of IL-10 are reduced in the cerebellum of rats injected with CCl_4 (Balzano et al., 2021).

6.1. Glial cells: microglia and astrocytes

Glial cells are a group of cells found in the CNS that are not excitable but have functions that help to define synaptic contacts and maintain the signaling abilities of neurons. There are several types of glial cells among them microglial cells and astrocytes exert immune functions and play a key role in regulation of neuroinflammation (Araque & Navarrete, 2010).

Microglial cells or microglia are myeloid cells that are part of the innate immune cells and are responsible for the primary immune surveillance and macrophage-like activities of the CNS. They can be found in the white matter and the gray matter of the brain and the spinal cord. They are overall 10% of the CNS population. Among their functions is the phagocytosis and elimination of particles that could be dangerous for the CNS such as microbes, dead cells and

protein aggregates. Microglia secrete cytokines, chemokines and neurotrophic factors that contribute to the immune responses and tissue repair of the CNS (Muzio et al., 2021).

Microglia structure is highly dynamic and depends on their activation state. In steady state, microglial cells present a small cell body and several ramifications, branches and processes. In response to injuries or inflammation, microglia change to an amoeboid shape with larger cell bodies and shorter processes. This state is highly activated and related to phagocytosis and proinflammatory function (Colonna & Butovsky, 2017).

Microglia activation is often differentiated between classical (M1) or alternative (M2) as happens with circulating and peripheric macrophages. M1 activation is known as proinflammatory and neurotoxic. For instance, M1 microglia will produce proinflammatory cytokines and chemokines, such as TNFα, IL-6, IL-1β, IL-12, and CCL2. In contrast, M2 activation is related to anti-inflammatory and healing functions for example releasing anti-inflammatory cytokines, such as IL-10 and TGF-β, inducing arginase 1, and secreting growth factors such as insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF), as well as neurotrophic factors such as brain derived neurotrophic factor (BDNF) and glial cell–derived neurotrophic factor (GDNF) (DiSabato et al., 2016; Colonna & Butovsky, 2017). Generally, activation of microglial cells is a way of protection of the CNS. However, an increased, chronic, or exaggerated microglial activation may produce brain dysfunction leading to neuropsychiatric symptoms such as depression or cognitive deficits (Norden & Godbout, 2013; DiSabato et al., 2016).

Astrocytes are a very numerous group of specialized glial cells. They contiguously cover the entire CNS and perform essential complex functions to maintain the CNS healthy responding to all types of CNS lesions. Classically it is considered that there are two main subtypes of astrocytes, protoplasmic and fibrous. Protoplasmic astrocytes are found in the grey matter and their morphology consists of several stem branches that give rise to many finely branching processes with a globoid distribution. In contrast, fibrous astrocytes can be found in the white matter and have many long processes similar to fibers (Sofroniew & Vinters, 2010).

Astrocytes functions include interactions at different levels. They actively participate in neuroprotection, neuronal metabolism, and synaptic plasticity (Colangelo et al., 2014). In the synapses, they collaborate with neurons mainly in the uptake of some molecules (as

neurotransmitters) and releasing others (neurotransmitters and their precursors, growth factors, energy substrates and neurosteroids among others). They also contact the Nodes of Ranvier and with blood vessels. In the blood vessels, they are also in charge of the uptake and release of different molecules and they have a function in the maintenance of the BBB (Sofroniew & Vinters, 2010).

In response to CNS injury and diseases, astrocytes suffer a group of molecular, cellular and functional changes. This response usually includes increased expression of glial fibrillary acid protein (GFAP, used as an astrocytes marker), morphological changes, proliferation and glial scar formation. Reactive astrogliosis is a physiological response to CNS injury that minimizes and repairs damage mainly during its earliest stages. Nevertheless, this neuroprotection is compromised during chronic neuroinflammation (Colangelo et al., 2014). Astrocytes dysfunction is produced in neurodegenerative diseases such as Alzheimer's disease, Huntington's disease and Parkinson's disease (Phatnani & Maniatis, 2015).

HE is able to induce microglial activation producing reactive nitrogen and oxygen species and secretion of cytokines. Microglial activation was only observed in the cerebral cortex from patients with cirrhosis and HE, and not in patients without HE. This suggests that neuroinflammation related to microglial activation is involved in the apparition of HE in cirrhotic patients (Zemtsova et al., 2011). On the other side, dysfunction of astrocytes plays a main role in the progression of the disease. Increased ammonia levels in the brain are detoxified mainly by astrocytes by the role of glutamine synthetase. This process leads to accumulation of glutamine, that leads to altered expression of proteins that can alter normal astrocytic functions and even to astrocytic swelling in more serious or acute increases of ammonia (Felipo & Butterworth, 2002).

Microglial and astrocytic activation has been also observed in cerebellum and hippocampus of cirrhotic patients. In fact, patients with steatohepatitis but without cirrhosis already show activation of microglia and astrocytes in hippocampus and in white matter and molecular layer of cerebellum (Balzano et al., 2018; Leone et al., 2023).

Animal models of HE also show activation of microglia and astrocytes. Hyperammonemic and BDL rats show activation of microglia in the cerebellum which is reversed when treated with Ibuprofen (Rodrigo et al., 2010). Hyperammonemia also induced increased activation of

microglia and astrocytes in rat hippocampus which was reversed by sulforaphane (Hernandez-Rabaza et al., 2016b). PCS rats show microglial activation in the cerebellum that is reversed by inhibiting p38 (Agusti et al., 2011). Rats with CCl_4-induced liver damage show activation of microglia and astrocytes in white matter and molecular layer of cerebellum and the hippocampus after only 4 weeks of administration of CCl_4, in rats with mild steatohepatitis (Balzano et al., 2021; Leone et al., 2022).

7. Neurotransmission. Alterations in HE

In HE, the imbalance between excitatory and inhibitory neurotransmission is a crucial factor in the development of cognitive dysfunction and neurological symptoms (Albrecht and Jones 1999, Cauli et al 2009). Altered neurotransmission has been reported in different animal models of HE. Glutamatergic and GABAergic neurotransmission are the most affected in HE (Cauli et al., 2009b), but alterations in other neurotransmitter systems as serotonergic and cholinergic systems were shown also associated with HE (Albrecht & Jones, 1999; Dhanda & Sandhir, 2015).

7.1. Alterations of glutamatergic neurotransmission

Glutamate is the main excitatory neurotransmitter of the CNS. Glutamatergic neurotransmission depends on different receptors including ionotropic receptors as N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and metabotropic glutamate receptors (mGluRs). Glutamatergic neurotransmission plays a main role in several cognitive functions such as learning and memory (Niciu et al., 2012). The hippocampus is a brain region that is very important for spatial navigation and long-term memory storage and contains NMDA and AMPA receptors which are involved in mediating long-term potentiation (LTP). LTP is a process that underlies the strengthening of synaptic connections between neurons in response to repeated stimulation and is considered as one of the primary mechanisms of memory formation. However, disturbances in glutamatergic neurotransmission can have deleterious effects on cognitive functions, affecting LTP (Luscher & Malenka, 2012).

In animal models of HE such as hyperammonemic and PCS rats, LTP in the hippocampus is altered (Monfort et al., 2005; Monfort et al., 2007). In hyperammonemia, glutamatergic neurotransmission is disrupted by the alteration of membrane expression and function of NMDA and AMPA receptors, which leads to cognitive alterations. Cabrera-Pastor et al, (2018) showed that hyperammonaemia increased GluA1 and reduced GluA2 (AMPA receptor subunits 1 and 2) and increased NMDA receptors membrane expression in hippocampus (Cabrera-Pastor et al., 2018). However, Taoro-Gonzalez et al, (2019) observed that in hippocampal slices from hyperammonemic rats, the membrane expression of GluA1 was reduced and membrane expression of GluA2 and GluN2B (NMDA receptor subunit 2B) was increased (Taoro-Gonzalez et al., 2019a). Hyperammonemia alters AMPA and NMDA receptors functions in hippocampus in a stimulation intensity dependent manner, measured in a multielectrode array (MEA) system. Concretely, AMPA response is decreased in hyperammonemic rats while NMDA response is increased (Sancho-Alonso et al., 2022b). In addition, neuroinflammation contributes to these alterations in glutamate receptors, as the reduction of neuroinflammation with anti-TNF-α (Dadsetan et al., 2016b; Balzano et al., 2020b), sulforaphane (Hernandez-Rabaza et al., 2016b) or the antagonist of IL-1 receptor (IL-1Ra) (Taoro-Gonzalez et al., 2019b) reversed most of the glutamate receptor alterations in PCS or hyperammonemic rats.

In rats with CCl_4 induced mild liver damage, glutamatergic neurotransmission is altered in hippocampus, with an increased membrane expression of the GluA2 and reduced membrane expression of GluN1 and GluN2A (1 and 2A subunits of NMDA receptors). These alterations were related to the impairment of spatial learning and memory and hippocampus function (Leone et al., 2022). In cerebellum, rats with mild liver damage show increased extracellular glutamate levels and a reduced membrane expression of glutamate transporter GLAST (glutamate aspartate transporter). This alteration of glutamatergic neurotransmission would contribute to the alteration of GABAergic neurotransmission that will impair motor coordination (Balzano et al., 2021).

7.2. Alterations of GABAergic neurotransmission

GABA is the primary inhibitory neurotransmitter in the CNS, and its dysregulation has also been related to the pathophysiology of HE. Alterations in GABAergic neurotransmission have been observed in animal models of HE, including hyperammonemic, BDL, and PCS rats. In

the cerebellum of MHE rat models, there is an increased GABAergic tone due to the increased extracellular GABA, altered neurosteroids levels and altered content of $GABA_A$ receptor (type A) subunits (Cauli et al., 2009a). Rats with mild liver damage and hyperammonemic rats show increased levels of extracellular GABA in cerebellum that will increase the inhibitory neurotransmission (Cabrera-Pastor et al., 2018; Balzano et al., 2021). Increased activation of $GABA_A$ receptors by the increase of extracellular levels of GABA induces motor incoordination (Hanchar et al., 2005). In fact, motor coordination is restored when treating the animals with bicuculine, a $GABA_A$ receptor antagonist, pregnenolone sulphate, a negative modulator of the $GABA_A$ receptor or golexanolone, an antagonist of the neurosteroid allopregnanolone (Cauli et al., 2009a; Gonzalez-Usano et al., 2014; Johansson et al., 2016; Mincheva et al., 2022).

The increased extracellular levels of GABA in the cerebellum are mainly due to reduced membrane expression of GABA transporter GAT-1 (GABA transporter 1) in neurons and increased membrane expression of the astrocytic transporter GAT3 (GABA transporter 3). This was observed in rats with mild liver damage that show motor incoordination in the rotarod (Balzano et al., 2021). In hippocampus of hyperammonemic rats, both transporters GAT-1 and GAT-3, show a decreased membrane expression which also contributes to the increase of extracellular GABA levels (Sancho-Alonso et al., 2022a).

GABA levels also depend on the enzyme glutamate decarboxylase (isoforms GAD65 and GAD67) that transforms glutamate into GABA. Hyperammonemic rats show increased levels of GAD65 and GAD67 in Purkinje neurons of cerebellum (Arenas et al., 2022a). On the other side, GABAergic neurotransmission is also altered when GABA receptors membrane expression or function is altered. In cerebellum of hyperammonemic rats, membrane expression of the γ2, α2 and β3 subunits of $GABA_A$ receptors is increased (Arenas et al., 2022a). In hippocampus of this same animal model, activation of $GABA_A$ receptors is enhanced when analysed in a MEA system (Sancho-Alonso et al., 2022a). Sancho-Alonso et al, (2022) observed an increased membrane expression of the $GABA_A$ receptor subunits α1, α2, γ2, β3, and δ that together with the increase extracellular GABA levels are the main contributors to the enhanced GABAergic neurotransmission in hippocampus of hyperammonemic rats that would induce the observed cognitive impairment (Figure 6).

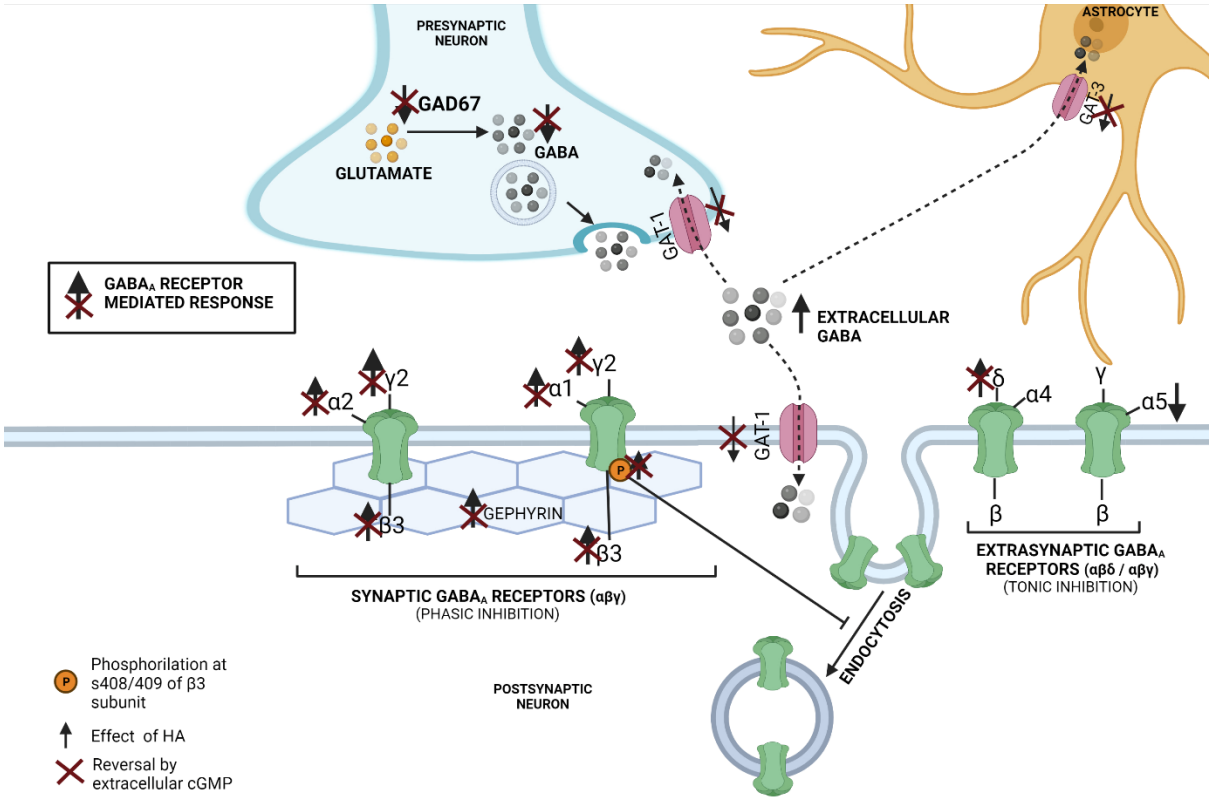

Figure 6. *Mechanism proposed by Sancho-Alonso et al (2022) by which hyperammonemia increases GABA$_A$ receptor response in the hippocampus. Chronic hyperammonemia reduces the membrane expression of GABA transporters (GAT-1 and GAT-3) leading to increased extracellular levels of GABA although the total content of GABA is reduced due to the reduced GAD67 content. Membrane expression of GABAA receptor is increased by increasing the phosphorylation of β3 subunit and the content of gephyrin (scaffolding element of GABA$_A$ receptors). The increased extracellular levels of GABA and membrane expression of GABA$_A$ receptors are the main contributors of the enhanced GABAergic response. From Sancho-Alonso et al (2022).*

As happens with glutamatergic neurotransmission, alterations of the GABA transporters are associated and induced, at least partially, by neuroinflammation in animal models such as PCS, hyperammonemia and rats with mild liver damage (Hernandez-Rabaza et al., 2016a; Agusti et al., 2017; Cabrera-Pastor et al., 2018; Balzano et al., 2021; Malaguarnera et al., 2021). Cabrera-Pastor et al, (2018) proposed a possible pathway by which neuroinflammation would increase extracellular GABA in cerebellum and impair motor coordination in hyperammonemic rats (Figure 7). Hyperammonemia induces neuroinflammation with activation of microglia and astrocytes and increased levels of TNF-α and the amount of its receptor (TNFR1) in the

membrane. This increases the activation of TNFR1 and leads to the translocation of the p50 subunit of NF-κB to the nucleus. Thus, the expression of glutaminase is increased, leading to increased levels of glutamate which increases the activation of its transporters (glutamate transporter 1 (GLT-1) and GLAST). Glutamate transporters also introduce Na+ in the cells while transporting glutamate so their increased activation also increases levels of intracellular Na+ which would reverse the function of GAT-3 in activated astrocytes resulting in release and increased extracellular concentration of GABA. This in turns leads to motor in-coordination.

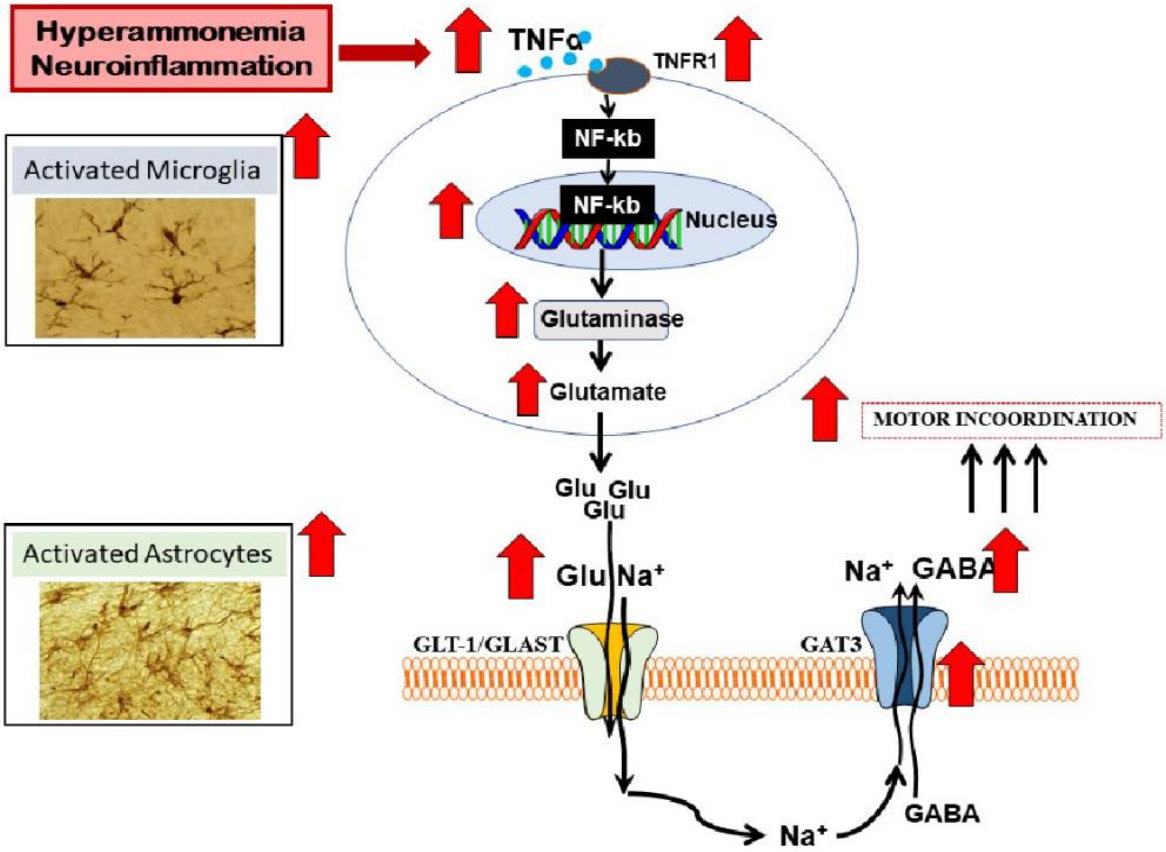

Figure 7*. Mechanism proposed by Cabrera-Pastor et al (2018) by which neuroinflammation alters neurotransmission in cerebellum in hyperammonemic rats. Red arrows: alterations produced by hyperammonemia; Glu: glutamate. Adapted from Cabrera-Pastor et al (2018).*

Arenas et al, (2022) proposed another possible pathway by which GABAergic neurotransmission would be affected by neuroinflammation in cerebellum, the TNFR1-S1PR2 (sphingosine-1-phosphate receptor 2)-CCR2 (CCL2 receptor)-TrkB (Tropomyosin receptor kinase B, BDNF receptor)-KCC2 (potassium chloride cotransporter 2) pathway (Figure 8). The content in the membrane of TNFR1 and S1PR2 and therefore their activation are enhanced in

Purkinje neurons of hyperammonemic rats, which leads to increased production of CCL2 by Purkinje neurons. CCL2 is released and activates its receptor CCR2 in microglia, leading to microglial activation and increased membrane expression of CCR2 and P2X4 (P2X purinoceptor 4) receptors, and increased formation of BDNF in microglia. Released BDNF activates TrkB-mediated signals in neurons, leading to higher content of KCC2 in the cell membrane, which alters the transmembrane chloride (Cl) gradient, resulting in altered GABAergic neurotransmission.

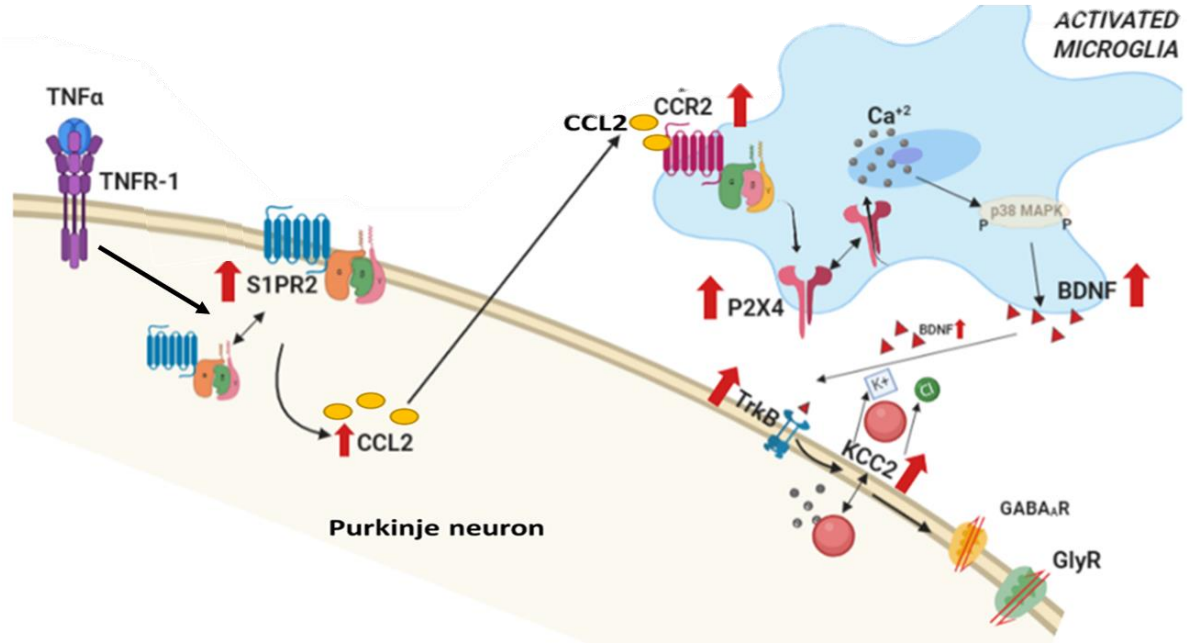

Figure 8. *Mechanism proposed by Arenas et al (2022) by which neuroinflammation alters GABAergic and glycinergic neurotransmission in cerebellum of hyperammonemic rats. Red arrows: alterations produced by hyperammonemia; $GABA_AR$: $GABA_A$ receptor; GlyR: glycine receptor.*

8. Treatments for HE and MHE

Hyperammonaemia is one of the main factors involved in the induction of the symptoms of HE, thus many treatments try to reduce blood ammonia levels. Protein intake is the main source of ammonia excess, but some components of the microbiota are also producers of ammonia, contributing to its increased levels. Some agents are used because of their demonstrated efficacy to reduce circulating ammonia levels. Many of them are used to reduce ammonia absorption as non-absorbable disaccharides (lactulose, lactitol), non-absorbable antibiotics as

rifaximin, probiotics and purgatives as polyethylene glycol (Butterworth, 2021). In this same direction, the transplantation of faecal microbiota from compatible donors has also been proposed to reduce the input of ammonia by some microorganisms of the gut microbiota (Bajaj et al., 2017).

The use of simple laxatives such as polyethylene glycol has been proposed as a therapy since there are studies that suggest that intestinal evacuation alone can improve the pathology (Rahimi et al., 2014). Similarly, non-absorbable saccharides are also used as purgatives, but they show a doubtful improvement of the HE and have some adverse effects as diarrhoea, flatulence, abdominal pain/cramping, nausea and anorexia (Blanc et al., 1992; Suraweera et al., 2016).

Probiotics are products that contain non-pathogenic microorganisms that modify the intestinal microbiota conferring beneficial effects to the host. The most frequently used probiotics are *Bifidobacterium* and *Lactobacillus* species. Both have been used for the treatment of hepatic disorders showing promising results in the improvement of liver function and the modulation of the inflammation. The benefits of the use of probiotics involve different mechanisms including the competition for the substrate against pathogenic and ammonia-producing bacteria, help to maintain epithelial barrier homeostasis, acidification of the intestinal lumen and stimulation of the immune response (Altamirano-Barrera et al., 2018).

Non-absorbable antibiotics as neomycin and rifaximin are another useful strategy to reduce the ammonia-producing bacteria in the gut. Neomycin has been extensively used in the treatment of HE because it is able to reduce the production of ammonia from glutamine and acts inhibiting glutaminase. However, it shows adverse effects such as ototoxicity and nephrotoxicity. Rifaximin is a synthetic antibiotic derived from rifamycin that acts as a bacteriostatic agent binding to the β subunit of RNA polymerase in aerobic and anaerobic gram-negative and gram-positive bacteria. Rifaximin decreases ammonia in blood, improves neuropsychiatric syndrome and also reduces peripheral inflammation (Miglio et al., 1997; Suraweera et al., 2016). Due to its safety, efficacy and tolerability rifaximin if preferred over neomycin (Suraweera et al., 2016). In rat liver damage models, rifaximin is able to improve motor incoordination, cognitive impairment and alterations in neurotransmission. Rifaximin reduces peripheral inflammation and prevents infiltration of immune cells in the brain, reducing neuroinflammation (Balzano et al., 2021; Leone et al., 2022).

9. Mesenchymal stem cells extracellular vesicles as treatment for neurodegenerative diseases

9.1. Mesenchymal stem cells properties

Mesenchymal stem cells or mesenchymal stromal cells (MSC) are a nonhematopoietic multipotent and heterogeneous population of cells with the potential to differentiate into diverse somatic lineages. They can be found in different human tissues such as bone marrow, adipose tissue, dental pulp, yellow ligament, peripheral blood, and placenta (Andrzejewska et al., 2019). Different MSC isolation techniques and characteristics of these cells have been lately reported, but cells must follow three criteria to be considered MSC. These criteria were proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy in 2006 (Dominici et al., 2006). According to this, MSC must be plastic-adherent in standard culture conditions in tissue culture flasks; the population must be positive for CD105, CD73 and CD90 surface markers and negative for CD45 (pan-leucocyte marker), CD34 (marker of endothelial cells and primitive hematopoietic progenitors), CD14 or CD11b (markers of monocytes and macrophages), CD79a or CD19 (markers of B cells) and HLA (human leukocyte antigen) class II; finally, the cells must have capacity for trilineage mesenchymal differentiation, proving their differentiation to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions (Dominici et al., 2006).

MSC specific properties include immunomodulatory, anti-inflammatory and regenerative effects. These advantages combined to their easy isolation and ex vivo expansion make them a candidate for different therapies. In fact, their potential as therapy for diseases involves different functions including differentiation to various lineages, immune modulation, cytokine secretion and direct cell to cell interaction (Rastegar et al., 2010). Their immunomodulatory properties are one of their main advantages. They have the ability to regulate the function of the main effector cells in the immune responses as PBMCs, neutrophils, mast cells, monocytes, NK cells, lymphocytes and dendritic cells. One of the main mechanisms by which MSC exert their effects is through paracrine factors, that can be referred to as the secretome involved in immunomodulatory function, anti-inflammatory effects and regenerative processes. Secretome composition includes cytokines, chemokines, components of the extracellular matrix, adhesion proteins, enzymes, activators, inhibitors, growth factors and binding proteins (Andrzejewska et al., 2019). For instance, MSC produce several factors related to immunomodulatory effects

such as TFG-β1, hepatocyte growth factor (HGF), IL-10, indoleamine 2,3-dioxygenase (IDO), PGE2, NO, IL-6 and HLA-G5 (Bruno et al., 2015). Due to their properties MSC have been proposed as a therapy for different diseases. The first clinical trial with cultured MSC was carried out in 1995 (Lazarus et al., 1995). Since then, several clinical trials have tested the effects of MSC therapy. The efficacy of MSC as a treatment has been demonstrated for diseases including stroke, acute myocardial ischemia, liver cirrhosis, amyotrophic lateral sclerosis and spinal cord injury (Honmou et al., 2012; Wei et al., 2013; Luger et al., 2017; Liau et al., 2020; Najafi et al., 2023).

Regarding liver injury, patients have been treated with MSC to improve liver cirrhosis. After autologous MSC injection, patients had their liver function improved with no side-effects, demonstrating the safety and efficacy of the treatment for end-stage liver disease (Mohamadnejad et al., 2007; Kharaziha et al., 2009; Wang, Huimin et al., 2023).

In recent years, the paracrine properties of MSC have gained importance due to their ability to secrete EVs including exosomes, microvesicles and apoptotic bodies. Generally, EVs cargo coincides with their cells of origin composition so their biological activity can be compared by the MSC activity itself. Moreover, some studies have demonstrated that MSC effects are exerted by the components of their secretome (including EVs) showing the importance of EVs as a feasible therapy for different diseases (Teixeira et al., 2017; Andrzejewska et al., 2019; Vilaca-Faria et al., 2021).

9.2.Mesenchymal stem cells extracellular vesicles

As mentioned before, EVs cargo composition and properties depend on their cells of origin. For instance, depending on their source, EVs may have pro- or anti-inflammatory effects (Yanez-Mo et al., 2015; Giebel et al., 2017). MSC-EVs have been proved to have similar properties compared to their parent cells as treatment for several diseases. This type of EVs express specific exosomal markers, such as CD9, CD81, CD63, CD107 and the 70 kilodalton heat shock protein (HSP70), but also MSC characteristic surface molecules as CD29, CD44, CD73 and CD105 (Bruno et al., 2015; Tan et al., 2021). Furthermore, MSC-EVs contain proteins associates with self-renewal and differentiation, messenger RNAs (mRNAs) related to control of transcription, cell proliferation, growth factors and immune-regulation; and micro RNA (miRNA) involved in angiogenesis, cellular transport apoptosis and proteolysis (Bruno

et al 2015). EVs derived from adipose tissue MSC have been also tested on stimulated T lymphocytes. An anti-proliferative effect has been observed for CD4+ and CD8+ cells (Blazquez et al., 2014).

Due to their properties MSC-EVs have been proposed as therapeutic approach for neurodegenerative diseases. Laso-García et al (2018) demonstrated that human adipose tissue MSC derived EVs attenuate motor deficits, reduced brain atrophy, increased cell proliferation in the subventricular zone, decreased neuroinflammation and showed immunomodulatory effects in a murine progressive model of multiple sclerosis (Laso-Garcia et al., 2018). This type of EVs also promote white matter integrity restoration in rats with intracerebral haemorrhage (Otero-Ortega et al., 2018) and functional recovery after stroke in rats (Rohden et al., 2021). Human bone marrow mesenchymal stem cells-derived EVs have been shown to improve liver fibrosis (Rong et al., 2019) and to ameliorate Alzheimer's disease alterations (Sha et al., 2021) in rat models through the Wnt/β-catenin pathway. This MSC-EVs also reduce neuroinflammation in rats with focal brain injury (Dabrowska et al., 2019).

MSC-EVs may present some advantages over their parent cells. EVs present less probability to produce pulmonary embolism after the intravenous injection. They have lower immunogenicity and they do not have tumorigenic potential (Ankrum et al., 2014; Liew et al., 2017; Turano et al., 2023). Because of their smaller size they arrive easily to the target tissues and are able to cross the BBB (Turano et al., 2023). They also admit modifications such as enriching them with a beneficial molecule. Because of these reasons they are a potential cell-free therapy (Garcia-Contreras & Thakor, 2021).

9.3.MSC EVs treatment for liver diseases and HE

MSC have been also suggested lately as an effective treatment for hepatic diseases. Several studies have demonstrated that bone marrow derived MSC are able to produce an improvement of hepatic fibrosis in activated HSC, animal models and patients (Jang et al., 2014; Eom et al., 2015). Adipose tissue derived MSC (ASC) are also able to improve hepatic fibrosis in a CCl_4-induced liver failure rat model (Fathy et al., 2020). MSC can potentially differentiate into hepatocytes, moreover, they therapeutic effects also depend on their immunomodulatory effects and the secretion of different factors. MSC regulate the immune response in liver in animal models and patients after tissue damage. They may also inhibit activation and induce

apoptosis of HSC. However, MCSs have fibrogenic potential and can differentiate to myofibroblasts that may act as scar-forming cells in the liver in some specific conditions (Zhang, Z. & Wang, 2013; Eom et al., 2015).

Factors released by MSC such as cytokines, growth factors and EVs can also improve liver damage and peripheral inflammation. EVs regulate pathways related to inflammation, liver fibrosis, integrin-linked protein kinase signaling and apoptosis (Psaraki et al., 2022). Li et al. (2013) showed that injection of MSC-EVs into mice with CCl_4-induced liver fibrosis reduced inflammatory cell infiltration in the liver, hepatocyte apoptosis, and damage in liver lobules (Li, T. et al., 2013). Ohara et al, (2018) demonstrated that ASC-EVs reduce inflammation and fibrogenesis in a rat model of NASH. ASC-EVs decrease the number of KC in the liver of rats with NASH and the mRNA expression levels of inflammatory cytokines such as TNFα, Il-1β and Il-6, and TGF-β. They also decrease fiber accumulation, KC number, and HSC activation in rats with liver fibrosis. In vitro, ASC-EVs inhibit KC and HSC activation (Ohara et al., 2018). EVs from human embryonic stem cells reduce fibrosis and collagen density, necrosis, caspase density, portal vein diameter, and transaminitis in thioacetamide-induced chronic rat liver injury. EVs also produced upregulation of anti-inflammatory cytokines TGF-β1 and IL-10 and down-regulation of fibrosis contributors collagen 1α, α-smooth muscle actin (αSMA), and TIMP metallopeptidase inhibitor 1 (TIMP1), and pro-inflammatory cytokines TNF-α and IL-2 (Mardpour et al., 2018).

Regarding HE, only some studies have analysed the effect of the improvement of liver function by MSC on encephalopathy symptoms. These studies suggest an improvement also of the neurological alterations (Elberry et al., 2016; Liu, P. et al., 2023). Izquierdo-Altarejos et al, (2023) reported reduction of neuroinflammation and improvement of cognitive function in hyperammonemic rats after treatment with MSC-EVs. However, the effect of MSC-EVs on HE due to liver injury has not been deeply studied yet and this is the main objective of this thesis.

HYPOTHESIS AND OBJECTIVES

1. Hypothesis

As detailed before, patients with liver cirrhosis may develop MHE with cognitive and motor impairment being hyperammonemia and peripheral inflammation the main contributing factors for the development of the neurological impairment. Moreover, in patients with chronic hepatic diseases, such as NAFLD, hyperammonemia and peripheral inflammation may induce alterations at cognitive and motor level even without reaching cirrhosis. In rats with mild liver damage, the pro-inflammatory environment in peripheral blood induces infiltration of immune cells in cerebellum and hippocampus which induces neuroinflammation. Neuroinflammation alters glutamatergic and GABAergic neurotransmission in cerebellum and hippocampus leading to cognitive and motor impairment.

Numerous studies show that EVs have a main role in intercellular communication, being related to many inflammatory and immune processes. Many studies point out the therapeutic potential of MSC-EVs, mainly in disorders related to inflammation. Injection of MSC-EVs in hyperammonemic rats, a model of HE without liver failure, reduces neuroinflammation in cerebellum and hippocampus and improves motor coordination and cognitive function. Although the mechanisms by which MSC-EVs exert their functions are not clear, TGF-β seems to have an important role in the EVs mediating its beneficial effects.

Our main hypothesis is that treatment with MSC-EVs may be useful to reduce peripheral inflammation, liver damage, neuroinflammation and cognitive and motor alterations found in rats with mild liver damage induced by CCl_4, which can support the therapeutic use of MSC-EVs for NAFLD and NASH to prevent progression of hepatic and neurologic damage. This hypothesis could be divided in:

1. Treatment with MSC-EVs may normalize cognitive and motor alterations observed in rats with mild liver damage.
2. Treatment with MSC-EVs may reduce neuroinflammation in cerebellum and hippocampus and normalize the alterations in glutamatergic and GABAergic neurotransmission observed in rats with mild liver damage.

3. Treatment with MSC-EVs may reduce peripheral inflammation and hepatic injury produced by CCl_4-induced mild liver damage in rats.
4. Effects of MSC-EVs in mild liver damage, peripheral inflammation, neuroinflammation in cerebellum and hippocampus and cognitive and motor impairment may be dependent on their content of TGF-β

2. Objectives

To assess this hypothesis, we have the following objectives:

1. To assess if treatment with MSC-EVs may reduce the alterations observed in rats with mild liver damage in cognitive and motor functions, neuroinflammation and glutamatergic and GABAergic neurotransmission in cerebellum and hippocampus, peripheral inflammation and liver structure and inflammation.

2. To analyse if TGF-β has an important role in the effects that MSC-EVs may have in the alterations produced by mild liver damage in rats.

3. To expand the knowledge about the underlying mechanisms by which MSC-EVs exert their therapeutic effect on mild liver damage in rats.

MATERIALS AND METHODS

1. Animal Model

These experiments were approved by the Ethic Committee of Animal Experimentation (*Comité de Experimentación y Bienestar Animal*) of *Centro de Investigación Príncipe Felipe* (Valencia, Spain) and the *Conselleria de Agricultura de la Generalitat Valenciana* (Valencian Community, Spain) with code 2020/VSC/PEA/0042 and they were performed in accordance with the guidelines of the Directive of the European Commission (2010/63/EU) for the care and handling of experimental animals. All experiments were performed according the ARRIBE guideline for animal experimentation.

For all the experiments, male Wistar rats (Charles River) with weight 150-180 g were used. Rats were allocated in standard conditions of temperature (22ºC) and humidity (40-60%) and light-dark cycle of 12:12 h, with standard diet and food and drink *ad libutum.*

To induce mild liver damage, half of the rats were intraperitoneally injected 3 times/week during 4 weeks with 1 mL/kg body weight of CCl_4. CCl_4 was prepared 1:10 (v:v) in corn oil as previously described (Lee et al., 2005; Balzano et al., 2021; Leone et al., 2022). Control rats were intraperitoneally injected with 1 mL/kg of corn oil.

2. Treatment with Mesenchymal stem cells derived extracellular vesicles

Rats were treated with MSC-EVs. ASC obtained from subcutaneous fat were used (HistoCell Ltd, Spain). Cells were cultured and expanded in growth medium (DMEM (Dulbecco's Modified Eagle Medium) medium enriched in glucose and supplemented with 20% fetal bovine serum (FBS), 100 u/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine). FBS was previously centrifuged at 100,000 g for 1 hour and filtered with 0.2 µm filters for EVs depletion. EVs were isolated from the sub-confluent culture media after 48 h of incubation through several centrifugations, as detailed in (Castro et al., 2019) The medium was collected and cell debris was removed by two centrifugations for 10 min at 300 g and 2000 g, respectively. The supernatant was centrifuged at 10000 g for 30 min to remove apoptotic bodies. Subsequently, the supernatant was centrifuged at 100000 g for 1 h and the pellet from this centrifugation was resuspended in phosphate-buffered saline (PBS) and centrifuged again

at 100000 g for 1 h to wash the vesicles. All centrifugations were performed at 4°C. The final pellet was resuspended in 100 µL of sterile PBS, the amount of protein was determined by colorimetric assay with bicinchoninic acid (BCA) and the EVs were frozen at -80°C.

To assess the role of TGF-β, two different types of EVs were obtained. EVS from control ASC (C-EVs) and EVs from ASC with silenced TGF-β gene (T-EVs). For silencing, cells were transfected with a lentiviral construct containing a vector pLL3.7 (plasmid #11795, Addgene) with the sequence of a silencing RNA that binds to the TGF-β transcript and prevents its translation into protein.

EVs were administered twice (at 2 and 3 weeks of CCl_4 treatment) as an intravenous injection at 50 µg per 300 µL of PBS. Vehicle rats were administrated with PBS. Experimental procedure planning can be observed in Figure 9.

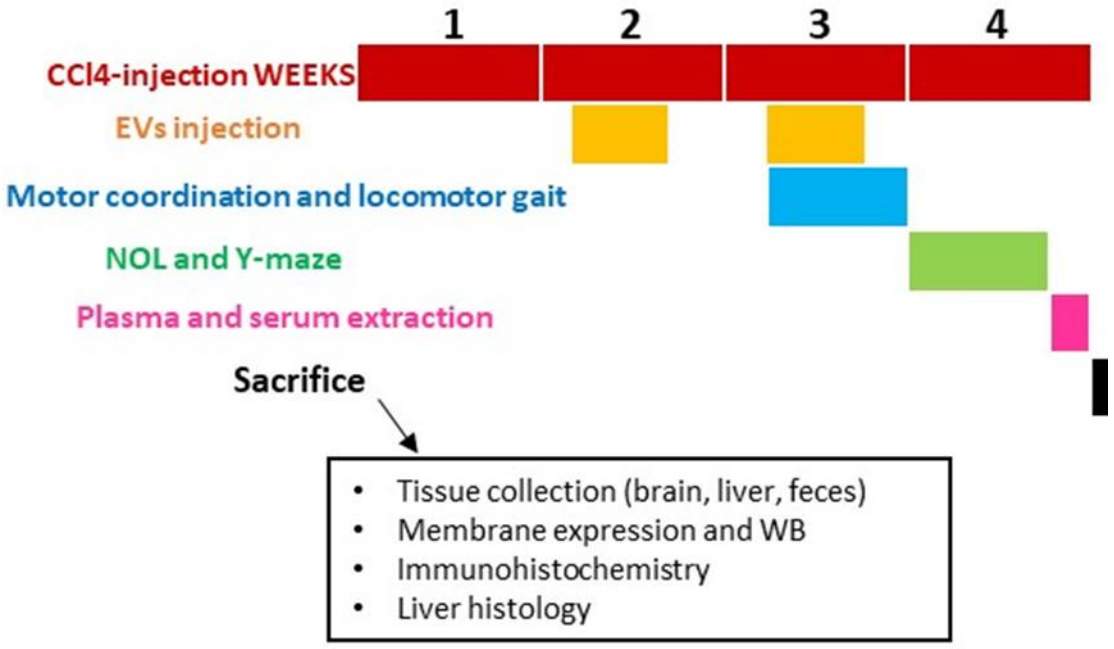

Figure 9. Experimental procedure

Rats were divided into six groups (n=32-38 rats per group; Table 1): Control-PBS (CV); CCl_4-induced liver damage-PBS (CC); Control-treated with C-EVs (CE); Control treated with T-EVs (CE-T); CCl_4-with C-EVs (CCE) and CCl_4-with T-EVs (CCE-T).

Table 1. *Experimental groups depending on the model and the treatment with EVs. Colours and group names are kept the same in all the document*

EXPERIMENTAL GROUP	CV	CE	CE-T	CC	CCE	CCE-T
INTRA-PERITONEAL INJECTION (Model)	Corn oil	Corn oil	Corn oil	CCl_4	CCl_4	CCl_4
INTRA-VENOUS INJECTION (Treatment)	PBS	C-EVs	T-EVs	PBS	C-EVs	T-EVs

3. Behavioural tests

Behavioural tests were performed after 3-4 weeks of CCl_4 administration.

3.1. Foot analysis of locomotor gait in the CatWalk

CatWalk XT is a video-based system for automatic gait analysis (Noldus, Wageningen, The Netherlands). The system consists of an enclosed walkway on a glass plate that animals have to cross from side to side (each cross is called a run) until reaching a dark box that connects to their housing cage, where they feel secure (Figure 10). The glass has a green light internally reflected that can only escape and be detected in the areas where the animal is touching it. When a part of the animal is in contact with the glass a digital image is created where the brightness of the pixel depends on the amount of light received and enables to detect differences of intensity. The software allows to discriminate between paws and other parts of the body and it is able to detect each different paw (front or hind and left or right). Three trials (runs) were recorded each day during three days. Gait analysis values are the mean of nine runs. Data were analysed using the CatWalk^{TM} analysis software (v 7.1). The parameters that were analysed were: Dual Stance, stride length, stand index, step cycle and regularity index. Dual Stance refers to the duration in seconds of ground contact with both hind paws simultaneously, being differentiated between the Initial Dual Stance (first time in the step cycle that there is a simultaneous contact of both hind paws) and the Terminal Dual Stance (second time in the step cycle). Stand index is a measure of the speed (calculated by the software) at which the paw

loses contact with the glass plate. The Step Cycle is the time in seconds between two consecutive contacts with the same paw, it is the time of stand and swing (paw in the air) of the same paw. These parameters detect akinesia and dyskinesia. Stride length is defined as the distance between successive placement of the same paw. Lastly, the Regularity Index represents the percentage of normal step sequence patterns relative to the total number of paw placements. Regularity Index can be used as measure of inter-paw coordination, in healthy animals its value is usually 100%.

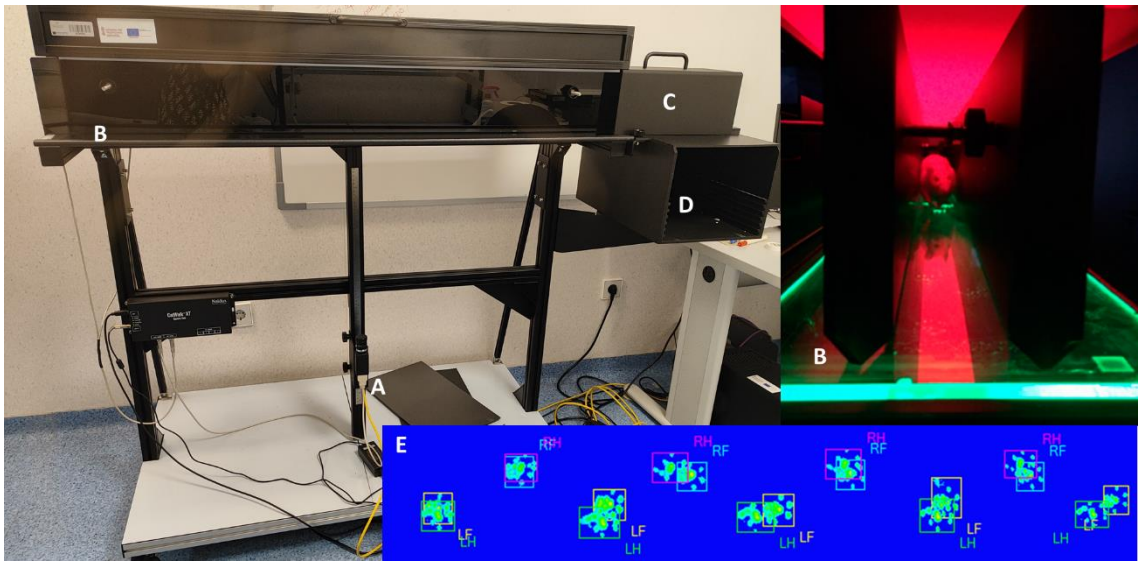

Figure 10. CatWalk XT apparatus and its components*: camera (A), glass plate (B), dark box (C), place for animal's cage (D) and analysis of paw prints in the CatWalk software (E)*

3.2. MotoRater

A kinematic analysis of motor coordination was conducted using MotoRater apparatus (TSE Systems, Germany) as in (Zorner et al., 2010). The system consists of a horizontal ladder that the animals have to cross (each time called a run) until reaching their own cage, while being recorder from three different angles (Figure 11). Each day four uninterrupted runs were recorded for each rat, over two days. The runs were analysed by counting and classifying the steps as correct or wrong paw placements (slips or misses). The results are expressed as percentage of total steps and are the mean of eight runs.

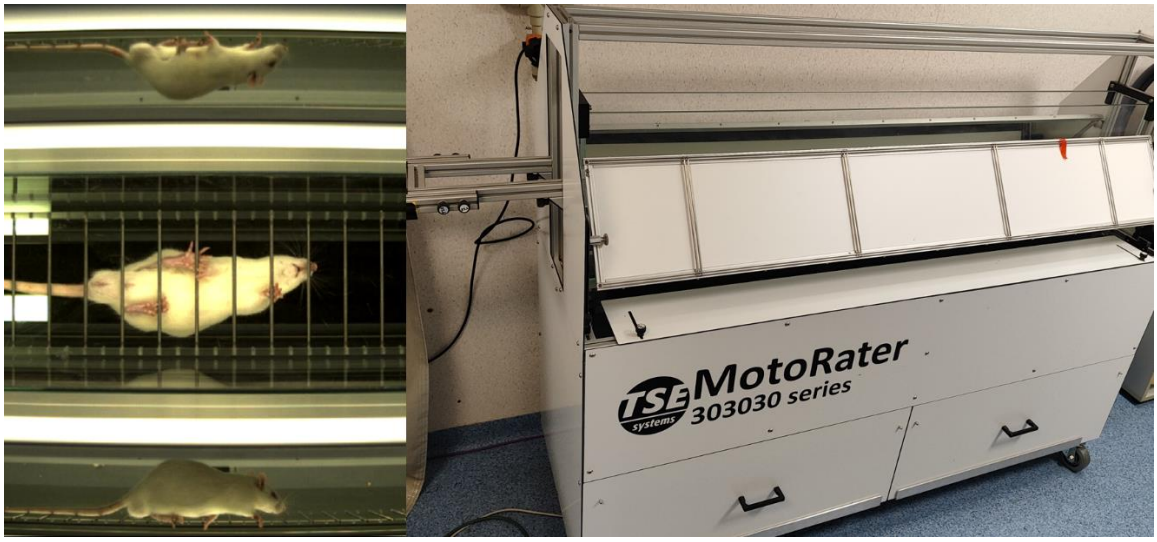

Figure 11. MotoRater system *(right) and example of view for quantification (left).*

3.3. Beam walking

The beam walking test was performed as in (Gonzalez-Usano et al., 2014).In this test, the animals have to cross a beam consisting of a wood strip of 20 mm in diameter and 1 m long, located 1 m above the ground and, reach a dark secure place (Figure 12). Rats were habituated to the beam the day before the test by training them to cross the beam and allowing them to stay in the dark box for 2 minutes. Each animal had to cross the beam three times, and the number of slips were counted. The test was repeated three times and the average of the three results was considered a measure of incoordination.

Figure 12. Beam walking

3.4. Rotarod

The rotarod (Ugo Basile, Comerio, Italy) assesses the ability of rats to stay on a rotating drum with progressive acceleration. It consists of a cylinder of 5 cm of diameter and 8.5 cm long (Figure 13). Before testing, each rat was placed on the rotarod at constant speed for 3 min for habituation. During the test, the rotarod speed was increased from 4 to 40 rpm over 300 s. Two trials were performed. Motor incoordination was calculated as the mean latency to fall with a maximum cut-off of 300 s.

Figure 13. Rotarod

3.5. Novel Object Location test

Location memory assessment in rats is based in their ability to remember a location stimulus that they have been exposed to previously, being able to differentiate if it is placed in a new position. The novel object location memory (NOL) was assessed. Rats were habituated in a wooden black box (70x70x40 cm) with visual clues in the walls. They were allowed to explore the box freely for 5 minutes during 5 days. On the sixth day the NOL test was performed. It consisted of two phases: a pre-test in which two identical objects were placed in the box and a test (2 hours after the pre-test) in which one of the objects was moved to a new location (Figure 14A). The objects were built with wooden pieces and painted in the same colour. Positions of the objects were changed between animals to avoid possible bias from external stimuli. In each phase, the animals were allowed to explore freely. The tests were quantified with the ANY-maze video tracking system (Stoelting Europe, Ireland) (Figure 14B). The time that the animal spent exploring each object was quantified (old vs new position).

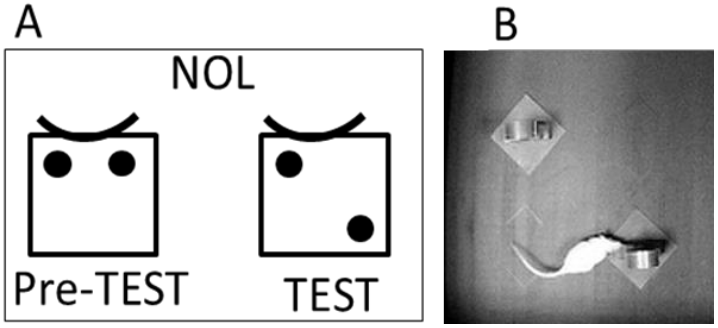

Figure 14. Possible configuration for NOL (A) and image of the box during the test (B). *The left box represents the configuration for the pre-test and the right box represents the test for the NOL (A).*

The discrimination ratio was calculated as the difference between the time spent with the object in the new location and the time spent with the object in the old location divided by the total time of exploration (Equation 2).

$$Discrimination\ ratio = \frac{time\ (new\ location) - time\ (old\ location)}{total\ exploration\ time} \qquad \textbf{Eq. 2}$$

3.6. Radial maze

The Radial maze was used to quantify spatial learning and working memory. As we previously found very mild affectation in this test in rats with mild liver damage, this test was performed during the eighth week of CCl_4 treatment, when rats injected with CCl_4, with high grade of hepatic steatohepatitis, show impaired learning and memory in this test. EVs treatment was continued by injection at five and seven weeks of CCl_4 administration. The maze consists of a black structure with a central area of 30 cm of diameter that connects eight arms of 70 cm long and 10 cm wide. Each arm has walls of 30 cm high in the central area and 5 cm high at the end. At the end of each arm there is a place for the placement of food rewards (Figure 15).

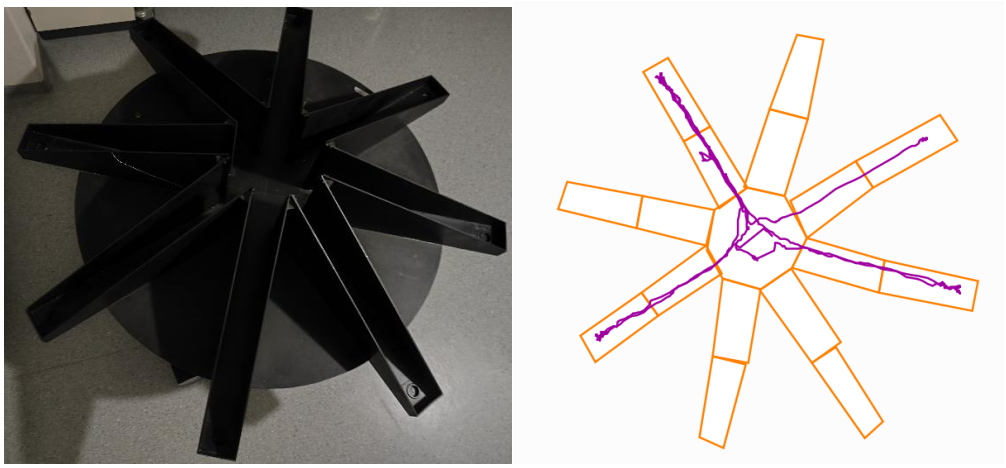

Figure 15. Radial Maze *(left) and an example of the animal's track in the Any-Maze software (right)*

Four days before the beginnig of the habituation, rats have their food restricted. This restriction is maintained until the end of the test. Rats weight is controlled daily during the test and a loss of more than 20% of weight in not allowed. For food reward, Dustless Precision Pellets® (Rodent Purified Diet, Bio-Serv) were used. Habituation is performed the day before the first day of tests. It consists of two sessions. In the first session, pellets were placed randomly over the whole maze and rats were allowed to explore the maze by pairs for 5 min. In the second session, food is only placed in its specific place (2 pellets per arm) at the end of the arms and rats are allowed to explore the maze individually for 5 min. The test was performed starting the following day for 4 days with 3 trials per day. For each trial, food is placed in four of the arms, two consecutive and two not consecutive (Figure 16). This configuration is kept for each animal for all the experiment but is changed between animals. Rats are able to learn this configuration and remember it throughout the days, using their spatial orientation and the spatial clues around the maze. The entrances to the arms was registered, considering an entrance if the animal passes half of the arm. Reference spatial errors were calculated as the number of times that the rat entered an arm without food. Working memory errors were quantified as the number of times that the rat visited an arm that it visited previously during the same trial. Learning errors were considered the number of times that the animal entered an arm without food for the first time. A spatial learning index was calculated as the difference between the correct choices and the learning errors.

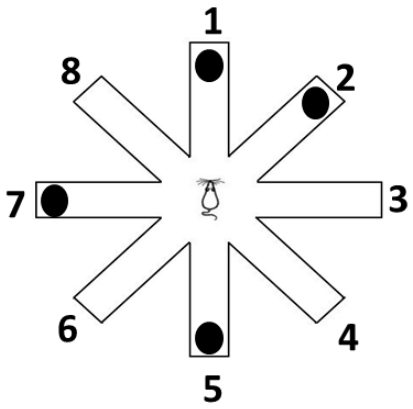

Figure 16. Example configuration *of the arms with food (black circle) in two consecutive arms (1 and 2) and two non-consecutive arms (5 and 7).*

3.7. Short-term spatial recognition memory

Short-term spatial recognition memory was measured in a Y-maze, a maze with two short arms and a longer arm (Figure 17). Each rat was placed into the long arm (start arm) and allowed to explore the maze with one of the short arms closed (new arm), for 2 min (training trial). This was repeated with 1 min of inter-trial interval. Then, after 1 min, the rat was placed in the maze for a third time but with all the arms opened for 2 min (test trial).

Figure 17. Y maze

The number of entries into and the time spent in each arm were registered during the test trial. The discrimination ratio was calculated (Equation 3).

$$Discrimination\ ratio = \frac{time\ (new\ arm) - time\ (old\ arm)}{total\ time\ in\ short\ arms} \qquad \textbf{Eq.3}$$

4. Analysis of liver damage markers in serum

Serum was extracted from the saphenous vein in serum specific blood collection tubes (BD Microtainer SST). Samples were sent to be analysed in *Laboratorio Dr. F. Echevarne, Analisis, S.A.* Liver damage markers GPT/ALT, GOT/AST, alkaline phosphatase, bilirubin and bile acids were analysed.

5. Analysis of protein content in plasma, liver, cerebellum and hippocampus

Plasma was obtained from the saphenous vein in ethylenediaminetetraacetic acid (EDTA) vials (BD Microtainer K2E tubes) and stored at -80 ºC. TNF-α and TGF-β in plasma were measured by ELISA kits (Invitrogen 88-7340-22; 88-50680) All other cytokines in plasma were analysed by Western blot. Total protein content in plasma samples was determined by BCA method (Pierce). Seventy-five μg of protein were loaded in 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and posterior immunoblotting was performed.

For tissue collection, rats were euthanized by decapitation. Liver sections, hippocampus and cerebellum were carefully extracted and stored at -80ºC. Tissues were homogenized in 5 (hippocampus) or 8 (cerebellum and liver) volumes of homogenate buffer (50 mM tris(hydroxymethyl)aminomethane-HCl (TRIS–HCl) pH 7.5, 50 mM NaCl, 10 mM ethylene glycol-bis(β-aminoethyl ether) (EGTA), 5 mM EDTA and protease and phosphatase inhibitors) for analysis by Western blot. Total protein content was determined by BCA method. Twenty-five μg of protein were loaded in 15 or 10% SDS-PAGE gels.

Proteins were separated by size with the discontinuous electrophoretic system SDS-PAGE with two differentiated gels, the stacking (25% Tris 0.5 M + 0.4% SDS pH=6.8 adjusted with HCl, 13% Acrylamide/Bisacrylamide (A0385, Panreac), 1% ammonium persulfate (APS) (c7727-54-0, Merck-Millipore) and 0.2% tetramethylethylenediamine (TEMED) (T9281, Sigma)) and the separating (25% Tris 1.5M + 0.4% SDS pH=8.8, 20% Acrylamide/Bisacrylamide, 0.33% APS y 0.07% TEMED) although, acrylamide concentration in the separating gel was adjusted in each case depending on the size of the protein as it determines the size of the pore in the gel and thus the distance that the proteins are able to travel. Loading volume of each sample was calculated with the protein content in order to load the same total quantity of protein of all the samples. Samples were diluted with loading buffer 2X (65% Tris-HCl 0.5 M pH=6.8, 21% glycerol, 4% SDS, 10% 2-Mercaptoethanol, 0.02% bromophenol blue) and they were boiled for 5 min at 100ºC. Electrophoresis was run with running buffer (Tris Base 25 mM, SDS 3.5 mM, Glycine 0.2 M) at 25 mA for 1.5 h. After that, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane with transfer buffer (Tris Base 25 mM, glycine 0.19 M, 20% methanol) at 250 mA for 2h at 4ºC. Membranes were blocked with 5% bovin serum albumin (BSA) in Tris-buffered saline-Tween (TBS-T) buffer (NaCl 150 mM, Tris Base 50 mM, 0.1%, Tween-20 pH 7.5) for 45-60 min. Then, the membranes were incubated with the primary antibody for each specific protein (Table 2) diluted to the indicated concentration in 5% BSA solution overnight at 4ºC. Membranes were washes (3 times 10 min with TBS-T) and incubated with the secondary antibody (1:4000) that are IgGs conjugated with alkaline phosphatase (Sigma, Madrid, Spain) for 1h at room temperature. After TBS-T washing, membranes were washed with substrate buffer (NaCl 100 mM, MgCl2 5 mM, Tris Base 100 mM pH=9.5). The revealing solution was prepared with 0.66% de NBT (4-Nitro blue tetrazolium chloride) and 0.34% de BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine) in substrate buffer, as colorimetric substrate for alkaline phosphatase. Revealing time depended on each protein, for all of them the reaction was stopped before saturation introducing the membranes in distilled water. Once dry, the images were captured using an Epson Perfection V39 scanner and band intensities were quantified using AlphaImager 2200 software (Cambridge, UK). Intensity of each band was quantified. β-Actin (Abcam, 1:5000) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Millipore, 1:15,000) were used as loading controls. Results were expressed as percentage of the average of control samples run in the same gel.

Table 2. *Primary antibodies used for analysis of protein content by immunoblot*

Primary antibody against:	**Dilution**	**Commercial brand and reference**	**Tissue of application**
GAD65	1:1000	Abcam ab26113	Brain
GAD67	1:500	Abcam ab26116	Brain
TNF-α	1:250	RD Systems AF-510-NA	Brain, Liver
Glutaminase I	1:1000	Novus NBP-1 76544	Brain
GAT-3	1:500	Abcam ab431	Brain
CCL2	1:2000	Proteintech 66272-1-lg	Brain
BDNF	1:1000	Invitrogen OSB00017W	Brain
TrkB	1:500	Abcam ab18987	Brain
YM-1(chitinase-like protein 3)	1:500	Abcam ab93934	Brain
IL-6	1:500	Biosource ARC0062	Plasma, Liver
IL-15	1:2000	BIOSS bs-1829R	Plasma
HMGB1	1:1000	Abcam ab18256	Plasma
IL-17	1:1000	Abcam ab79056	Plasma, Liver
IL-21	1:500	Cloud-clone PAB688Ra01	Plasma, Liver
IL-4	1:1000	Abcam ab9811	Plasma
Collagen I	1:1000	Novus NB600-408	Liver
α-SMA	1:500	Invitrogen MA5-11547	Liver
TGF-β	1:1000	Invitrogen PA5-99186	Liver

5.1. Protein expression on the cell membrane surface

Membrane surface expression of receptors and transporters in hippocampus and cerebellum was analysed as described in (Boudreau et al., 2012). Rats were euthanized by decapitation and each dissected cerebellum or hippocampus was put into ice-cold Krebs buffer (in mmol/L): NaCl 119, KCl 2.5, KH_2PO_4 1, $NaHCO_3$ 26.2, $CaCl_2$ 2.5, and glucose 11, aerated with 95% O_2 and 5% CO_2 at pH 7.4. Transversal 400 μm thick slices were obtained with a chopper (hippocampus) or a vibratome (cerebellum). Slices were added to tubes containing ice-cold Krebs buffer with or without 2 mM bis(sulfosuccinimidyl)suberate (BS3) (Pierce, Rockford,

IL, USA) and incubated for 30 min at 4 °C. Cross-linking was terminated by adding 100 mM glycine (10 min, 4 °C). The slices were homogenized by sonication for 20 s. Protein content of samples treated with or without BS3 was determined by BCA and the same amount of total protein of both type of samples was separated in the same SDS-electrophoresis gel to further perform immunoblot with antibodies shown in Table 3 and as described before. The surface expression of receptor subunits was calculated as the difference between the intensity of the bands without BS3 (total protein) and with BS3 (non-membrane protein) (Krasnow et al., 2017).

Table 3. *Primary antibodies used for membrane expression analysis by immunoblot*

Primary antibody against:	**Dilution**	**Commercial brand and reference**	**Tissue of application**
GluN1	1:1000	Cell signaling 5704S	Hippocampus
GluN2A	1:1000	Millipore 04-901	Hippocampus
GluN2B	1:1000	Millipore 06-600	Hippocampus
GluA1	1:1000	Millipore 04-855	Hippocampus
GluA2	1:2000	Millipore ab1768-I	Hippocampus
$GABA_A$-α1	1:1000	Abcam ab8341-50	Hippocampus
$GABA_A$-α2	1:1000	Bioss BS-12061R	Hippocampus
$GABA_A$-α5	1:1000	Genetex GTX31004	Hippocampus
$GABA_A$-α6	1:1000	Thermofisher OPA1-04114	Cerebellum
$GABA_A$-β3	1:1000	Abcam ab98968	Hippocampus
$GABA_A$-δ	1:1000	Novus NB 300-200	Cerebellum
$GABA_A$-γ2	1:500	Abcam ab16213	Hippocampus
KCC2	1:1000	Millipore 07-432	Hippocampus
NKCC1	1:1000	Abcam ab59791	Hippocampus
GAT-1	1:500	Abcam ab426	Cerebellum
GAT-3	1:500	Abcam ab431	Cerebellum
P2X4	1:2000	Invitrogen PA5-41080	Cerebellum

6. Histological staining and immunohistochemistry

For obtention of tissue sections for immunohistochemistry and histological staining, rats were anaesthetized with sodium pentobarbital (1 mL/kg body weight) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains and livers were removed and post-fixed in the same fixative solution for 24 h at 4 °C (for brains) or for 48h (for livers). Five-micrometer thick, paraffin-embedded sections (5 µm) were cut in a microtome and mounted on coated slide glasses.

6.1. Immunohistochemistry

Sections were deparaffinized for 1 hour at 62ºC, rehydrated and antigen retrieved (Tris-EDTA buffer pH=9 or sodium citrate buffer pH=6, depending on antibody brand recommendation). After that, sections were incubated with 3% H_2O_2 for 15 min to block endogenous peroxidases, and with 5% normal goat serum (NGS) in PTA (PBS-Triton-albumin buffer 0.1 M PBS with 0.1% BSA and 0.1% Triton-X100) for 1 h to block unspecific binding. Sections were incubated with primary antibodies diluted in PTA with 1:100 NGS overnight at 4°C. Primary antibodies used for the study and are detailed in Table 4. Sections were washed and incubated with biotinylated secondary antibodies goat anti-rabbit and goat anti-mouse (Vector Laboratories, 1:200) for 1 h at room temperature. After that, they were incubated with Avidin Biotin Complex (ABC) (Vector Laboratories) for 30 min, which contains avidin and biotinylated horse-raddish peroxidase (HRP). Finally, diaminobenzidine (DAB, colorimetric substrate for HRP) was added for a maximum of 10 min. Mayer's hematoxylin (DAKO S3309; Ready to use) for 1 min was used to counterstain nuclei. Slides were then dehydrated and mounted in an AutoStainer (Leica).

***Table 4**. Antibodies and dilutions used for immunohistochemistry*

Primary antibody against:	Dilution	Antigen Retrieval Buffer	Commercial brand and reference	Tissue of application
IBA1	1:300	High	Wako 019-19741 (RB)	Brain
GFAP	1:400	High	Sigma G3893 (MS)	Brain
IL-1β	1:300	Low	Abcam ab9722 (RB)	Brain
GAD65	1:200	High	Abcam ab26113 (MS)	Brain
GAD67	1:1000	High	Abcam ab26116 (MS)	Brain
GABA	1:200	High	Millipore ABN131 (RB)	Brain
Collagen I	1:200	Low	Novus NB600-48 (RB)	Liver
α-SMA	1:100	High	Invitrogen MA5-11547 (MS)	Liver

6.2. Histology

Liver damage grade was determined by histopathological analysis in hematoxylin and eosin (H&E) and Masson trichrome liver stains. The H&E staining was performed with an AutoStainer (Leica) with the following steps: hydration, Harris hematoxylin (5 min), water wash, bluing (1 min), water wash, 70% ethanol + acetic acid (2 min), eosin Y (1,5 min) and dehydration. Masson's trichrome stain carried out the laboratory of *Universidad Católica de Valencia.* Liver damage was graded using a scoring system as in (Kleiner et al., 2005) The histological features considered were steatosis (0–3), lobular inflammation (0–3), and fibrosis grade (0–3).

6.3. Analysis of Astrocytes and Microglia Activation

Sections stained with Iba1(ionized calcium-binding adapter molecule 1) and GFAP were scanned with Aperio Versa system (Leica Biosystems) and images were taken with ImageScope software (Leica Biosystems).

Iba1 was used to assess microglial activation in the cerebellum and the hippocampus. Iba1 marker stains macrophages and microglia cytoplasm. Microglia cells show a ramified structure

when they are not activated and a rounder and ameboid like shape with less ramifications when they are activated. The measure of the perimeter was used as a measure of activation meaning a smaller perimeter more activation. Images were taken in all the hippocampus and white matter of cerebellum (10 random fields per animal at 40X). The perimeter of individual $Iba1^+$ cells was measured with ImageProPlus software. The results were expressed as the mean perimeter of microglia per animal.

GFAP marker stains cytoplasm of astrocytes. When activated, astrocytes show a more ramified structure. Images were taken in hippocampus and white matter of cerebellum (10 random fields per animal at 40X). Astrocytic activation was measured as percentage of GFAP stained area with Image J software.

6.4. Analysis of IL-1β in hippocampus

In the hippocampus, the number of IL-1β positive cells were manually counted in 10 random fields of the whole area (20X) per animal. The results were expressed as number of positive cells per mm^2 as percentage of the control group. Besides, the intensity of staining in the CA1 area was measured with the image J ROI tool selecting specifically the area. The mean intensity of the background was used as a blank. The results were expressed as the mean intensity of the images per animal.

6.5. Analysis of GABA, GAD67 and GAD65 in Purkinje neurons

The content of GABA, GAD65 and GAD67 in Purkinje neurons was quantified by staining intensity in 10 fields 20X per rat with the Image J ROI tool. The blank (mean intensity of background) was subtracted to the mean intensity of the Purkinje neurons. Results were expressed as mean intensity of the images per rat.

6.6. Analysis of Collagen I and α-SMA in liver sections

Collagen I and α-SMA in liver sections were measured as % of stained area in the Image J. These analyses were performed with the Color threshold tool selecting the specific staining in each case and processing the images with a macro.

7. Statistical Analysis

GraphPad Prism software v. 9.5.0 was used for statistical analysis and graph drawing. Data are expressed as mean ± SEM. Statistical analysis was carried out using one-way ANOVA of four groups. Groups were analysed four by four to analyse the effects of each type of MSC-EVs separately. To analyse the effect of C-EVs groups CV, CE, CC and CCE were included, while the effects of T-EVs were analysed with the groups CV, CE-T, CC and CCE-T. For multiple comparisons Tukey's test and uncorrected Fisher's LSD test were used. Two-way ANOVA with repeated measures and Bonferroni's multiple comparisons test were used when appropriate. Data that did not pass the normality test (D'Agostino and Pearson or Kolmogorov–Smirnov tests) were analysed with the nonparametric Kruskal–Wallis test, followed by Dunn's test (or uncorrected Dunn's test) for multiple comparisons. When standard deviations (SDs) were not equal, Welch's ANOVA was applied followed by Dunnett's T3 multiple comparisons test or unpaired t tests with Welch's correction. A confidence level of 95% was considered as significant.

8. Analysis of fecal microbiota

8.1. DNA extraction and amplicon-based library sequencing

Rat feces were extracted after the sacrifice and immediately frozen at -80ºC until being processed. The DNA from rat feces was extracted using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with prior treatment on FastPrep-24™ 5G bead beating grinder (MP Biomedicals, Santa Ana, CA, USA) using InhibitEX Buffer (kit extraction reagent) and 0.1 mm diameter zirconium/silica beads. The DNA (deoxyribonucleic acid) concentration was measured by Qubit 4.0 and the Qubit double strand DNA (dsDNA) HS Assay Kit (Thermo Fisher Scientific, USA). The V3-V4 hypervariable regions of the 16S ribosomal RNA (rRNA) gene were amplified using ~20 ng DNA and 25 PCR cycles consisting of the following steps: 95 ºC for 20 sec., 55 ºC for 20 sec. and 72 ºC for 20 sec. NZYProof DNA polymerase (NZYTech, Lisbon, Portugal) and the 7-mer barcoded primers, S-D-Bact-0341-b-S-17 (TAGCCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (ACTGACTACHVGGGTATCTAATCC) targeting a wide diversity of bacterial

16S rRNA genes (Klindworth et al., 2013) were used for polymerase chain reaction (PCR). Dual-barcoded PCR products were purified from duplicate reactions with the NZYGelpure purification Kit (NZYTech, Lisbon, Portugal) and quantified through Qubit 4.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). The samples were multiplexed by combining equimolar quantities of V3-V4 amplicons (~40 ng per sample) and sequenced in two lanes of the Illumina MiSeq platform with 2x300 PE configuration (CNAG, Barcelona, Spain).

8.2 Amplicon data analysis

Raw data was delivered in fastq files and pair ends with quality filtering were assembled using Flash software (Magoc & Salzberg, 2011). Assessment of alpha and beta diversity analyses was performed using the Operational Taxonomic Unit (OTU)-picking approach as follows. Sample de-multiplexing was completed with sequence information from barcoded forward/reverse primers and the SeqKit tool (Shen et al., 2016). After de-multiplexing and barcodes/primers removal, the chimera detection was performed with UCHIME algorithm (Edgar et al., 2011) and the SILVA reference set of 16S rRNA sequences (Release 138) (Quast et al., 2013). The OTU information was retrieved by using the UCLUST algorithm implemented in USEARCH v8.0.1623 (Edgar, 2010). The alpha diversity descriptors such as Chao's index, evenness, Shannon index, Simpson's reciprocal index, Dominance index, and phylogenetic distance (PD) were computed using QIIME v1.9.1 (Caporaso et al., 2010) from a subset of 15,000 sequences randomly selected after shuffling (10,000X). For phylogenetic-based metrics the OTUs sequences were aligned with PyNAST algorithm and the SILVA aligned reference database. Tree topology reconstruction was retrieved with the FastTree algorithm (Price et al., 2009) using the generalized time-reversible (GTR) model and gamma-based likelihood. The community structure across the sample groups was performed with the Vegan R package through interpretative multivariate appraisal based on non-metric multidimensional scaling method - NMDS (vegan::metaMDS function and "bray" distance).

For taxonomy assessment, the complete set of sequences obtained after chimera removal were used. Following the OUT-picking approach (UCLUST algorithm), the transformation of compositional microbiota-derived data was achieved by applying the centered log-ratio (clr) algorithm implemented in CoDaSeq::codaSeq.clr with prior execution of zCompositions:: cmultRepl function supporting a Bayesian-Multiplicative replacement of count zeros.

Taxonomy identification for OTUs of interest was assisted by utilization of SINA aligner (Pruesse et al., 2012) and SILVA database (Release 138).

Generalized- or linear mixed models (GLMM/LMM) allowing covariate control (e.g. weight-gain, sequencing pool library) was used to test differences in treatment groups when compared to respective controls across the alpha diversity descriptors, with prior evaluation of normal distribution of variables (Shapiro-Wilk test). Differences in the microbial community structure were assessed by interpretative approaches using the permutation-based vegan::adonis2 function. Differential abundance of OTUs across groups was also assisted by application of GLMM or LMM with covariate control. Additionally, pairwise Wilcoxon Rank Sum test with Benjamini-Hochberg post hoc correction was also completed for those categories detected to have meaningful differences in distribution across the groups. Highly divergent OTUs in terms of abundance, and associated with EVs treatment, were selected by applying estimation statistics relying on size-effect (dabestr::dabest R function). Correlation analysis to assess the association between microbiota and behavior variables was completed by computing Kendall's ρ (rho) parameter (stats::cor.test R function). Graphs and plots were drawn using ggplot2 R package (R distribution V4.1.2.)

RESULTS

1. Effects of mesenchymal stem cells derived extracellular vesicles on spatial memory and learning in rats with mild liver damage

Spatial learning was analysed in the radial maze. The calculated learning index showed a significative effect of time ($p<0.0001$) indicating learning of the test along days, but there was not any significant difference between groups. However, a significative interaction between both factors was observed ($p=0.0099$), meaning that there were significative differences between groups only at some specific times (Figure 18A). Significant differences between groups were found at day 4. Rats with mild liver damage showed a decreased learning index at this time (CC 1.94±0.27; $p<0.05$) compared with the control group (CV 4.18±0.87). C-EVs did not restore this alteration in rats with mild liver damage (CCE 1.48±0.15; $p<0.01$ compared with CV,) but even worsened learning index at day 4 in the control rats (CE 1.46±0.16, $p<0.05$). Rats with mild liver damage treated with T-EVs showed a partial improvement in learning index at day 4 (CCE-T 3.04±0.79), although difference with CC group do not reached significance.

CCl_4 treated rats also showed impaired working memory, measured as total working errors (four days) in the radial maze. Rats with mild liver damage showed more working errors (22.0±4.1) compared with the control group (12.5±1.9; $p=0.0392$). As in the learning index, treatment with C-EVs did not restore this alteration (CCE 23.3±2.8; $p=0.0067$ compared with CV) and C-EVs treatment in control rats increased total working errors (CE 22.3±2.2 errors, $p=0.0167$). However, CCl_4 rats treated with T-EVs (CCE-T 16.7±1.5) had fewer working errors than CCl_4 rats without treatment, although the difference was not significant (Figure 18B).

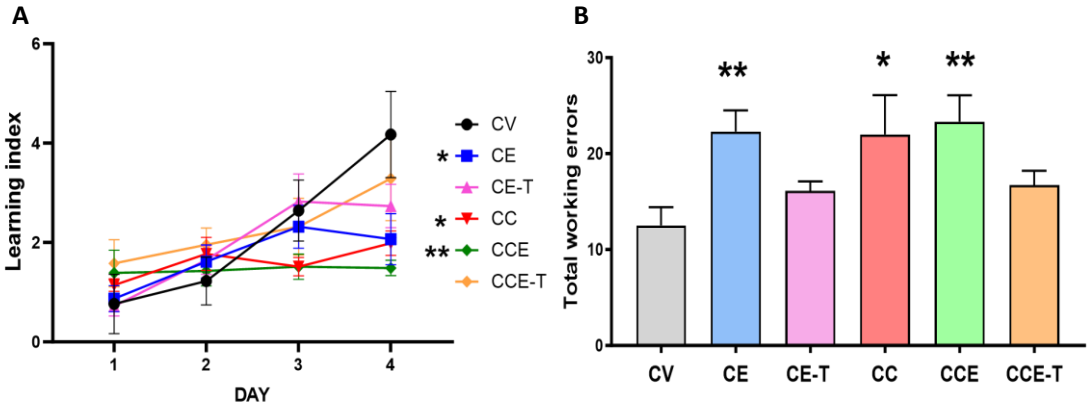

Figure 18. Effects of MSCs-EVs on spatial learning and working memory. *Learning index (A) and Total working errors (B) were measured in the radial maze at 8 weeks of CCl_4 treatment. Values are expressed as mean ± SEM of 7-12 rats per group. (A) Learning index was analysed with a two-way ANOVA followed by Bonferroni's multiple comparisons test F (15, 139) = 2,173, p=0.0099. (B) One-Way ANOVA was used (C-EVs F=3.256, p=0.0343; T-EVs F=3.002, p=0.00472) followed by the Uncorrected Fisher's LSD. Asterisks indicate significant difference compared with the control group (CV) *p<0.05, **p<0.01.*

Rats with mild liver damage showed worse object location memory (NOL test) than control rats. Discrimination ratio was 0.06±0.03 for CCl_4 rats compared to 0.22±0.03 in control rats (p=0.0123). Treatment with C-EVs in rats with mild liver damage significantly increased discrimination ratio (0.21±0.04, p=0.012 compared with CCl_4 rats without treatment). In contrast, T-EVs were not able to improve object location memory (-0.02±0.06, p=0.0038 compared with controls) (Figure 19).

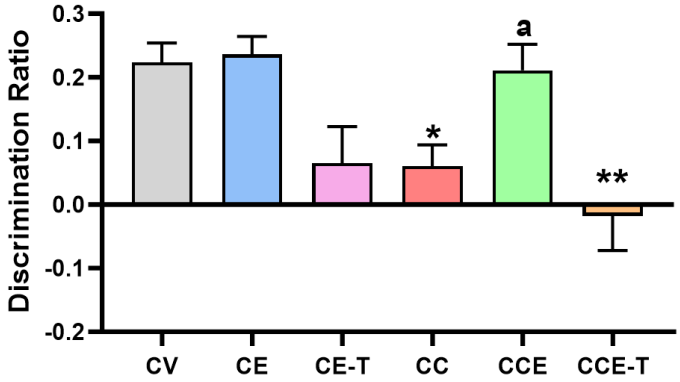

Figure 19. Effects of MSCs-EVs on NOL test. *The test was performed in the third week of CCl_4 treatment. Values are expressed as mean ± SEM of 8-14 rats per group. Data were analysed with One-Way ANOVA (C-EVs F=6.403; p=0.0011; T-EVs F=5.015; p=0.0054) followed by Tukey's multiple comparisons test. Asterisks indicate a significant difference compared with CV group *p<0.05, **p<0.01; "a" compared with CC group a, p<0.05.*

Rats with mild liver damage also showed impaired short-term memory measured in the Y maze (Figure 20). This group showed a decreased discrimination ratio (CC 0.47±0.06) compared with the control group (CV 0.73±0.08, p=0.0295). Treatment with C-EVs or T-EVs did not restore this alteration (CCE 0.46±0.04, p=0.0325; CCE-T 0.49±0.03, p=0.0382 compared with CV). Moreover, treatment with both types of EVs reduced the discrimination ratio in control rats (CE 0.43±0.04, p=0.0072; CE-T 0.43±0.08, p=0.0267).

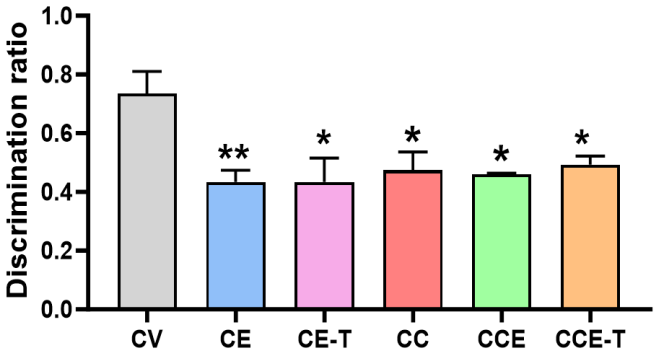

Figure 20. Effects of MSCs-EVs on Short-term memory test measured in the Y maze. *The test was performed in the third week of CCl_4 treatment. Values are expressed as mean ± SEM of 4-7 rats per group. Data were analysed with the One-Way ANOVA (C-EVs F=6.107, p=0.0047; T-EVs F=4.050, p=0.0231) followed by the Tukey's multiple comparisons test. Asterisks indicate a significant difference compared with CV group *p<0.05, **p<0.01.*

2. C-EVs restore membrane expression of GluN2B receptor subunit, while T-EVs restore membrane expression of GluA2 receptor subunit in hippocampus of rats with mild liver damage

To assess if the learning and memory improvements by MSCs-EVs is associated to restoration of alterations in glutamate receptors membrane expression in the hippocampus, we analysed membrane expression of different NMDA and AMPA glutamate receptors subunits: GluN1, GluN2A, GluN2B, GluA1 and GluA2. Rats treated with CCl_4 showed decreased membrane expression of NMDA receptor subunits GluN1 (35.1±6.8% of CV, p<0.0001), GluN2A (83.8±15.5% of CV, p=0.0446) and GluN2B (63.3±12.9% of CV, p=0.003) in hippocampus, compared with control rats (Figure 21). Treatment with C-EVs only normalized membrane expression of GluN2B subunit in rats with mild liver damage (99.3±11.1% of CV, p=0.0325 compared with CC) while they did not normalize alterations in GluN1 (48.2±9.1% of CV,

p=0.0018 compared with CV) nor GluN2A (65.4±11.9 % of CV, p=0.0077 compared with CV). In contrast, T-EVs administration to rats with mild liver damage did not restore any of the alterations in membrane expression of NMDA receptor subunits (GluN1 50.6±13.0% of CV, p=0.032; GluN2 73.5±17.4 % of CV, p=0.0028; GluN2B 45.6±5.6 % of CV p<0.0001, compared with CV). In fact, these modified EVs decreased membrane expression of GluN2B subunit in control rats (CE-T 58.7±16.1 % of CV, p=0.0138) (Fig. 2C).

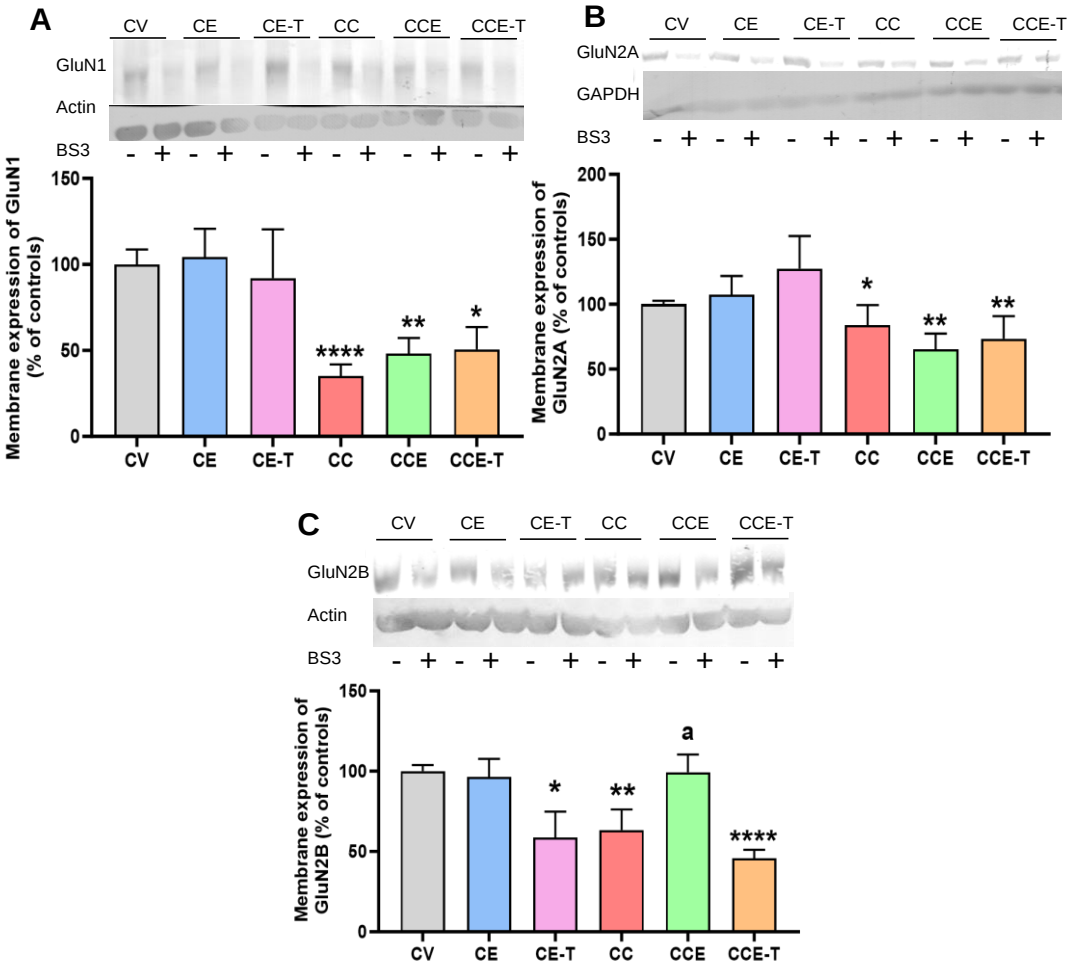

Figure 21. Effects of MSCs-EVs on membrane expression of NMDA receptor subunits. *Data of GluN1 (n=8-17, A), GluN2A (n=19-32, B) and GluN2B (n=13-28, C) membrane expression in hippocampus are expressed as mean ± SEM. (A) Data were analysed with Welch's ANOVA test followed by the Dunnett's T3 multiple comparisons test: C-EVs W(3.000,31.01)=14.05 (p<0.0001), T-EVs W(3.000,18.93)=11.40 (p=0.0002). (B) Kruskal-Wallis test was followed by Dunn's test (C-EVs statistic=13.34, p 0.004; T-EVs statistic=15.4, p=0.0015). (C) Kruskal-Wallis test was followed by uncorrected Dunn's test (C-EVs statistic=9.957, p 0.0189; T-EVs statistic=20.19, p=0.0002). Asterisks indicate a significant difference in the multiple comparison tests compared with CV group *p<0.05, **p<0.01, ****p<0.0001 and "a" compared with CC group; a, p<0.05.*

Regarding AMPA receptor subunits, membrane expression of GluA1 subunit was not altered in rats treated with CCl_4 nor by EVs administration (Figure 22 A). Rats with mild liver damage show reduced membrane expression of GluA2 subunit when compared to the control group (74.4±6.8 % of CV, p=0.0397). This alteration was restored by treatment with T-EVs (123.4±17.0 % of CV, p=0.0413) but not with C-EVs (67.7±8.6 % of CV, p=0.0156 compared with CV). Treatment with EVs did not alter membrane expression of control rats (Fig. 2E).

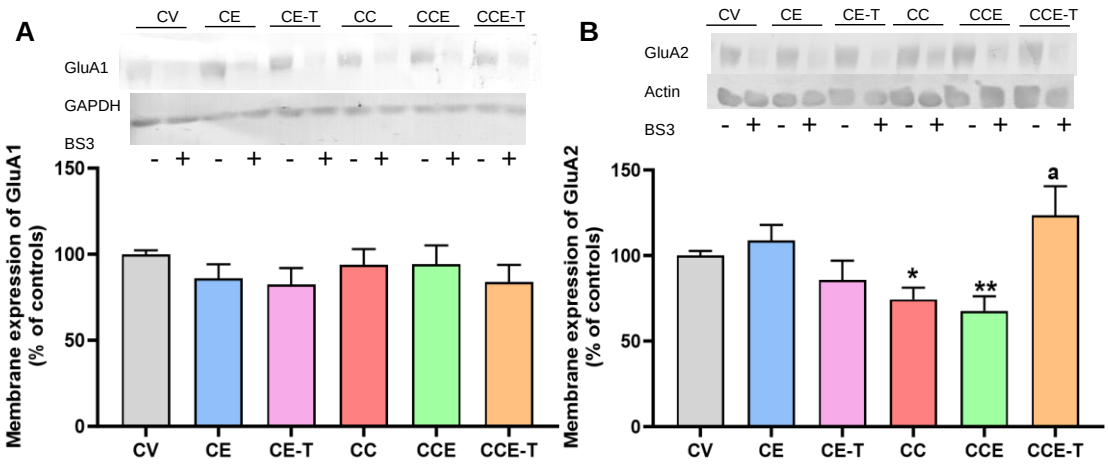

Figure 22. Effects of MSCs-EVs on membrane expression of AMPA receptor subunits. *Data of GluA1 (n=17-32, A) and GluA2 (n=14-29, B) membrane expression in hippocampus are expressed as mean ± SEM. In B, data were analysed with Kruskal-Wallis test followed by Dunn's test (C-EVs statistic=18.86, p=0.0003; T-EVs statistic=12.56, p=0.0057). Asterisks indicate a significant difference in the multiple comparison tests compared with CV group *p<0.05, **p<0.01, and "a" compared with CC group; a, p<0.05.*

3. Effects of MSC-EVs on proteins associated to GABA neurotransmission in the hippocampus

We also analysed alterations in GABA neurotransmission system in the hippocampus and the effects of treatment with MSCs-EVs, by assessing the content of the GABA synthesizing enzymes GAD65 and GAD67 and membrane expression of $GABA_A$ receptor subunits.

The content of the glutamic acid decarboxylase isoforms GAD65 and GAD67, responsible for the GABA synthesis was analysed by western blot. In the hippocampus of rats with mild liver damage, the content of both isoenzymes was significantly decreased compared with controls (GAD65 75.3±7.3 % of CV, p=0.0468; GAD67 73.8±6.3 % of CV, p=0.031). The content of GAD65 was restored by treatment with both types of EVs (CCE 103.0±9.9 % of CV, p=

0.0283; CCE-T 99.9±8.0 % of CV, p=0.0473; compared with CC), whereas the content of GAD67 was not affected by any type of EVs (CCE 72.7±5.9 % of CV, p= 0.0171; CCE-T 70.5±6.8 % of CV, p=0.0132; compared with CV) (Figure 23).

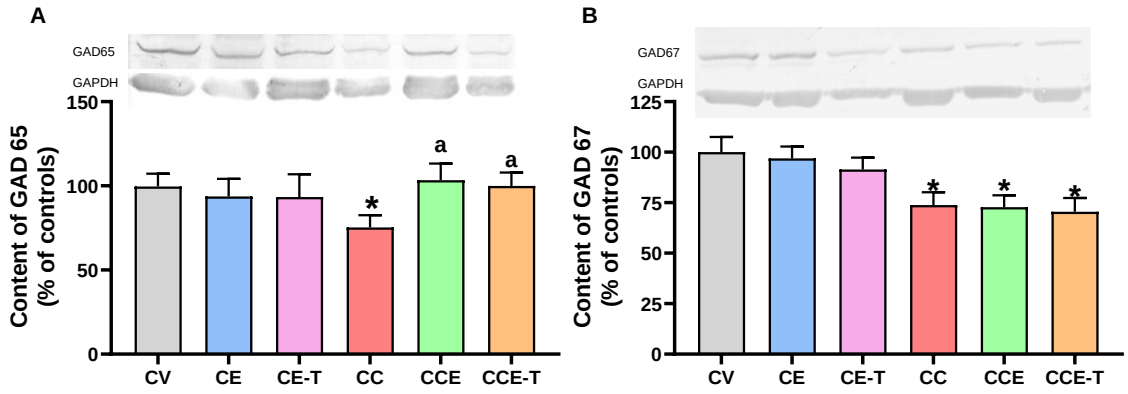

Figure 23. Effects of MSCs-EVs on content of GAD isoenzymes in the hippocampus. *Data of GAD65 (A), GAD67 (B) content are expressed as mean ± SEM of 11-16 rats per group. (A) Data were analysed with One-Way ANOVA (C-EVs F= 1,968, p=0.129; T-EVs F=1,835. P=0.152) followed by the Uncorrected Fisher's LSD test. (B) Data were analysed with One-Way ANOVA (C-EVs F=5.054, p=0.004; T-EVs F=4.504, p=0.0072) followed by the Tukey's multiple comparisons test. Asterisks indicate a significant difference (p<0.05) in the multiple comparison tests compared with CV group, and "a" compared with CC group.*

GABAergic neurotransmission in hippocampus also depends on the content of GABA receptor subunits in the membrane. The membrane expression of some subunits of the $GABA_A$ receptor was measured in hippocampus and expressed as percentage of values in control rats (Table 5).

Table 5. *Results of membrane expression of $GABA_A$ receptor subunits expressed as mean±SEM.*

GROUP	GABAA-α1 (N=11-22)	GABAA-α2 (N=6-14)	GABAA-α5 (N=11-26)	GABAA- β3 (N=7-10)	GABAA-γ2 (N=7-16)
CV	100.0±3.5	100.0±10.9	100.0±5.3	100.0±0.0	100.0±3.6
CE	96.3±10.6	101.2±20.3	102.1±10.9	**24.7±12.9*****	101.7±25.1
CE-T	**68.5±7.2****	92.6±128.1	102.5±13.2	**57.7±7.2***	**60.1±11.6***
CC	92.3±11.3	**154.4±21.4***	**77.1±8.1***	**42.3±14.3****	**153.8±17.0***
CCE	**60.3±7.0****	**154.0±25.7***	94.4±11.6	**4.8±5.9*****	**67.8±10.7*aa**
CCE-T	**63.2±7.6****	95.7±10.4 a	130.8±22.4 a	**32.5±16.4****** a	**50.5±12.2**aa**

Note: *Asterisks indicate a significant difference in the multiple comparison tests compared with CV group *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and "a" compared with CC group a, p<0.05; aa, p<0.01.*

Membrane expression of $GABA_A$-α5 and $GABA_A$-β3 subunits was significantly reduced (p=0.0454 and p=0.005 respectively, compared with controls) in rats with mild liver damage (Figure 24C and 24D). These rats also showed increased membrane expression of $GABA_A$-γ2 (p= 0.0146) and $GABA_A$-α2 (p=0.022) (Figure 24B and 24E), whereas membrane expression of $GABA_A$-α1 was not altered.

Treatment with T-EVs in rats with mild liver damage normalized the membrane expression of $GABA_A$-α5 subunit (p=0.0269 compared with CC) and $GABA_A$-α2 (p=0.0332) and reduced membrane expression of $GABA_A$-γ2 subunits below control values (p=0.0072 compared with CV, and p<0.0001 compared with CC) inducing the opposed effect to that observed in CCl_4 rats without treatment. The membrane expression of $GABA_A$-α1 was also reduced in rats with mild liver damage treated with T-EVs, (p=0.0024, compared with controls), whereas T-EVs treatment did not alter the decreased membrane expression of $GABA_A$-β3 (p=0.0005, compared to CV) in CCl_4 injected rats. Moreover, T-EVs administration in control rats also reduced the membrane expression of $GABA_A$-α1 (p=0.0077), $GABA_A$-β3 (p=0.0164) and $GABA_A$-γ2 subunits (p=0.0264), compared with control rats.

Treatment with C-EVs in rats with mild liver damage reduced membrane expression of $GABA_A$-α1 (p=0.0028 compared with CV, p=0.0427 compared with CC), inducing a synergistic effect with CCl_4 administration, of $GABA_A$-β3 (p=0.0002), increasing the effect observed in untreated CCl_4 rats and of $GABA_A$-γ2 subunits (p=0.0406 compared with CV, but p=0.0001 compared with CC), inducing the opposed effect than those observed in untreated CCl_4 rats. Membrane expression of the $GABA_A$-α2 subunit was not restored by C-EVs but membrane expression of $GABA_A$-α5 was increased, normalizing the decrease in CCl_4 injected rats, although differences between both groups (CC and CCE) were no significant. Besides, C-EVs administration in control rats reduced membrane expression of $GABA_A$-β3 subunit (p=0.001) in hippocampus.

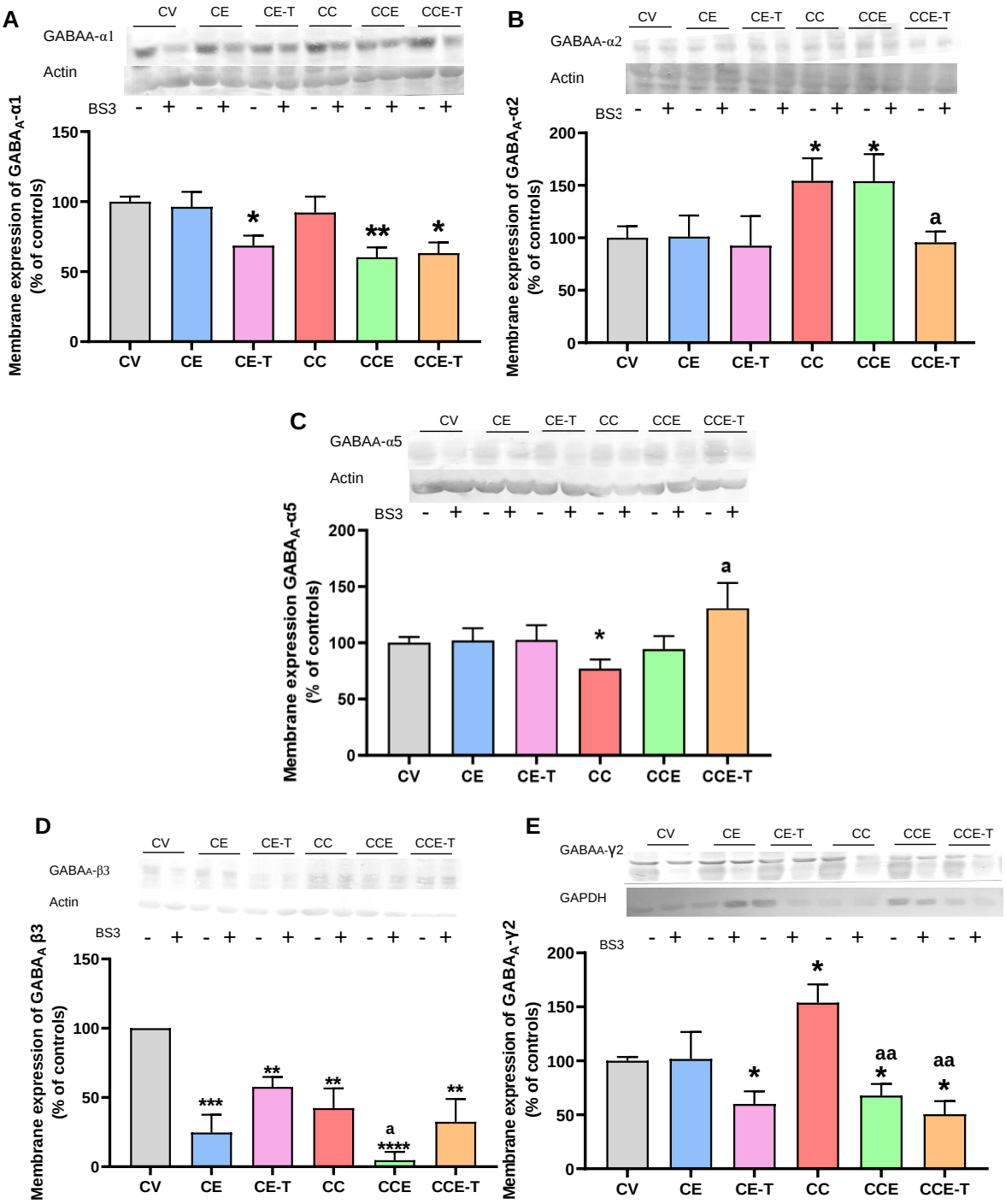

Figure 24. Effects of MSCs-EVs on membrane expression of GABA$_A$ receptor subunits in hippocampus. *Data of content of GABA$_A$-α1 (n=11-22, A), GABA$_A$-α2 (n=6-14, B), GABA$_A$-α5 (n=11-26, C), GABA$_A$-β3 (n=7-10, D), and GABA$_A$-γ2 (n=7-16, E) in cell membranes of hippocampus* ***(****mean ± SEM) are represented. (A) Data were analysed with Kruskal-Wallis test followed by the Dunn's test: C-EVs statistic=13.86 (p=0.0031), and the Welch's ANOVA test followed by the Dunnett's T3 multiple comparisons tests: T-EVs W(3,000, 26,16)= 9,284 (p=0.0152). (B) One-Way ANOVA was followed by the Uncorrected Fisher's LSD test (C-EVs) and Welch's ANOVA followed by unpaired t test with Welch's correction (T-EVs), ANOVAs*

*were not significant. (C) Kruskal-Wallis test (not significant) followed by the uncorrected Dunn's test. (D) Kruskal-Wallis test followed by the Dunn's test: C-EVs statistic=17.52 (p=0.0006), T-EVs statistic=14,63 (p=0.0002). (E) Kruskal-Wallis test followed by the Dunn's test: C-EVs statistic=16.00 (p=0.0011), T-EVs statistic=24.89 (p=0.0001). Asterisks indicate a significant difference in the multiple comparison tests compared with CV group *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and "a" compared with CC group a, p<0.05, aa, p<0.01.*

Another factor that modulates the intensity of the GABAergic response is the chloride gradient through the cell membrane. This gradient is mainly modulated by chloride co-transporters in the cell membrane, mainly KCC2 in neurons and NKCC1 in glia. Membrane expression of KCC2 was not altered in the CCl_4-injected rats (Figure A). However, treatment with C-EVs (47.1±13.5 % of CV, p= 0.0255) or T-EVs (40.8±17.6 % of CV, p=0.0451) in these rats reduced KCC2 membrane expression compared to the control rats (Figure A). Besides, membrane expression of NKCC1 was increased in rats with mild liver damage (265.5±69.3% of CV) compared to the control group (p=0.0486) (Figure B). This alteration was reversed with the treatment with T-EVs (86±30 % of control group, p=0.0399 compared with CC) but not C-EVs (246.4±58.0 % of CV, p=0.0398 compared with CV).

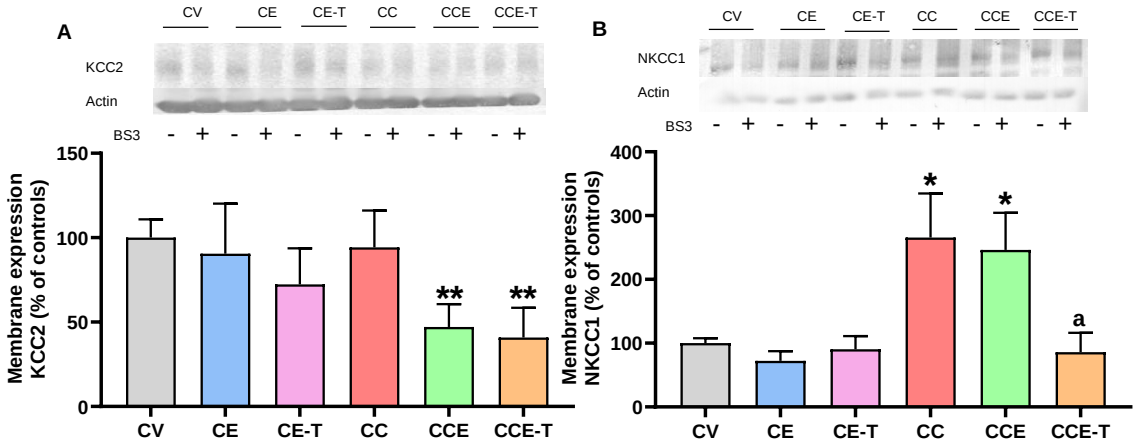

Figure 25. Effects of MSCs-EVs on chloride channels in hippocampus. *Membrane expression of KCC2 (A) and NKCC1 (B) represented as mean ± SEM of 6-11 animals per group. (A) Data were analysed with Kruskal-Wallis (not significant) test followed by the Uncorrected Dunn's test. (B) Data were analysed with Welch's ANOVA (C-EVs W(3.000, 14.09)=4.681, p=0.0181) followed by the Unpaired t test with Welch correction. Asterisks indicate a significant difference compared with CV group *p<0.05, **p<0.01, and "a" compared with CC group a, p<0.05.*

4. C-EVs reduce microglial activation and T-EVs reduce astrocytic activation in hippocampus of rats with mild liver damage

Alterations in neurotransmission systems are often induced by neuroinflammation. Therefore, we assessed if effects of MSCs-EVs on neurotransmission are mediated by modulation of neuroinflammation in hippocampus. Neuroinflammation in hippocampus was analysed by the assessment of microglial and astrocytic activation. Microglial activation was measured as the perimeter of microglia cells that is reduced when the cell is activated. Astrocytic activation was measured as percentage of stained area of the cytosolic marker GFAP, that is increased when the cell is activated due to the multiple ramifications that are created.

Rats with mild liver damage show increased microglial activation (perimeter 273.2±31.3 μm) compared to the control group (446.3±62.1 μm, p=0.0320). Activation of microglia was significantly reduced by the treatment with C-EVs (458.3±51.6 μm, p=0.0107) but not with T-EVs (297.1±50.1 μm) (Figure 26A and 26C).

Rats injected with CCl_4 also showed astrocytic activation (GFAP stained area 112.5±5.1 % of CV, p=0.0098) in the hippocampus. Astrocytic activation was not reduced by C-EVs (114.4±3.5 % of CV, p=0.0044 compared with CV) although it was partially reduced by treatment with T-EVs, as CCE-T group (104.3±6.8 % of CV) was not different to the control group although neither the rats with mild liver damage. Treatment with EVs increased astrocytic activation in the control groups (CE 116.2±6.0 % of CV p=0.0134, CE-T 122.1±3.1 % of CV, p=0.0049) (Figure 26B and D).

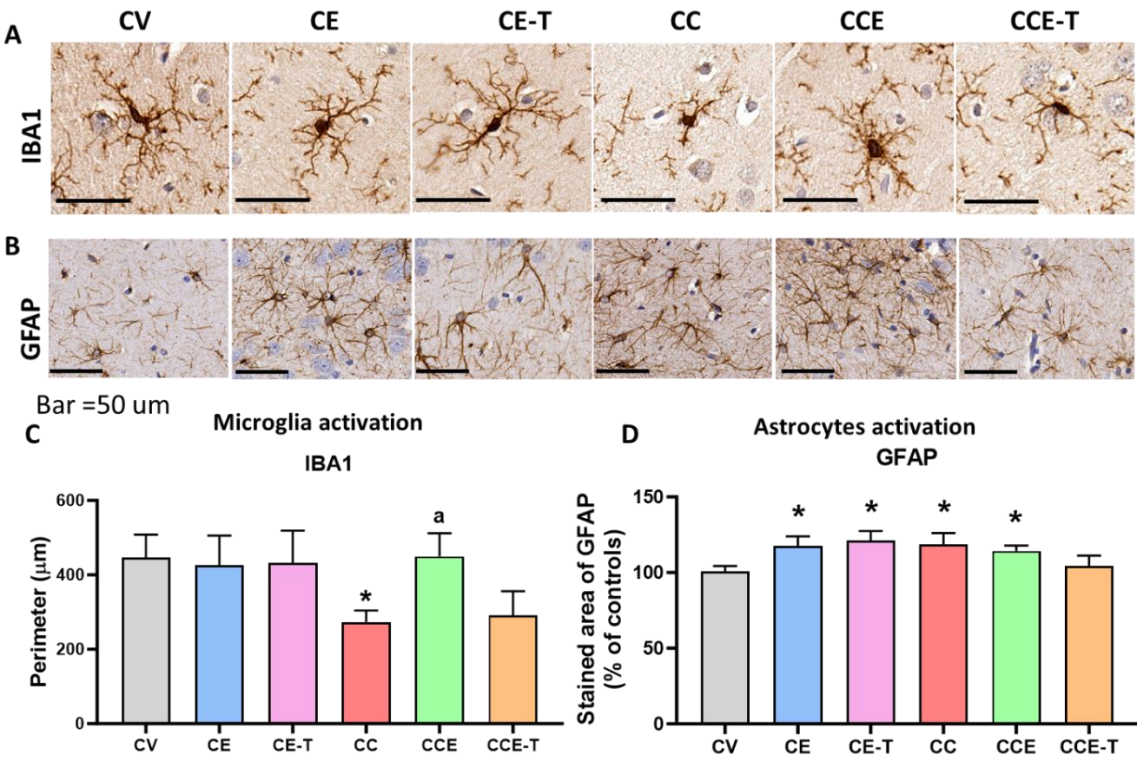

Figure 26. Effects of MSCs-EVs on microglial and astrocytic activation in hippocampus. *Representative images of microglia marked with Iba-1 (A) and astrocytes marked with GFAP (B) in hippocampus. Values are mean ± SEM of 5-6 (microglia, C) and 3-6 (astrocytes, D) animals per group. Data were analysed by One-Way ANOVA followed by the Uncorrected Fisher's LSD test. (C) T-EVs F=3.267, p=0.047; C-EVs was not significant; (D) C-EVs F=4.745, p=0.0139, T-EVs F=3.920, p=0.0283. Asterisks indicate a significant difference compared with CV group *p<0.05, **p<0.01, and "a" compared with CC group a, p<0.05.*

5. Effects of C-EVs and T-EVs on IL-1β and TNFα content in the hippocampus

We also measured proinflammatory cytokine content in the hippocampus to analyze neuroinflammation. IL-1β was measured by immunohistochemistry. Rats injected by CCl_4 show increased number of IL-1β+ cells in hippocampus (188.6±17.4 % of CV) compared to the control group (p=0.0036) (Fig. A and C). This alteration was normalized by the treatment with T-EVs (112.1±7.1 % of CV, p= 0.0289 vs CC group). Treatment with C-EVs tended to reduce the number of IL-1β+ cells although this reduction was not complete (148.3±16.2 % of CV).

Neurons from CA1 also express IL-1β in inflammatory conditions. Then, the intensity of the IL-1β staining in the CA1 region of hippocampus was also measured (Fig. B and D). Rats with mild liver damage show reduced IL-1β staining in CA1 (22.6±0.6) compared to the control rats (27.7±0.8, p=0.0039). This alteration was normalized by the treatment with C-EVs (26.6±1.2, p=0.0267 compared with CC) but not T-EVs (23.9±1.0, p=0.0161 compared with CV).

TNF-α was measured by Western Blot (Fig. E). Rats with mild liver damage show increased levels of TNF-α in hippocampus (148.4±15.9 % of CV, p=0.0113). Treatment with C-EVs normalizes TNF-α levels in rats with mild liver damage (87.7±14.4 % of CV, p=0.0028). T-EVs were not able to reduce these levels (195.2±32.6 % of CV, p= 0.0103 compared with CV). Moreover, C-EVs and T-EVs administration increased TNF-α levels in control rats (CE 140.4±16.0 % of CV, p=0.0326, CE-T 208.9±34.2 % of CV, p=0.0141).

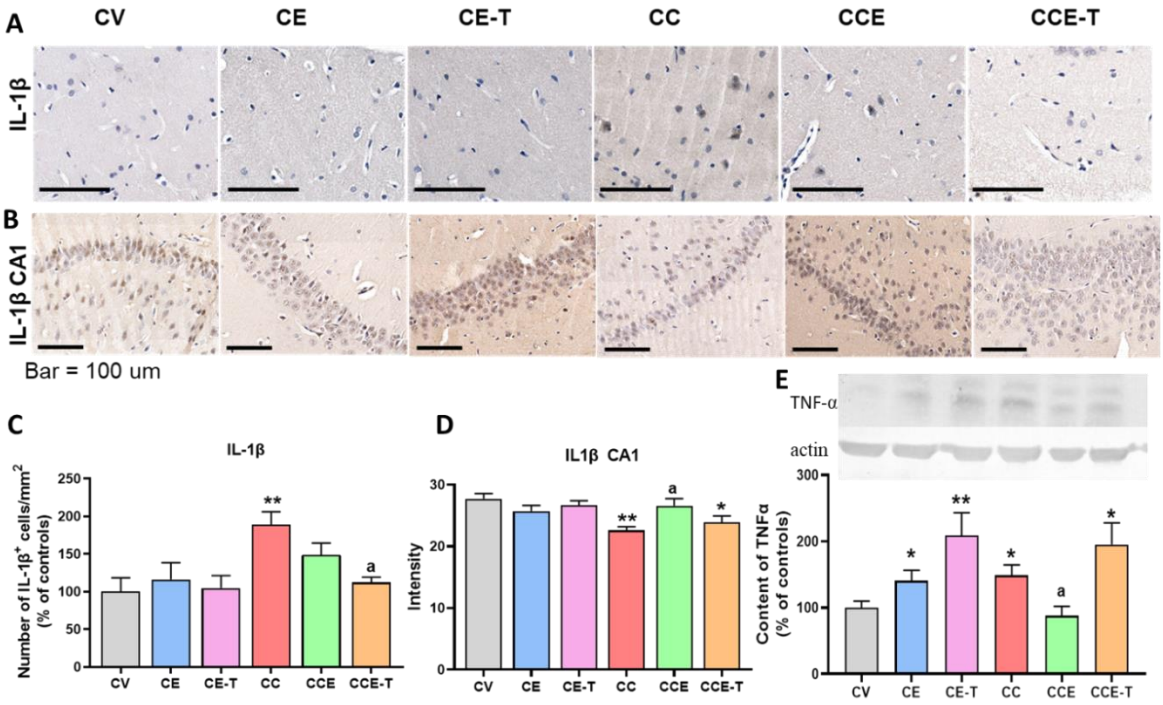

Figure 27. Effects of MSCs-EVs on proinflammatory cytokines in hippocampus. *Representative images of IL-1β immunostaining in hippocampus (A) and IL-1β staining in CA1 region (B). (C) Number of IL-1β+ cells is expressed as percentage of control rats and represented as mean ± SEM of 3-8 animals per group. Data were analysed by One-Way ANOVA followed by the Tukey's multiple comparisons test, T-EVs F=6.436, p=0.0034 (D) Values of IL-1β mean intensity in CA1 are represented as mean ± SEM of 3 animals per group (5 images per animal). One-way ANOVA (C-EVs F=3.854, p=0.0209; T-EVs F=6.865, p=0.0015) was followed by Tuckey's (C-EVs) or Fisher's LSD (T-EVs) tests. (E) Content of*

*TNF-α.in hippocampus measured by WB is expressed as mean± SEM of 7-16 animals per group. One-way ANOVA (C-EVs F=4.629, p=0.0064; T-EVs F=5.141, p=0.0039) was followed by Tuckey's (T-EVs) or Fisher's LSD (C-EVs) tests. Asterisks indicate a significant difference in the multiple comparison tests compared with CV group *p<0.05, **p<0.01, and "a" compared with CC group a p<0.05.*

6. Rats with mild liver damage show impaired motor coordination and locomotor gait, which are improved by EVs from MSCs

Motor coordination was analysed in the MotoRater, the rotarod and the beam walking tests. In addition, the regularity index analysed in the CatWalk is also a measure of motor coordination. CCl_4-injected rats showed increased wrong foot placement percentage (CC 4.38±0.48% of wrong foot placements, CV 2.59±0.35%, p=0.0180) in the MotoRater (Figure 28A), remained less time in the rotarod (CC 134.8±7.0 s, CV 168.9±9.2 s, p=0.0351) (Figure 28B) and showed an increased number of slips than control rats in the beam walking (CC 1.62±0.17 slips, CV 0.94±0.15 slips, p=0.013) (Figure 28C). Moreover, rats injected with CCl_4 showed reduced regularity index (93.18±0.60%) compared to the control rats (97.14±0.73%, p=0.0014) (Figure 28D).

Treatment with C-EVs in CCl_4-injected rats reversed the impairment of motor coordination in the MotoRater (2.84±0.19 %, p=0.0184), rotarod (168.8±9.6 s, p=0.0274) and beam walking (1.02±0.10 slips, p=0.0347) tests and the regularity index (95.94±0.60%, p=0.027), returning to values similar to control rats (Figures 28 A-D).

Treatment with T-EVs in CCl_4-injected rats also reversed motor incoordination in the MotoRater (2.57±0.23 %, p=0.0083) and regularity index (95.62±0.65%, p=0.0125) (Figures 28A and 28D). Concerning motor coordination assessed in the rotarod and the beam walking, T-EVs reversed them partially, as the time in the rotarod (153.0±9.0 s) and the number of slips in the beam walking (1.08±0.15 slips) were not different from control rats, but no statistical significance was reached compared to CCl_4-injected rats without treatment (Figure 28B and 28C). T-EVs impaired motor coordination in control rats in the MotoRater (4.15±0.36 %, p=0.013 compared with the controls) (Figure 28A).

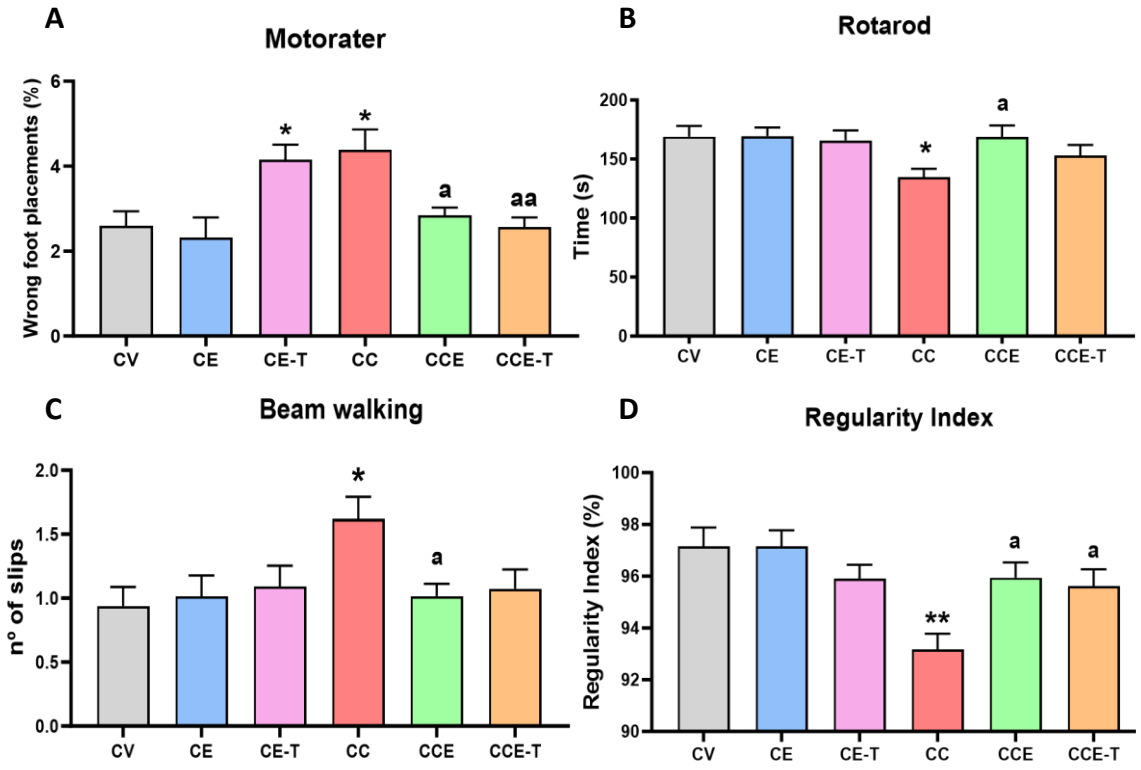

Figure 28. Effects of MSCs-EVs on motor coordination. *Motor coordination was assessed in the MotoRater (A), the Rotarod (B), the beam walking (C) tests and the CatWalk regularity index (D). The tests were performed in the third week of CCl_4 administration. Mean ± SEM is represented. For A (n=5-7), data were analysed with Kruskal-Wallis test followed by uncorrected Dunn's tests to assess the effect of C-EVs (statistic=9.893, p=0.0195) and T-EVs (statistic 13.24, p=0.0041). In B (n=17-24), C (n=12-22), and D (n=4-7) One-Way ANOVA was used to analyse the data. In B (F=4.027, p=0.0101), and C (F=4.424, p=0.0065) the ANOVA was significant for C-EVs but not T-EVs and was followed by the Tukey's multiple comparisons test. In D, the effect of C-EVs (F=8.660, p=0.0008) was additionally analysed by the Tukey's multiple comparisons test and the effect of T-EVs (F=6.477, p=0.0033) was followed by the Uncorrected Fisher's LSD test. Asterisks indicate a significant difference compared with CV group *p<0.05, **p<0.01 and "a" compared with CC group a p<0.05, aa p<0.01.*

Besides regularity index, different parameters of locomotor gait were analysed in the CatWalk XT (Table 6). The CatWalk XT software calculates each parameter for each paw, but as paws of the same animal had similar results, the mean of the paws was used to analyze the differences between groups (except for dual stance). Rats with mild liver failure showed increased initial dual stance in the left hind (p=0.0088; Figure 29A), terminal dual stance in the right hind

(p=0.02), and step cycle (p<0.0001, Figure 29D) and reduced stand index front (p=0.0061; Figure 29C) and stride length (p<0.0001, Figure 29B).

Table 6. *Results of locomotor gait analysed in the CatWalk (mean±SEM).*

GROUP	Initial dual stance left hind	Terminal dual stance right hind	Stride length	Stan index front	Step cycle
CV	0.08±0.01	0.08±0.01	11.9±0.3	-8.57±0.44	0.39±0.02
CE	0.09±0.02	0.09±0.02	**12.4±0.9****	-8.17±0.52	0.37±0.03
CE-T	0.09±0.01	0.09±0.003	12.0±0.2	-7.63±0.27	0.39±0.01
CC	**0.12±0.01***	**0.12±0.01***	**11.3±0.5******	**-7.04±0.42****	**0.43±0.03******
CCE	**0.08±0.01 a**	**0.08±0.01 a**	**12.5±0.6**aaaa**	-7.94±0.34	**0.39±0.02 aaaa**
CCE-T	**0.12±0.01***	0.10±0.01	**12.3±0.6**aaaa**	-6.84±0.47	**0.44±0.02******

*Asterisks indicate a significant difference in the multiple comparison tests compared with CV group *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and "a" compared with CC group a p<0.05, aa p<0.01.*

Treatment with C-EVs in rats with mild liver damage reversed the impairment of dual stance (initial p=0.0202 and terminal p=0.038 Figure 29A) and step cycle (p<0.0001 Figure 29D). They also increased stride length (p<0.0001 compared with CC) even higher than the control group (p=0.0012, Figure 29B), and partially normalized stand index front (Figure 29C). Treatment with T-EVs increased stride length (p<0.0001 compared with CC) to higher levels than the control rats (p=0.0043), but did not improve dual stance, step cycle, nor stand index front in rats with mild liver damage (Figure 29A-D).

Concerning the effects on control rats, C-EVs did not affect any of the parameters analysed except for stride length (p=0.0039), which was increased compared to the CV group (Figure 29B). T-EVs did not affect any parameter analysed in the CatWalk in control rats.

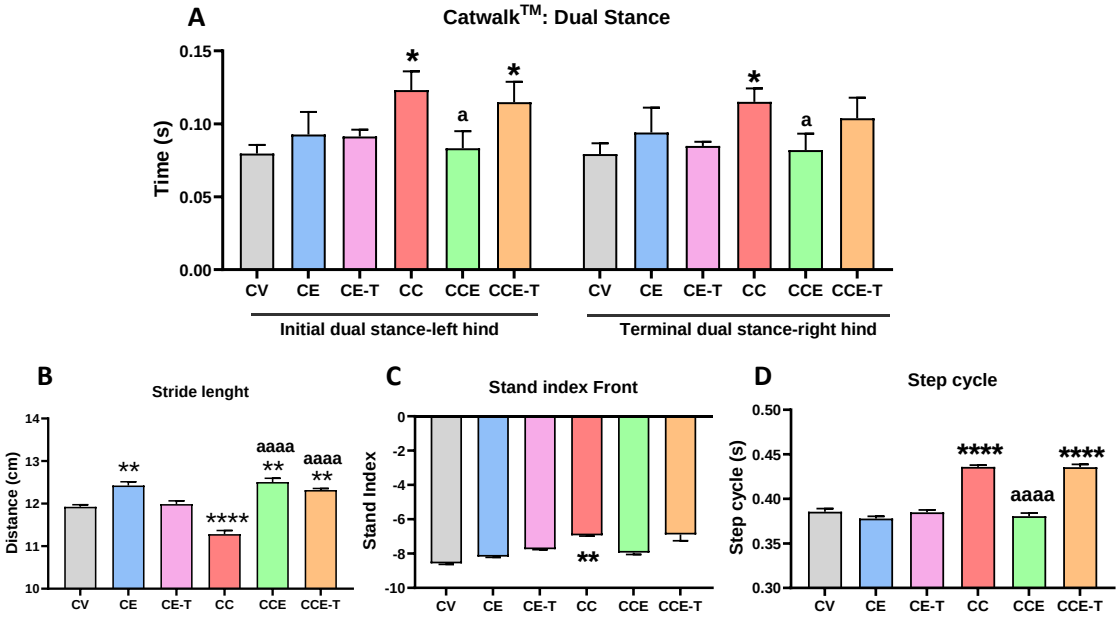

Figure 29. Effects of MSCs-EVs on locomotor gait. *Locomotor gait was assessed with some parameters from the CatWalk XT: Dual Stance (A), Stride length (B), Stand Index front (C) and Step cycle (D)**.** This analysis was performed in the third week of CCl_4 administration. Data are represented as mean ± SEM of 5-7 animals per group. (A) Data were analysed with a One-Way ANOVA test followed by the Uncorrected Fisher's LSD test. Initial dual stance left hind C-EVs F=3.008 (p=0.0456), T-EVs F=3.972 (p=0.021). (B) Stride length was analysed with One-Way ANOVA (C-EVs F=50.07; T-EVs F=46.43; both p<0.0001) followed by Tukey's multiple comparisons test. (C) Stand index front was analysed with Welch's ANOVA (C-EVs W(3.000,2.159)=127.40, p=0,0057; T-EVs W(3.000,2.020)=103.8, p=0,0092) followed by Dunnett's T3 multiple comparisons test. (D) Step cycle was analysed with One-Way ANOVA (C-EVs F=89.24; T-EVs F=101.2; both p<0.0001) followed by Tukey's multiple comparisons test. Asterisks indicate a significant difference in the multiple comparison tests compared with CV group *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and "a" compared with CC group a p<0.05, aaaa p<0.0001.*

7. Rats with mild liver damage show altered GABAergic neurotransmission in the cerebellum that is reversed by MSC-EVs

Impaired motor coordination is usually a consequence of altered GABAergic neurotransmission in cerebellum. We therefore analysed the main parameters modulating it. As in the hippocampus, the content of glutamate decarboxylases GAD65 and GAD67 was

analysed as main factors involved in GABA levels. As shown in Figures 30A and 30B, the content of GAD65 and GAD67 was not altered in the cerebellum of rats with mild liver failure nor by the treatment with both types of EVs.

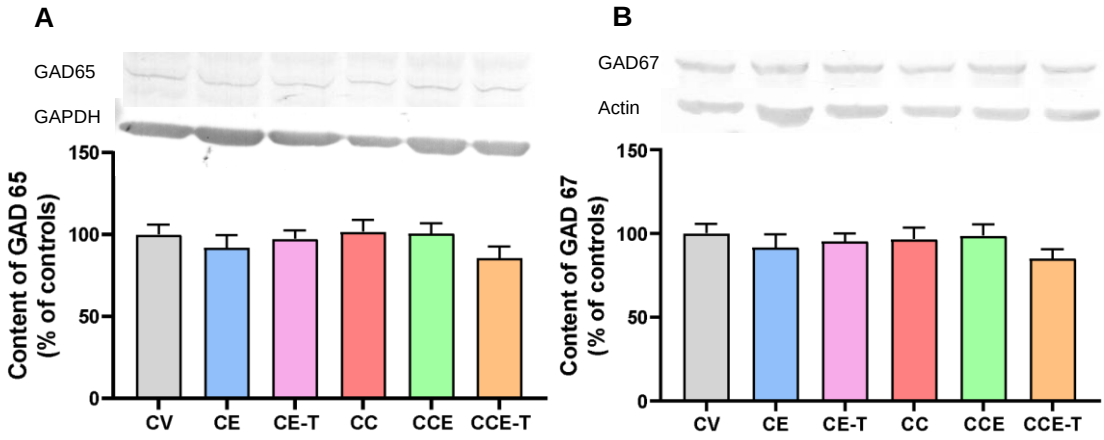

Figure 30. Effects of MSCs-EVs on GAD content measured by Western Blot in cerebellum. *Values are expressed as percentage of control rats. Mean ± SEM of 10-17 animals per group of GAD65 (A) and GAD67 (B) content is represented. Data were analysed by One-Way ANOVA test followed by the Uncorrected Fisher's LSD test but significant differences were not found.*

GAD65 and GAD67 content was also analysed by immunochemistry in Purkinje neurons of cerebellum, which are the main GABAergic neurons in cerebellum (Figure 31). Rats injected with CCl_4 showed increased intensity of immunostaining of GAD67 in Purkinje neurons (96.02±8.18 arbitrary units a.u., p=0.0095) compared to control rats (64.85±8.91 a.u.). This alteration was normalized with the administration of T-EVs (67.25±4.47 a.u., p=0.0149). Treatment with C-EVs partially reduced the intensity of GAD67 in Purkinje neurons of rats with mild liver damage (83.54±7.76 a.u) as this group was not significantly different to the CCl_4 injected group nor the control group (Figure 31C). The intensity of GAD65 in Purkinje neurons was not altered by the administration of CCl_4 but, treatment with T-EVs increased intensity of GAD65 in Purkinje neurons in both rats with mild liver damage (36.56±3.2 a.u., p=0.0351) and control rats (35.00±4.94 a.u., p=0.0388) (Figure 31B).

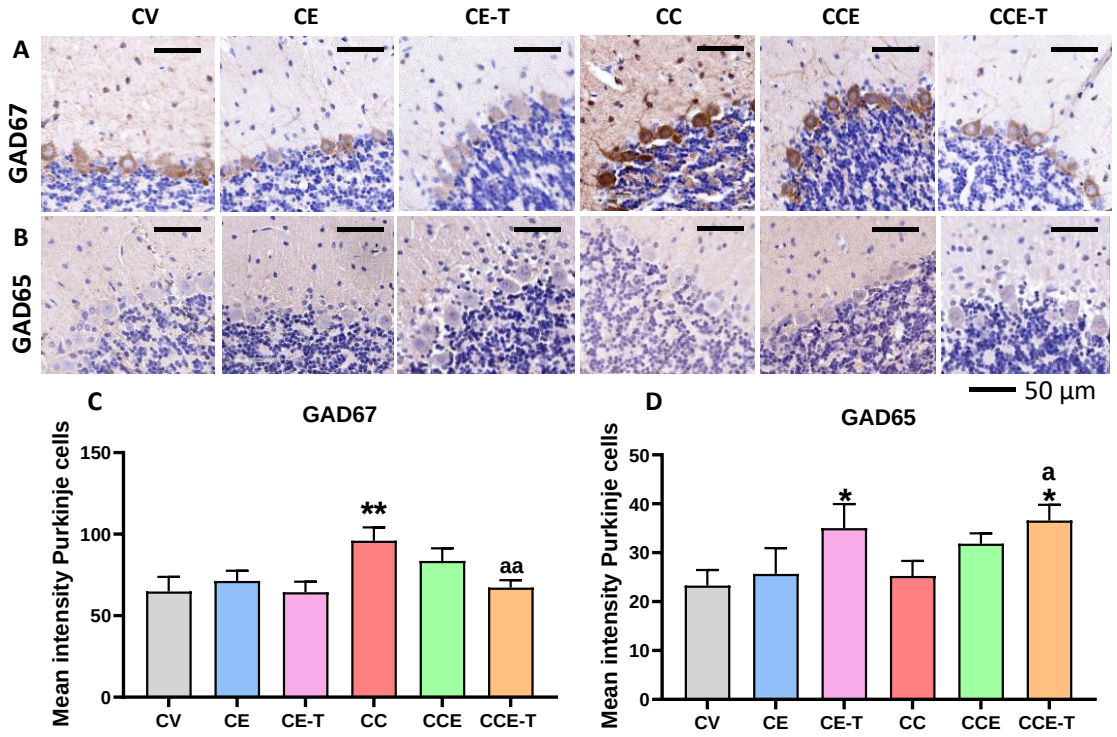

Figure 31. Effects of MSCs-EVs on GAD content in Purkinje neurons of cerebellum. *Representative images of GAD67 (A) and GAD65 (B) immunostaining. Mean ± SEM of 4-5 animals per group of GAD65 (C) and GAD67 (D) are represented. Values are expressed as mean intensity (arbitrary units, a.u.) in Purkinje neurons. Data were analysed by One-Way ANOVA test followed by the Uncorrected Fisher's LSD, only T-EVs had significant effects. (C) F=4.725, p=0.0193 (D) F=3.651, p=0.0371. Asterisks indicate a significant difference in the multiple comparison tests compared with CV group *p<0.05, **p<0.01, and "a" compared with CC group a p<0.05, aa p<0.01.*

Content of GABA was also analysed in Purkinje neurons by immunohistochemistry. Rats with mild liver damage showed increased content of GABA in Purkinje neurons (44.9±2.8 a.u., p=0.032) compared with the control rats (33.1±4.6 a.u.). Treatment with both types of EVs normalized GABA content in cerebellum (C-EVs 33.8±2.6 a.u., p=0.0328; T-EVs 30.7±5.2 a.u., p=0.0392) (Figure 32).

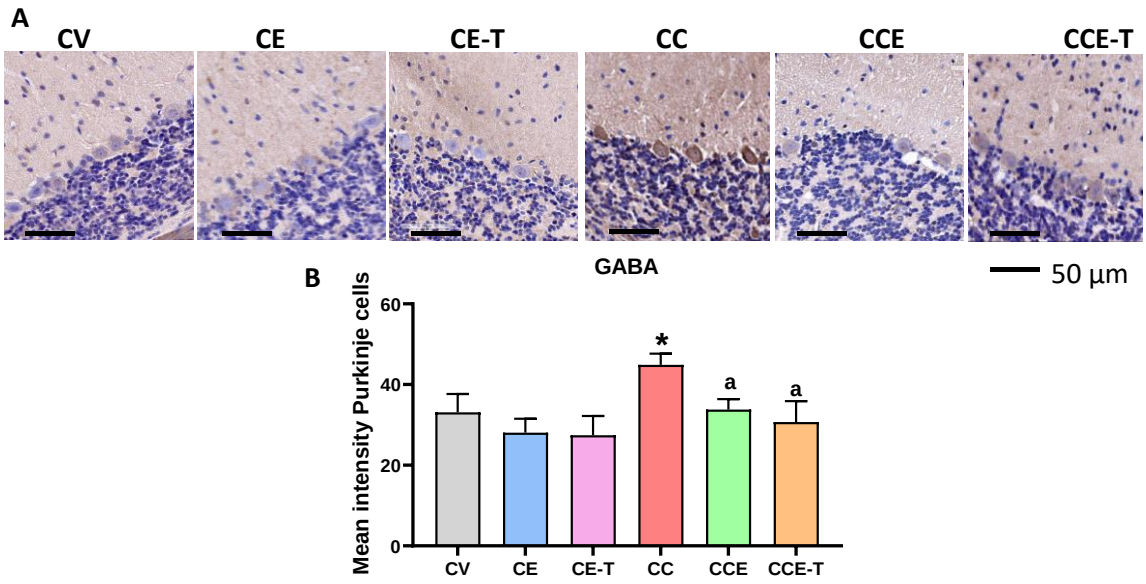

Figure 32. Effects of MSCs-EVs on GABA content in Purkinje neurons of cerebellum. *Representative images (A) and quantification results (B) of GABA mean intensity in Purkinje neurons of cerebellum. Graph represents mean ± SEM of 3-5 rats per group. Data were analysed by One-Way ANOVA test followed by the Uncorrected Fisher's LSD, C-EVs F=4.195, p=0.0279, T-EVs F=2.938, p=0.0806. Asterisks indicate a significant difference p<0.05 in the multiple comparison tests compared with CV group, and "a" compared with CC group.*

The extracellular concentration of GABA is mainly modulated by the GABA transporters GAT-1 and GAT-3 present in the cell membrane. We analysed the membrane expression of these transporters. Mild liver damage reduced the membrane expression of GAT-1 to around 50% of control rats (p=0.039, Figure 33A), possibly leading to increased levels of extracellular GABA in the cerebellum. However, membrane expression of GAT-3 was not altered in CCl_4-injected rats (Figure 33B). Treatment with C-EVs completely reversed the decrease in membrane expression of GAT-1 (p=0.0218). In contrast, treatment with T-EVs did not affect the reduction of GAT-1 in the membrane in CCl_4-injected rats (p=0.0036, compared with CV) but significantly reduced membrane expression of GAT-3 in rats with mild liver damage (p=0.0349, compared with CV).

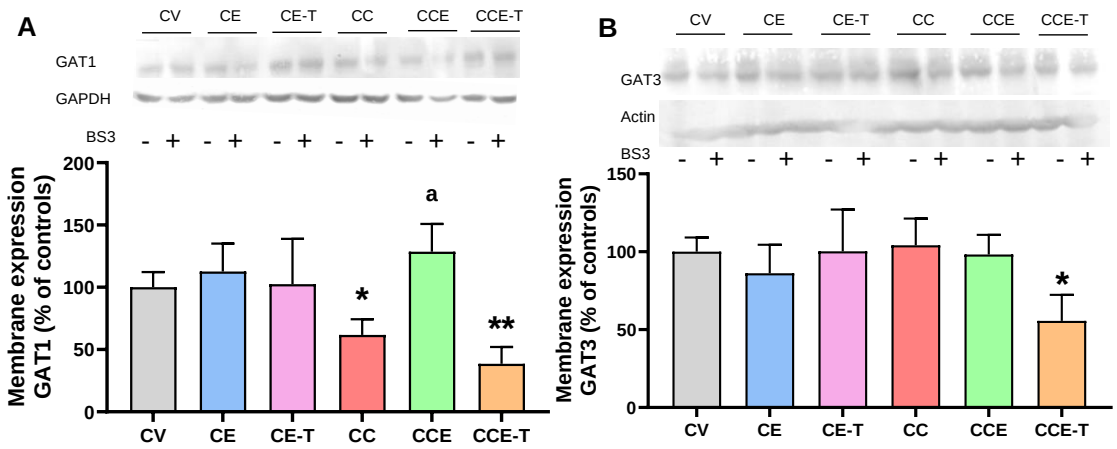

Figure 33. Effects of MSCs-EVs on GABA transporters in cerebellum. *Membrane expression in cerebellum of GAT-1 (A) and GAT-3 (B). Represented data are the mean ± SEM of 8-20 animals per group. Data were analysed with Welch's ANOVA test followed by the Uncorrected Fisher's LSD, GAT-1: C-EVs W(3.000, 22.77) =3.079, p=0.0478, T-EVs W(3.000, 19.83) =3.982, p=0.0226; ANOVA for GAT-3 was not significant. Asterisks indicate a significant difference compared with CV group *p<0.05, **p<0.01, and "a" compared with CC group a p<0.05.*

The intensity of the GABAergic response also depends on the number of receptors present in the cell membrane. Membrane expression of some $GABA_A$ receptor subunits was analysed. Mean±SEM of the indicated number of rats for each subunit are shown in Table 7. Rats injected with CCl_4 showed decreased membrane expression of $GABA_A$-β3 subunit (p=0.0256) compared to the control group. This alteration was partially reversed by treatment with C-EVs which was not significantly different to the control nor the liver damage groups. $GABA_A$ subunits α2, α6, γ2 and δ membrane expression was not altered in rats with mild liver damage. Membrane expression of subunits α2 (p=0.0161), β3 (p=0.0008) and γ2 (p=0.0062) was decreased in control rats treated with C-EVs, while $GABA_A$-δ membrane expression was decreased in control rats treated with T-EVs (p=0.0102).

Table 7. *Results of membrane expression of $GABA_A$ receptor subunits in cerebellum expressed as percentage of control rats.*

GROUP	$GABA_A$-α2 (N=9-18)	$GABA_A$-α6 (N=14-20)	$GABA_A$-β3 (N=8-13)	$GABA_A$-δ (N=7-11)	$GABA_A$-γ2 (N=22-32)
CV	100.0±3.3	100.0±10.9	100.0±0.0	100±3.5	100.0±4.9
CE	**58.5±16.3***	177.2±41.5	**34.2±10.3*****	90.7±13.8	**55.1±12.3****
CE-T	119.5±17.9	98.7±13.4	100.9±24.9	**48.8±17.3***	93.6±18.8
CC	102.4±20.1	90.0±22.7	**51.7±16.9***	63.7±14.8	91.8±19.9
CCE	153.3±33.7	110.9±16.7	110.8±40.1	115.8±17.6	103.1±23.1
CCE-T	94.3±13.6	95.2±14.1	**40.4±7.9****	94.0±15.9	109.6±15.8
p-value C-EVs statistic C-EVs	0.0231 9.52	- -	0.0059 12.49	- -	0.0095 11.45
p-value T-EVs Statistic T-EVs	- -	- -	0.0085 11.70	0.0318 F=3.339	- -

*Data were analysed by Kruskal-Wallis test and Dunn's test, except for β3 subunit in which the uncorrected Dunn's test was used for C-EVs and the δ subunit in which one-way ANOVA was used followed by the uncorrected Fisher's LSD test. Asterisks indicate a significant difference in the multiple comparison tests compared with CV group *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and "a" compared with CC group a p<0.05, aa p<0.01.*

8. Rats with mild liver damage show activation of microglia and astrocytes in cerebellum, which are reversed by C-EVs but not T-EVs

Rats injected with CCl_4 showed activation of microglia in white matter of the cerebellum since microglia cells showed reduced perimeter (75.1±4.8 % of control group, p=0.0014) compared with control rats (Figure 34A and C). Rats injected with CCl_4 also show activation of astrocytes, with increased (p=0.0463) area stained by GFAP (107.9±2.5 % of control group). Treatment with C-EVs reversed the activation of both microglia (90.6±5.1 % of control group, p=0.0246 compared with CC) and astrocytes (101.4±1.4 % of control group, p=0.0344) in rats with mild liver damage. In contrast, T-EVs did not reverse activation of microglia (82.6±2.8 % of control group, p=0.0211 compared with CV) nor astrocytes (120.3±8.6 % of control group, p=0.0112 compared with CV). Both types of MSCs-EVs induced astrocytic activation in control rats (CE 109.6±3.4 p=0.0089, CE-T 125.3±6.4 p=0.0007) (Figures 34B and D).

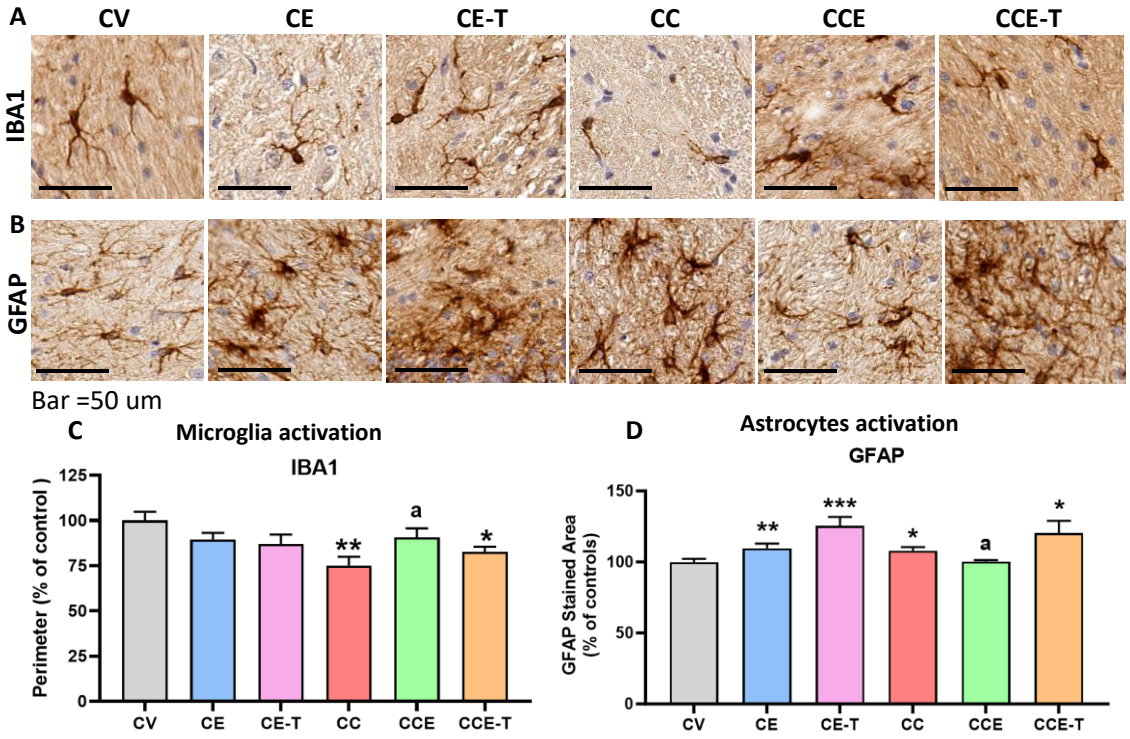

Figure 34. Effects of MSCs-EVs on microglial and astrocytic activation in cerebellum. *Representative images of microglia marked with Iba-1 (A) and astrocytes marked with GFAP (B) in white matter of cerebellum. Mean ± SEM of 5-8 (microglia, C) and 3-7 (astrocytes, D) animals per group are represented in graphs. Data were analysed by One-Way ANOVA followed by the Uncorrected Fisher's LSD test. (C) C-EVs F=4.463, p=0.0121; T-EVs F=5.235, p=0.0079; (D) C-EVs F=4.257, p=0.0185, T-EVs F=6.944, p=0.0037. Asterisks indicate a significant difference compared with CV group *p<0.05, **p<0.01, ***p<0.001 and "a" compared with CC group a p<0.05.*

Microglia can be polarized towards classical (M1) or alternative (M2) type, being M1 considered pro-inflammatory and M2 anti-inflammatory. Levels of YM-1 (chitinase-3-like-3, pro-survival factor released by M2 microglia and a specific marker used to distinguish M2-microglia) in the cerebellum were assessed by western blot. Content of YM-1 is reduced (63.8±12.3 % of CV, p=0.0331) in CCl_4-injected rats, compared to the control rats (Figure 35) showing that microglia are closer to M1 type (pro-inflammatory). Treatment with T-EVs, but not C-EVs, reversed the alteration in YM-1 levels of rats with mild liver damage (108.3±14.9 % of CV, p=0.0106), indicating that this treatment increases proportion of M2 anti-inflammatory microglia in these rats.

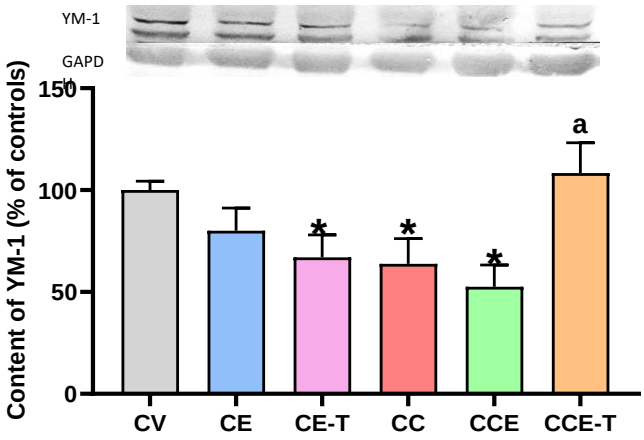

Figure 35. Effects of MSCs-EVs on YM-1 content in the cerebellum. *Values obtained by WB are expressed as percentage of control rats and mean ± SEM of 5-7 animals per group is represented. Data were analysed by One-Way ANOVA (F=3,931, p=0,0226) followed by the Uncorrected Fisher's LSD test. Asterisks indicate a significant difference p<0.05 in the multiple comparison tests compared with CV group, and "a" compared with CC group.*

9. MSC-EVs reduce TNF-α content in cerebellum and inhibits pathways that lead to GABA neurotransmission alteration

One of the pathways involved in neuroinflammation-induced increased levels of extracellular GABA is the TNF-α – glutaminase – glutamate transporters – GAT-3 pathway. TNF-α activates its TNRF1 receptor and this leads to activation of NF-kB that induces transcription of glutaminase. The increase of glutaminase activity increases glutamate levels and this can lead to activation of glutamate transporters that affect GABA transporters by modifying the gradient of sodium. Specifically, the content of GAT-3 and its activity is affected, as inflammation induce an alteration in GAT-3 activity that leads to release of GABA instead of GABA uptake (Cabrera-Pastor et al, 2018). Then, glutamate and GABA levels are both altered. Some key proteins of this pathway were analysed by western blot. Rats injected with CCl_4 showed increased levels of TNF-α (129.9±10.4 % of control group; p=0.0125) and glutaminase (144.0±16.1 % of control group; p=0.0098) compared to control rats (Figure 36A and 36B). TNF-α levels were reversed by both types of EVs (CCE 102.7±6.7 % of control group, p=0.0356; CCE-T 94.6±8.9 % of control group, p=0.0137; both compared with CC) (Figure 36A). Glutaminase levels were reduced by T-EVs (92.9±10.8 % of control group, p=0.0258) but not by C-EVs (130.7±12.6 % of control group) (Figure 36B). Interestingly T-EVs were

able to increase TNFα (122.0±9.4 % of control group, p=0.0391) and Glutaminase levels (147.9±13.9 % of control group, p=0.0409) in control rats. GAT-3 content in the cerebellum was not altered in rats with mild liver damage, although treatment with T-EVs in CCl_4-injected rats decreased GAT-3 content (49.2±9.9% of control group, p=0.0192) (Figure 36C). These results indicate that extracellular GABA levels could be reduced in rats with mild liver damage treated with T-EVs by reduction of the release of GABA by the GAT-3 transporter.

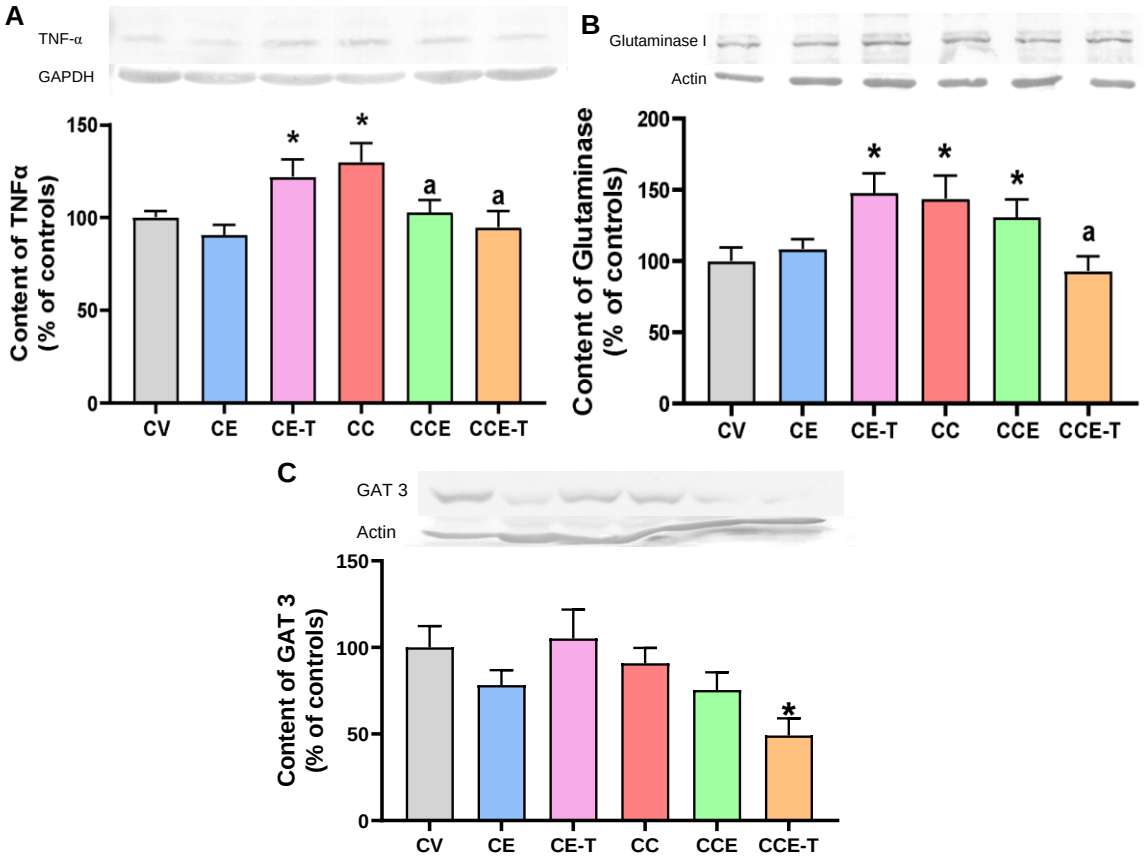

Figure 36. Effects of MSCs-EVs on TNF-α, glutaminase and GAT-3 content in cerebellum. *Data of content of TNF-α (n=16-28, A), glutaminase I (n=12-16, B) and GAT-3 (n=7-9, C) in cerebellum, analysed by western blot, are expressed as percentage of controls. Mean ± SEM is represented. (A) Data were analysed with Welch's ANOVA test followed by the Unpaired t with Welch correction test: C-EVs W(3000,37.93)=3.588 (p=0.0223), T-EVs W(3000,38.09)=3.945 (p=0.0152). (B) One-Way ANOVA was followed by the Uncorrected Fisher's LSD test (C-EVs F=2.987, p=0.0398) and by Tukey's multiple comparisons test (T-EVs F=5.410; p=0.0026). (C) One-Way ANOVA was followed by Tukey's multiple comparisons test (T-EVs F=4.695; p=0.0084). Asterisks indicate a significant difference p<0.05 in the multiple comparison tests compared with CV group, and "a" compared with CC group.*

Another pathway by which neuroinflammation may modulate GABAergic neurotransmission is the TNFα-TNFR1-CCl2-BDNF-TrkB pathway (described in the Introduction 7.2). TrkB activation modulates membrane expression of $GABA_A$ receptor subunits (Arenas et al, 2022). The main components of this pathway were analysed. As has already been shown TNF-α levels are increased by mild liver damage and this alteration is reversed by treatment with both types of EVs (Figure 36A). Moreover, mild liver damage enhances the activation of this pathway, increasing the levels of CCL2 (142.9±17.1 % of control group, p=0.0327; Figure 37A); the membrane expression of P2X4 (152.1±20.2 % of control group; Figure 37B); the content of BDNF (119.0±7.6 % of control group, p=0.0428; Figure 37C) and the content of its receptor TrkB (130.9±12.8 % of control group, p=0.0301; Figure 37D).

Treatment with C-EVs in CCl_4-injected rats reversed the membrane expression of P2X4 (88.2±12.3 % of control group, p=0.0062); the content of BDNF (90.5±7.3 % of control group, p=0.0118) and the content of TrkB (90.2±13.1 % of control group, p=0.0125); while treatment with T-EVs reversed all the components of the pathways that were analysed: CCL2 (89.8±9.7 % of control group, p=0.0138), membrane expression of P2X4 (91.7±11.9 % of control group, p=0.0163), content of BDNF (73.0±9.3 % of control group, p<0.0001 compared with CC, although p=0.0112 compared with CV) and TrkB (95.7±9.5 % of control group, p=0.0258). T-EVs increased content of CCL2 in control rats (159.2±13.9 % of control group, p=0.0013) (Figures 37A-D).

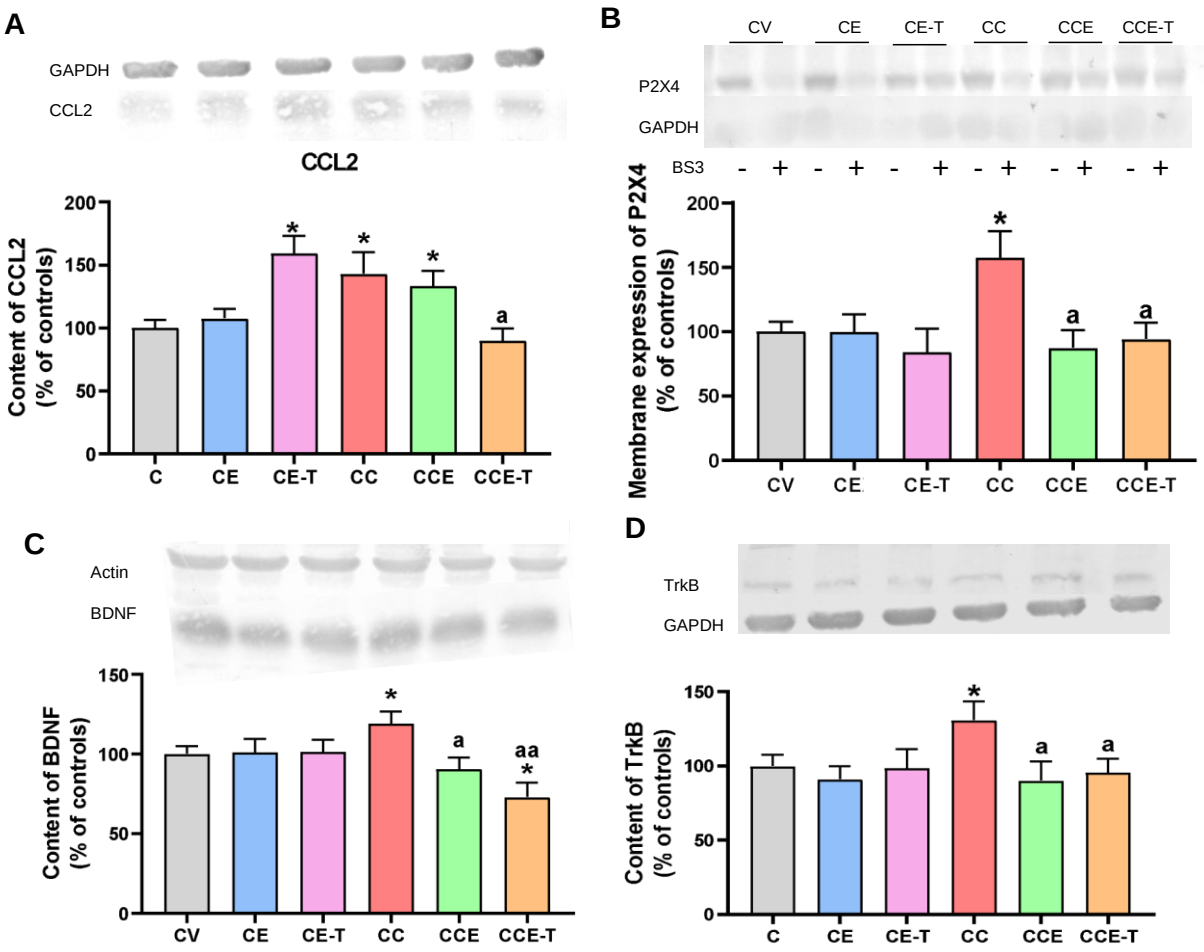

Figure 37. Effects of MSCs-EVs on TNFα-TNFR1-CCl2-BDNF-TrkB pathway. *Data of content of CCL2 (n=13-17, A), membrane expression of P2X4 (n=12-21, B), content of BDNF (n=11-15, C) and TrkB (n=9-15, D) in cerebellum are expressed as percentage of control rats and mean ± SEM is represented. (A) Data were analysed with Welch's ANOVA test followed by the Unpaired t with Welch correction test: C-EVs W (3000,27.25) =3.071 (p=0.0445), T-EVs W (3000,27.27) =7.207 (p=0.0010). (B) Brown-Forsyhte ANOVA was followed by the Dunn's Uncorrected test (T-EVs F (3.000,39.97) =4.683, p=0.0068). For the C-EVs the Kruskal-Wallis test was used (p=0.0509). (C) One-Way ANOVA was followed by Uncorrected Fisher's LSD test (T-EVs F=6.388; p=0.0010). (D) One-Way ANOVA was followed by the Uncorrected Fisher's LSD test (C-EVs F=3.175, p=0.0344). Asterisks indicate a significant difference compared with CV group *p<0.05, and "a" compared with CC group a p<0.05, aa p<0.01.*

10. Effects of MSCs-EVs on peripheral inflammation in rats with mild liver damage

Some pro- and anti-inflammatory factors were analysed in plasma to assess peripheral inflammation (Figure 38). Cytokine levels in peripheral blood were disrupted in rats with mild liver damage. Rats injected with CCl_4 showed increased levels of pro-inflammatory cytokines TNF-α (154.7±21.1 % of CV, p=0.0479), IL-6 (139.2±9.5 % of CV, p=0.0089) and IL-15 (129.5±12.7 % of CV, p=0.0375), and of HMGB1 (136.5±14.3 % of CV, p=0.0371) and reduced levels of TGF-β (70.0±4.6 % of CV, p=0.0467), IL-17 (83.3±5.7 % of CV, p=0.048), and IL-21 (73.0±5.1 % of CV, p=0.0105).

In general, treatment with MSCs-EVs reversed these alterations. Both types of MSCs-EVs, C-EVs, and T-EVs were able to normalize levels of TNF-α (CCE 75.2±12.8 % of CV, p=0,0268; CCE-T 27.9±14.8 % of CV, p=0.0012), TGF-β (CCE 100.7±10.9 % of CV, p=0,0493; CCE-T 117.4±16.0 % of CV, p=0.0114), IL-17 (CCE 116.0±6.5 % of CV, p=0,0003; CCE-T 112.2±8.3 % of CV, p=0.005), and IL-21 (CCE 105.9±5.5 % of CV, p=0,0018; CCE-T 10582±6.7 % of CV, p=0.0058). IL-6 levels were normalized by T-EVs (93.7±7.1 % of CV, p=0.0057) and partially by C-EVs (123.8±11.1 % of CV). HMGB1 levels were normalized by C-EVs (98.7±10.4 % of CV, p=0.049), but not by T-EVs. However, IL-15 levels were not normalized by any type of MSCs-EVs. MSCs-EVs also had some effect on control rats. C-EVs reduced levels of HMGB1 (76.8±7.8 % of CV, p=0.0389) and IL-17 (84.0±5.5 % of CV, p=0.047) while T-EVs increased levels of IL-15 (137.1±14.7 % of CV, p=0.0165).

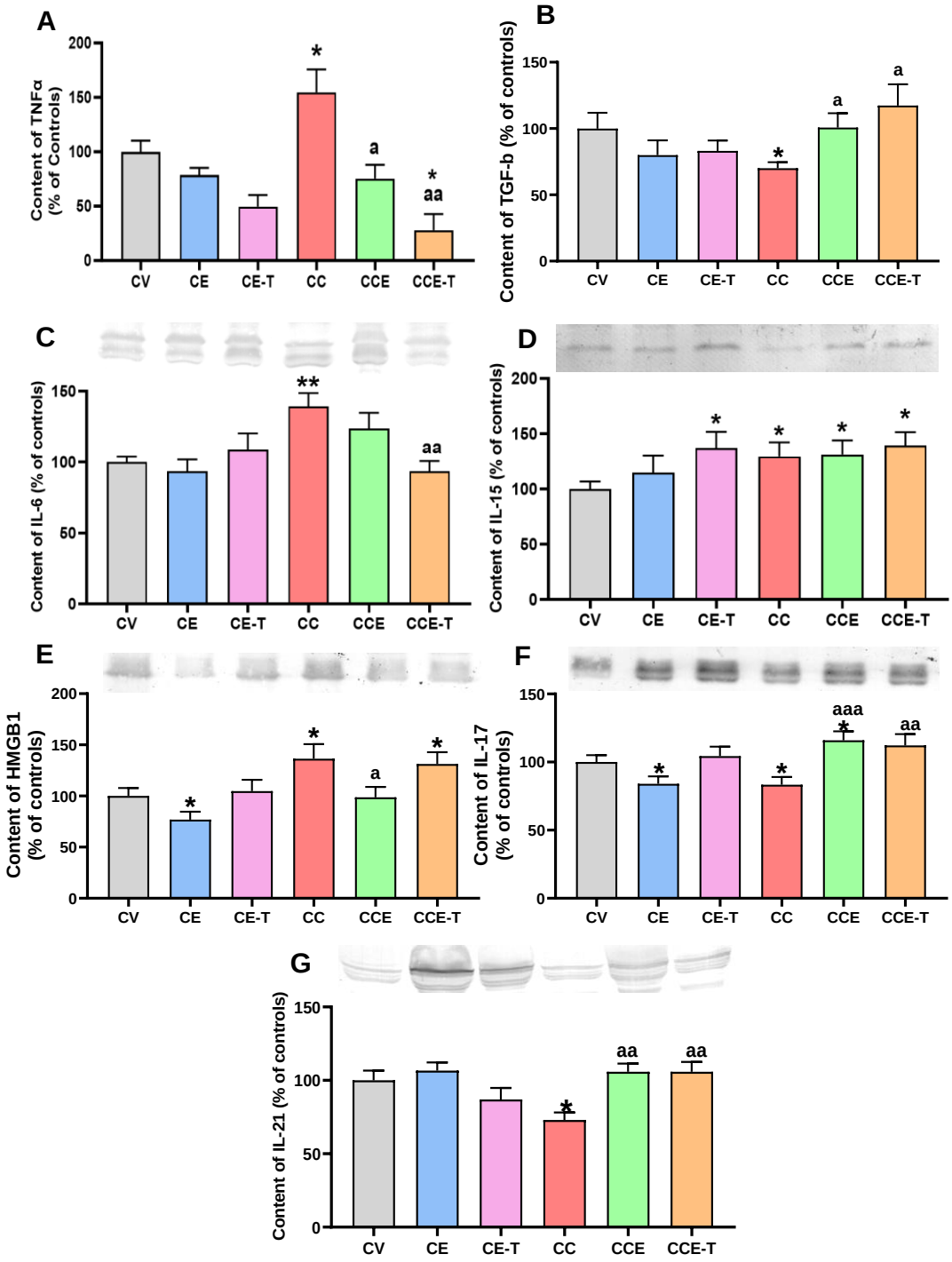

Figure 38. Effects of MSCs-EVs on peripheral inflammation. *Data obtained by ELISA of TNF-α (A, n=4-16) and TGF-β (B, n=11-15) and data obtained by Western blot of IL-6 (C, n=11-19), IL-15 (D, n=7-13), HMGB1 (E, n=9-19), IL-17 (F, n=12-17), and IL-21 (G, n=8-12) are expressed as percentage of control and mean ± SEM is represented. (A) Data were analysed with Welch's ANOVA test followed by the Dunnett's T3 multiple comparisons test C-EVs W(3.000, 22.29)=4.473 (p=0.0113) and One-way ANOVA followed by Tuckey test T-EVs F=7.933 (p=0.0004). (B) Welch's ANOVA test was followed by the Unpaired T test with*

*Welch's correction (T-EVs W(3.000, 24.91) =4.082, p=0.0173). (C) Welch's ANOVA test followed by the Dunnett's T3 multiple comparisons test T-EVs W(3000,24.35)=5.370 (p=0.0056) (D) One-Way ANOVA was followed by the Uncorrected Fisher's LSD test (T-EVs F=3.238, p=0.0345). (E) Kruskal-Wallis (statistic=12.73, p=.0052) test was used followed by uncorrected Dunn's test. (F) One-Way ANOVA was followed by the Uncorrected Fisher's LSD test (C-EVs F=7.106, p=0.0004; T-EVs F=3.035, p=0.0369). (G) One-way ANOVA followed by Tuckey test (C-EVs F=6.874, p=0.009; T-EVs F=4.942 (p=0.0055). Asterisks indicate a significant difference compared with CV group *p<0.05, **<0.01 and "a" compared with CC group a p<0.05, aa p<0.01, aaa p<0.001.*

11. Effects of MSCs-EVs on liver damage

Liver damage was assessed histologically in liver sections stained with H&E and also Masson staining was used to assess liver fibrosis in other sections. A scale from 1 to 4 grade, was used to quantify steatosis, lobular inflammation and fibrosis grade (Kleiner et al., 2005)

After 4 weeks of CCl_4 injection, rats show liver steatosis (1.17±0.11 p<0.001), lobular inflammation (1.50±0.20; p=0.0024) and fibrosis in liver (1.15±0.25 p=0.0214) compared with control rats (0.0±0, 0.50±0.17, 0.36±0.15, respectively) (Figures 39A-E). C-EVs treatment did not improve liver steatosis in rats (0.83±0.17) that was high compared to the control rats (p=0.0036). However, rats with mild liver damage treated with T-EVs (0.64±0.15) show significantly reduced steatosis compared with CCl_4-injected rats without treatment (p=0.0144) although it remains increased compared with the control group (p=0.0301) (Figure 39B). Histologically analysed inflammation and fibrosis were not affected by MSCs-EVs treatments. EVs did not improve lobular inflammation which was higher than in the control rats in liver damage rats treated with both types of EVs (CCE 1.17±0.21 p=0.032; CCE-T 1.71±0.20 p=0.0327). Treatment with both types of EVs was not able to improve liver fibrosis that was similar to that in untreated CCl_4 injected rats (CCE 1.08±0.26 p=0.0398 and CCE-T 1.25±0.22 p=0.0092, compared with CV) (Figures 39C and 39E). Lobular inflammation tended to be increased in control rats treated with T-EVs (1.25±0.35, p=0.07) (Figure 39C).

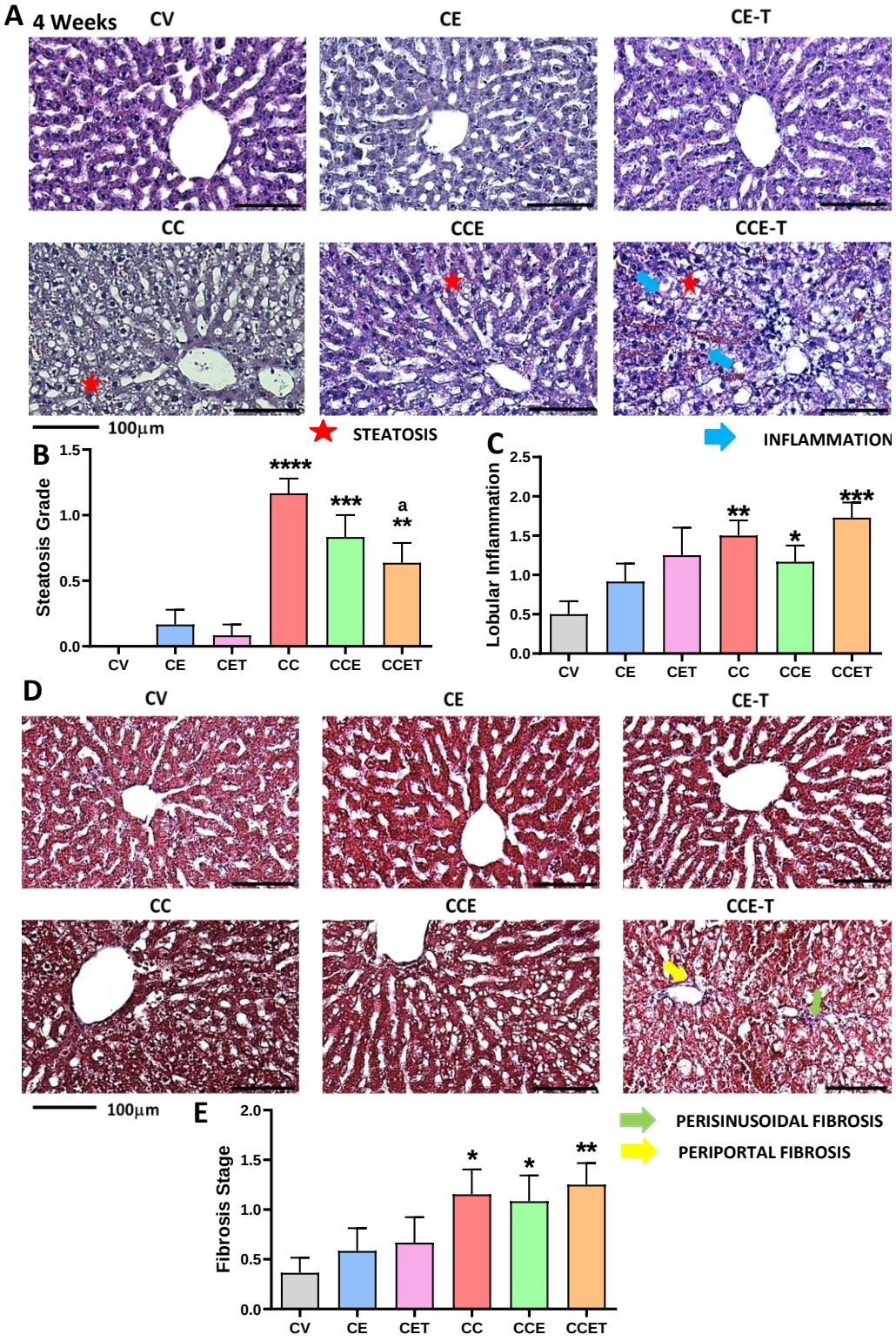

Figure 39. Effects of MSCs-EVs on mild liver damage. *Representative images of steatosis and lobular inflammation (A) and fibrosis (D) in liver at 4 weeks of CCl_4 administration. Score of steatosis (B), lobular inflammation (C) and fibrosis (E). Mean ± SEM of 10-13 animals per group is represented. Analysis was made with Kruskal-Wallis test followed by Dunn's test (B) or uncorrected Dunn's test (C and E). (B) C-EVs statistic=30.08 (p<0.0001) T-EVs*

*statistic=31.54 (p<0.0001). (C) C-EVs statistic=9.958 (p=0.0189) T-EVs statistic=12.28 (p=0.0065). (E) T-EVs statistic=8.936 (p=0.03). Asterisks indicate a significant difference compared with CV group *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001 and "a" compared with CC group a p<0.05.*

Macroscopic tissue damage was not observed in livers of all groups at 4 weeks of CCl_4 administration (Figure 40A). However, livers from rats sacrificed at 12 weeks of CCl_4 administration showed some macroscopic alterations that could be detected by eye. As can be observed in Figure 40B, livers from rats injected with CCl_4 with and without EVs treatment (Groups CC, CCE and CCE-T) were browner and paler in color, while rats injected with corn oil had more reddish livers. Moreover, rats injected with CCl_4 showed livers with an altered texture (Figure 40B).

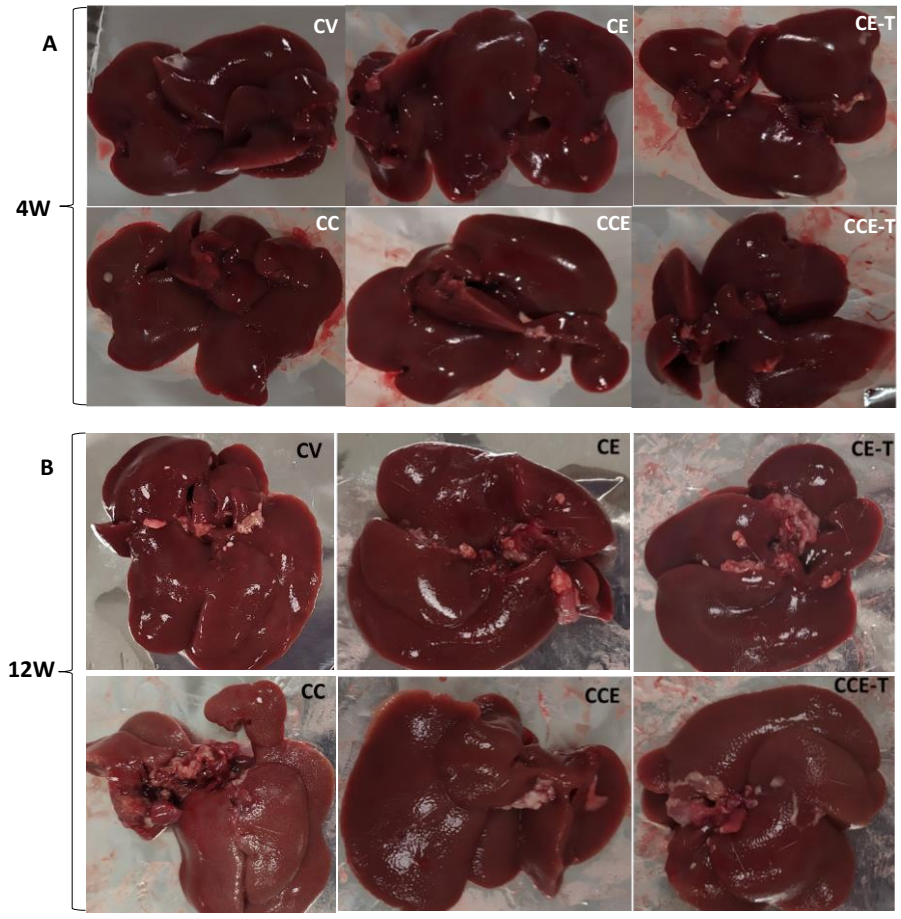

***Figure 40. Representative images of liver at 4 (A) and 12 (B) weeks of CCl4 administration**. Differences in color and texture can be observed in the livers from rats injected with the toxin (groups CC, CCE and CCE-T) compared with the rats injected with corn oil (CV, CE and CE-T) at 12 weeks (B).*

After 12 weeks of CCl_4 administration, CCl_4 injected rats did not show a significant increase in the grade of steatosis compared with the control rats. Moreover, treatment with EVs did not alter steatosis (Figure 41A and 41B). However, CCl_4-injected rats presented increased grade of lobular inflammation (2.4±0.4 p=0.0146) compared to control rats (0.3±0.3) (Figure 41C). Any of the EVs treatments were not able to reduce this alteration (CCE 2.7±0.3 p=0.0128; CCE-T 2.7±0.3 p=0.0157 compared with CV). Rats injected with CCl_4 also had a higher grade of fibrosis (2.4±0.7 p=0.0392) than control rats, in which was zero (Figure 41D and 41E). Fibrosis stage at 12 weeks was not reduced by any of the EVs treatments (CCE 3.0±0.0 p=0.019; CCE-T 3.0±0.0 p=0.0137 compared with CV).

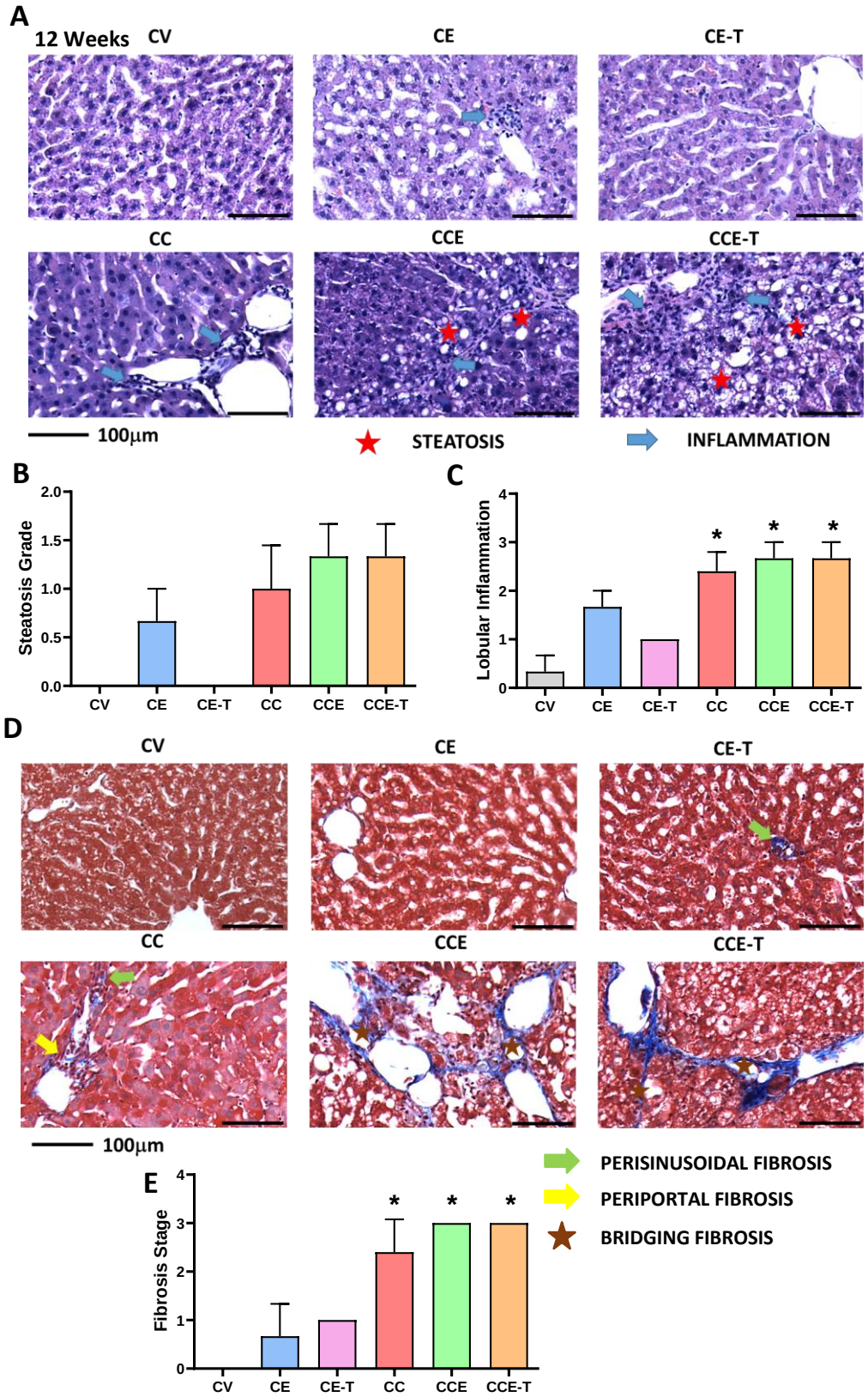

Figure 41. Effects of MSCs-EVs on advanced liver damage. *Representative images of steatosis and lobular inflammation (A) and fibrosis (D) in liver at 12 weeks of CCl_4 treatment. Score of steatosis (B), lobular inflammation (C) and fibrosis (E). Data are mean ± SEM of 2-5 animals per group. Analysis was made with Kruskal-Wallis test followed by uncorrected*

*Dunn's tests. (C) C-EVs statistic=8.12 (p=0.0176) T-EVs statistic=8.482 (p=0.0127). (E) C-EVs statistic=8.089 (p=0.0218) T-EVs statistic=7.459 (p=0.0321). Asterisks indicate a significant difference compared with CV group *p<0.05.*

Liver fibrosis can be also assessed by immunohistochemistry or western blot as an increase of the presence of collagen, the main extracellular protein forming fibers in liver. In addition, the content of α-SMA is also increased in liver fibrosis. Therefore, liver fibrosis markers collagen I and α-SMA in the liver were analysed by Western blot (Figure 42) and by immunohistochemistry (Figure 43).

Rats injected with CCl_4 showed increased levels of collagen I (158.7±17.7 % of CV, p=0.0217, Figure 42A) and α-SMA (126.1±11.3 p=0.0283, Figure 42B) in liver at 4 weeks of administration. T-EVs but not C-EVs were able to normalize completely the content of Collagen I (104.3±12.4 % of CV, p=0.0143 compared with CC) and α-SMA (91.9±7.2 % of CV, p=0.0067) in rats with mild liver damage. C-EVs also reduced the increase of α-SMA in the liver of rats treated with CCl_4 (106.7±8.0 % of CV), although no significant difference with untreated CCl_4 injected rats was reached.

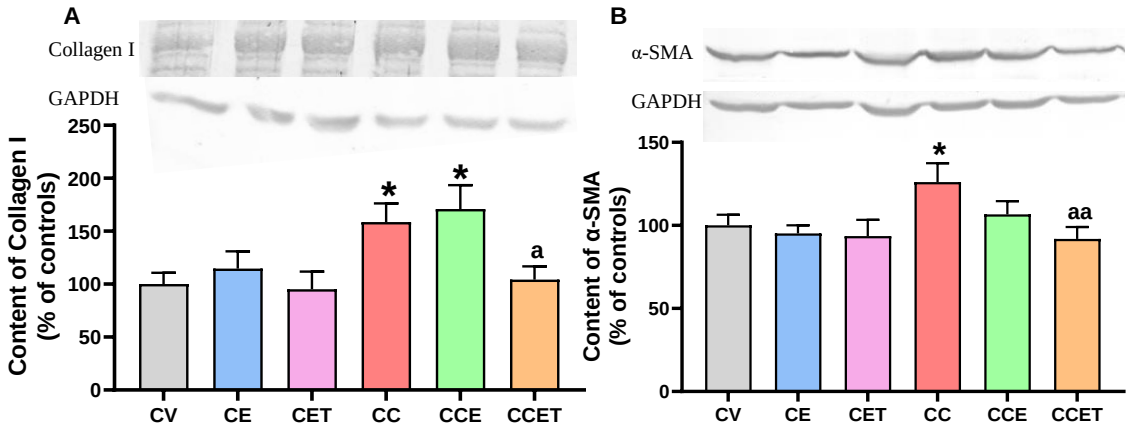

Figure 42. Effects of MSCs-EVs on markers of liver fibrosis. *Content of collagen I (A, n=8-11) and α-SMA (B, n=14-21) in liver was analysed by western blot are represented as mean±SEM. Analysis was made with One-Way ANOVA followed by the Uncorrected Fisher's LSD test. (A) C-EVs F=3.928 (p=0.0157); T-EVs F=4.338 (p=0.011). (B) T-EVs F=3.266 (p=0.0269). Asterisks indicate a significant difference compared with CV group *p<0.05, and "a" with the CC group a p<0.05, aa p<0.01.*

These same liver fibrosis markers were analysed in liver sections by immunohistochemistry (Figure 43). α-SMA showed increased intensity of immunostaining in rats with mild liver damage (13.5±1.6 % of stained area, p=0.0002) compared to the control group (1.4±0.4 % of stained area). These values were reduced by both C-EVs (6.3±0.4 % of stained area, p=0.0027) and T-EVs (7.9±2.2 % of stained area, p=0.025). However, intensity of immunostaining of α-SMA in both groups of CCl_4-injected rats treated with MSCs-EVs was higher than in the control group (C-EVs p=0.0276, T-EVs p=0.0218). In addition, C-EVs increased α-SMA levels in the control rats (5.7±1.2 % of stained area, p=0.0463). Intensity of staining of collagen I was not significantly altered by CCl_4 injection nor by treatment with MSC-EVs (Figure 43B).

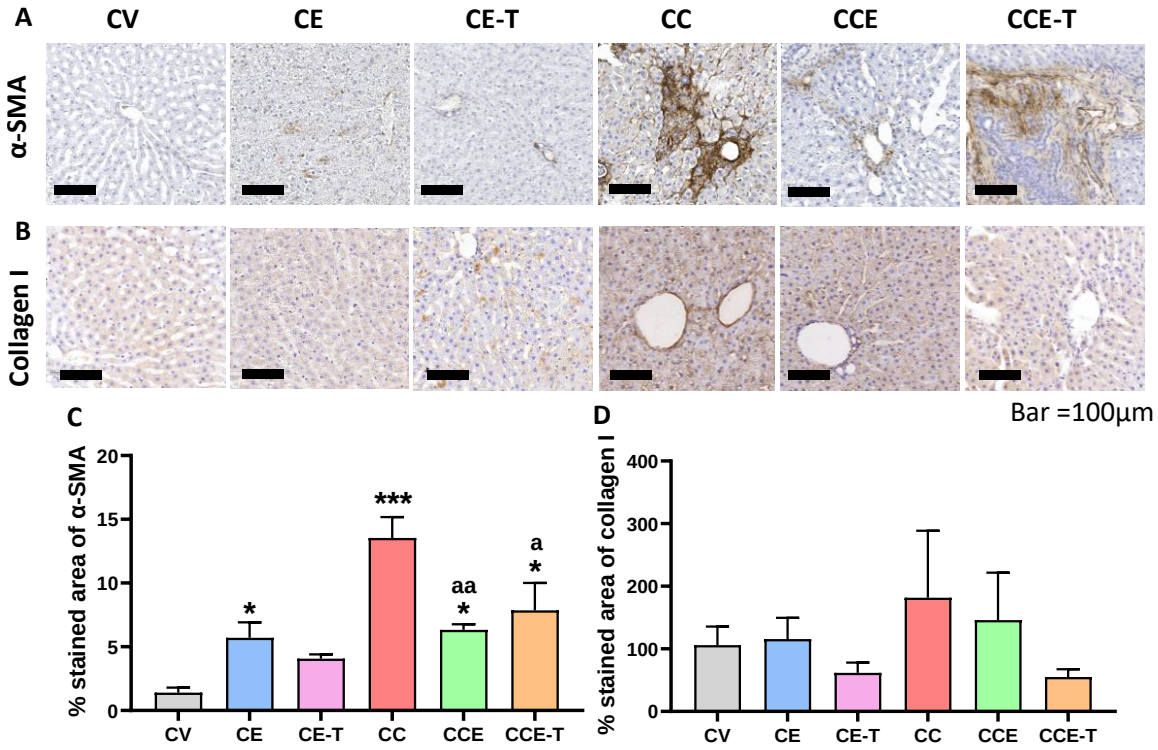

Figure 43. Effects of MSCs-EVs on markers of liver fibrosis by immunohistochemistry. *Representative images of α-SMA (A) and collagen I (B) immunostaining. The percentage of stained area with α-SMA (C, n=3) and collagen I (D, n=3-5) in liver is represented as mean ± SEM. Analysis was made with One-Way ANOVA followed by the Uncorrected Fisher's LSD test. (C) C-EVs F=17.25 (p=0.0013); T-EVs F=16.52 (p=0.0026). Asterisks indicate a significant difference compared with CV group *p<0.05, **p<0.01, ***p<0.001 and "a" with the CC group a p<0.05, aa p<0.01.*

Liver function was assessed by measuring activity of the hepatic enzymes AST, ALT, and alkaline phosphatase in serum, and serum content of bilirubin and bile acids. Rats with mild liver damage show a significant increase of ALT (CC 45.9±5.8 U/L compared with 23±2 U/L in CV; p=0.0173, Figure 44A) and alkaline phosphatase (CC 287.1±10.9 vs CV 216.3±7.9 U/L; p=0.0039, Figure 44C) activities and bile acids content (CC 23.8±2.8 µM, CV 10.2±2.3 µM, p=0.0239, Figure 44E) in serum after 4 weeks of CCl_4 administration.

C-EVs treatment in rats with mild liver damage did not reverse the increase of ALT (47.2±6.1 U/L; p=0.0146 compared with CV, Figure 44A), bile acids content (29.1±4.8 U/L; p=0.0022 compared with CV, Figure 44E) nor alkaline phosphatase activity (349.8±12.8 U/L; p<0.0001 compared with CV) that was even potentiated by C-EVs treatment in rats with mild liver damage (p=0.0162 compared with CC) and was also significantly increased in control rats (278.8±18.7 U/L, p=0.0148) (Figure 44C). In addition, C-EVs increased AST activity in serum of CCl_4-injected rats (181.0±19.5 U/L, Figure 44B) compared with the control rats (109.0±12.7 U/L; p=0.0348).

In contrast, rats with mild liver damage treated with T-EVs showed reduced ALT activity (33.2±3.4 U/L) compared with the untreated rats, although this effect was not statistically significant (Figure 44A). It was also observed a mild reduction of bile acids content (18.5±3.1 U/L) (Figure 44E).

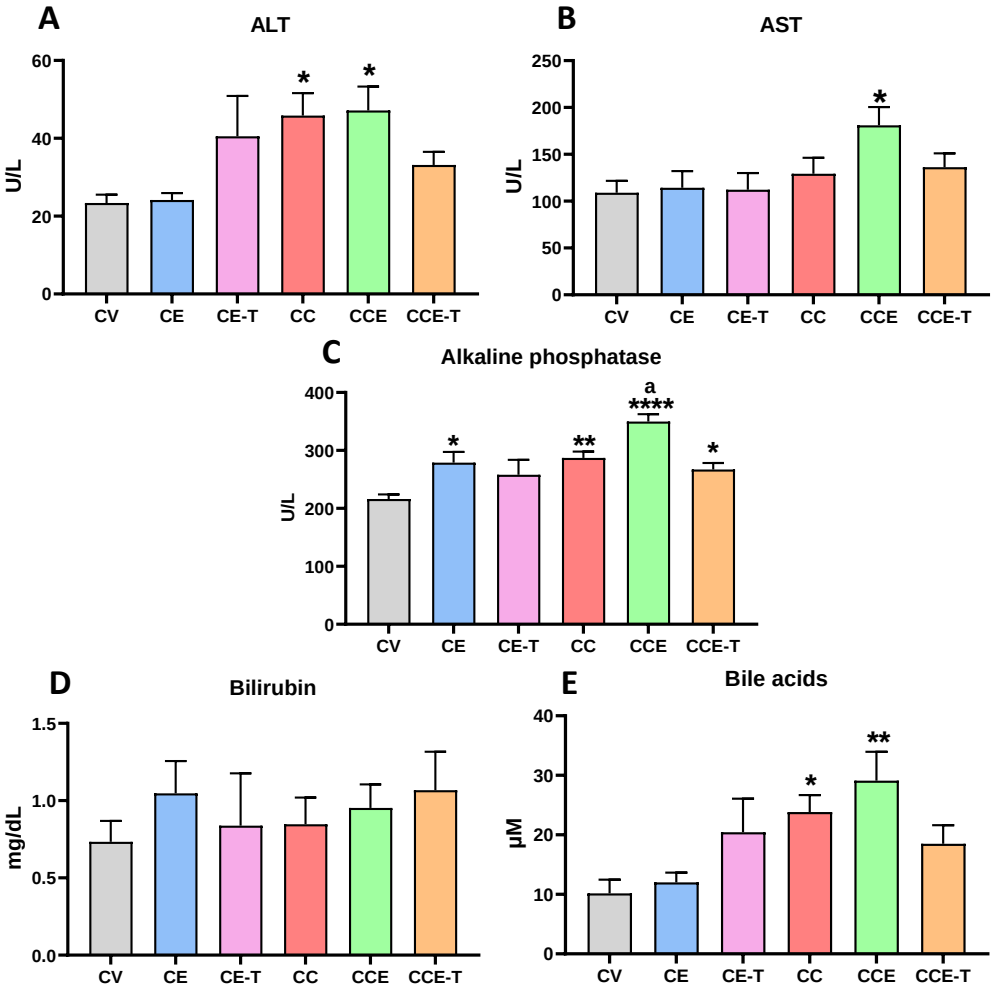

Figure 44. Effects of MSCs-EVs on serum markers of liver damage. *The mean ± SEM of 4-7 animals per group is represented: ALT (A), AST (B), alkaline phosphatase (C), bilirubin (D) and bile acids (E). Data were analysed with One-Way ANOVA test followed by Tukey's multiple comparisons test. (A) C-EVs: F=7.55, p=0.0014 (B) C-EVs: F=3.745, p=0.0259 (C) C-EVs F=15.84; p<0.0001 and for T-EVs the Welch ANOVA (W(3,000, 8,746)= 9,303, p=0.0044) was followed by the Dunnett's T3 multiple comparisons tests. (D) ANOVA was not significant. (E) C-EVs F=8.347, p=0.0008). The ANOVA of T-EVs were not significant. Asterisks indicate a significant difference compared with CV group *p<0.05,**p<0.01, ****p<0.0001 and "a" compared with CC group a p<0.05.*

Some cytokines were measured in the liver by Western blot to assess liver inflammation (Figure 45). Rats injected with CCl_4 showed increased levels of TNF-α (143.2±15.7 % of CV) compared to the control group (p=0.024). The TNF-α levels were normalized by treatment with C-EVs (99.0±9.5 % of CV, p=0.0292) but not T-EVs (193.1±24.3 % of CV, Figure 45A).

Content of IL-6 and TGF-β was not altered by CCl_4 injection nor by treatment with any of the EVs (Figures 45B and 45C)

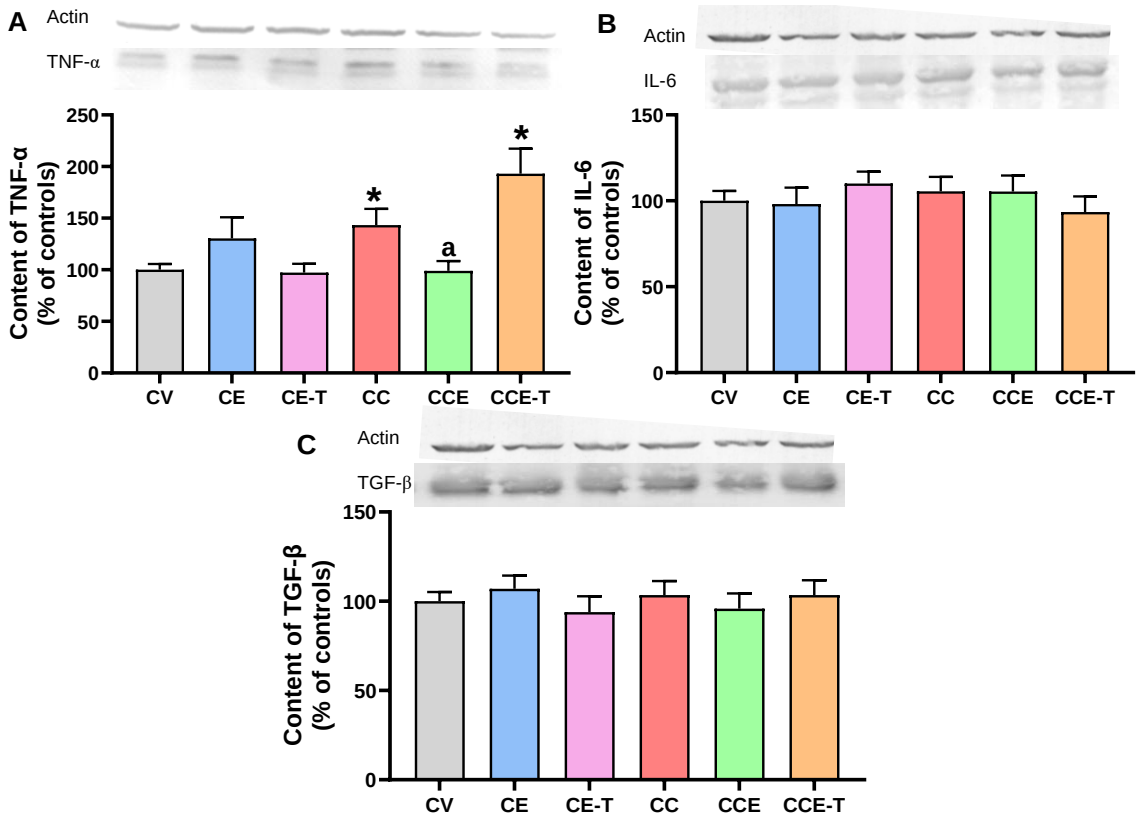

Figure 45. Effects of MSCs-EVs on liver inflammation measured by Western Blot. *Data of content of TNF-α (n=10-13, A), IL-6 (n=12-21, B), and TGF-β (n=11-15, C) are expressed as percentage of control rats and mean ± SEM is represented. Data were analysed with Welch's ANOVA test followed by the Unpaired t with Welch correction test. (A) C-EVs W(3,000, 19,73)=2,664, p=0.0761 T-EVs W(3000,21.22)=6.463 (p=0.0028). Statistical analysis in (B) and (C) did not show any significant difference. Asterisks indicate a significant difference compared with CV group *p<0.05, and "a" compared with CC group a p<0.05.*

12. Effects of MSCs-EVs on gut microbiota in rats with mild liver damage

The analysis of individual fecal microbiota composition indicated that none of the alpha diversity descriptors assessed showed important shifts across the experimental groups (Figure 46A). However, when this analysis was achieved at the microbial community structure level (beta diversity), meaningful different composition among treatment groups was detected. This microbial community composition was mainly explained by the treatment itself (Adonis R2=0.129, p=0.006). The two covariates explored during analysis, weight gain and sample sequencing pool, exhibited almost null impact on the community structures (Figure 46B). The evaluation of differential abundance across experimental groups was completed by assessing the distribution of more than 3,500 OTUs. We detected significant signals of differential abundance in almost 200 OTUs, 79 of which were prioritized to have the most prominent variation based on LMM score with covariate control (weight gain and sequencing pool). To narrow down the list of OTUs to be associated with treatments, the focus was placed on those with reliable taxonomy identification (genus level) and those exhibiting the reversion of changes seen group with mild liver damage (CC) versus the control group (CV). A predominant presence of OTUs identified as members of the *Bacteroides* genus was detected as well as OTUs identified as *Intestinimonas, Acetatifactor*, *Anaerostipes* and multiple *Muribaculacea/Muribaculum* species. To contribute to disambiguating in the *Bacteroides* taxonomy annotation, we further assessed OTU identity by using SINA aligner (SILVA database) and Blast (NCBI Taxonomy database) automatic servers. We found that *Bacteroides* species likely match with OTU24, OTU129, and OTU148 were *B. eggerthii, B. cellulosilyticus, and B. caccae*, respectively. These *Bacteroides* OTUs were increased in rats with mild liver damage and decreased by C-EVs treatment towards abundance seen for the control group (Figure 46C). On the other hand, the OTU123 (likely *Anaerostipes faecis*) was also increased in rats with mild liver damage but exclusively reduced by treatment with T-EVs (Figure 46C). The comparison of microbiota and cognitive and motor function data using rank-based approaches distinguishing more accurate concordant and discordant pairs of data indicates that OTUs 24 and 148 correlate positively with dual stance (Kendall > 0.22, $p < 0.04$), OTU148 correlates negatively with stride length (Kendall = -0.25, $p = 0.022$), and OTU24 correlates positively with step cycle (Kendall = 0.23, $p = 0.033$) being all locomotor gait parameters measured in the CatWalk.

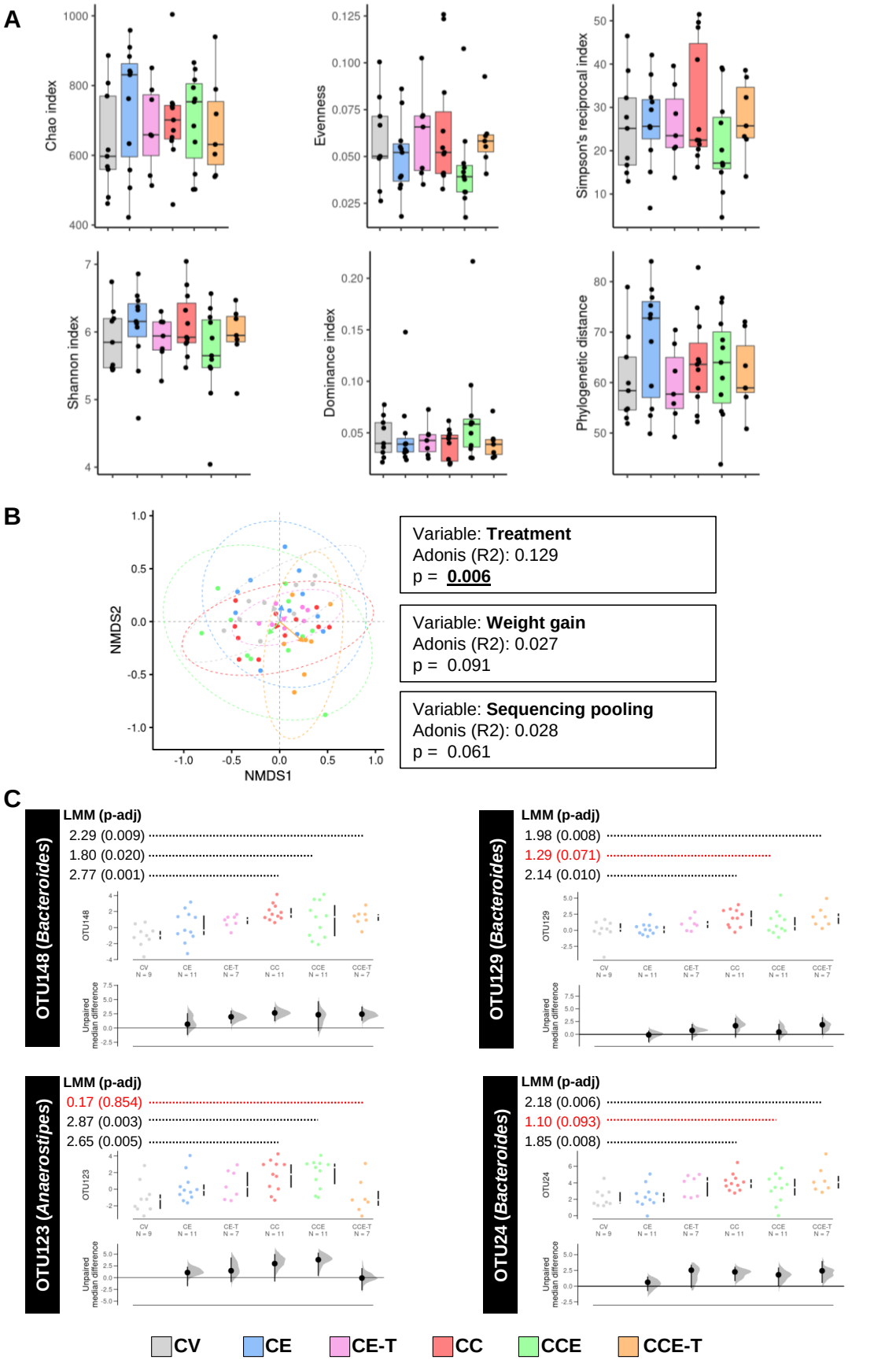

Figure 46. Gut microbiota analysis of rats with mild liver damage and effects of MSC-EVs treatment. *A) The alpha diversity assessment including study of the Chao's index, Simpson's*

evennees index, Simpson's reciprocal index, Shannon index, Dominance index, and PD descriptors. Color legend and group labelling is shown in the legend at bottom. Alpha diversity data is presented in a boxplot manner (N = 58 samples) no significantly different data distributions were obtained following Kruskal-Wallis statistical appraisal and Wilcoxon Rank Sum test pairwise comparisons. B) Beta diversity evaluation of the fecal microbial community structure is provided using distance-based redundancy analysis (dbRDA). The two gradients of dataset dispersion in ordination space explaining more variation of this constrained approach are shown in a scatter-plot fashion. Dashed lines circumscribe the confidence interval (95%) for distribution of respective grouped samples. The result of the Adonis test is depicted in the text boxes embedded. Arrows' heads point out the respective centroids of data dispersion. C) Gardner-Altman estimation plots. Clr-normalized abundance of DNA reads for selected OTUs was processed with Dabestr R package to obtain graphics based on shared control analysis, median difference as size effect, and CI 95%. CV data was assumed as reference data. Estimate of LMM score from comparison between control and CCl_4 groups (CC, CCE and CCE-T) and exact adjusted p-values are shown at top of respective panels, respectively.

DISCUSSION

As previously described, liver cirrhosis may lead to MHE in patients, a syndrome that courses with cognitive and motor impairment and may lead to clinical HE (Felipo, 2013). Patients with chronic liver diseases may show cognitive and motor deficits even without reaching cirrhosis (Gimenez-Garzo et al., 2021) In addition, patients deceased with steatohepatitis showed neuroinflammation and neuronal loss in cerebellum and hippocampus (Balzano et al., 2018; Leone et al., 2023). Then, it is of great interest to analyse mechanisms leading to these alterations in preclinical animal models of mild liver damage as steatohepatitis or even fatty liver disease. In addition, it is necessary to test therapeutic options that reduce, prevent or delay neurological alterations in animal models of mild liver damage, which can be translated to patients with steatohepatitis or fatty liver disease, which present a decreased quality of live due to mild cognitive impairments and motor deficits (Colognesi et al., 2020b; Kjaergaard et al., 2021).

In recent years, the interest in the use of MSC-EVs as a treatment for several diseases has increased. The first objective of this project was to assess if MSC-EVs are able to reduce the alterations produced by mild liver damage in a rat model. As has been shown, MSC-EVs are able to reduce several alterations produced in cognitive and motor functions, neuroinflammation in the cerebellum and hippocampus, and to reverse alterations in glutamatergic and GABAergic neurotransmission in both areas. MSC-EVs also reduce peripheral inflammation, liver steatosis and fibrosis, and liver inflammation. The second objective of the project was to analyse if TGF-β could have an important role in the effects of MSCs-EVs treatment. Our results showed several different effects dependent on the type of EVs used as a treatment, thus meaning there are involved mechanisms dependent on TGF-β in the effects of EVs and that this cytokine can have a main role modulating the alterations induced by mild liver damage.

1. MSCs-EVs improve cognitive impairment and alterations in the hippocampus of rats with mild liver damage. Role of TGF-β

Generally, impaired cognitive function such as altered spatial learning and memory are related to alterations in neurotransmission in the hippocampus. Moreover, neuroinflammation largely contributes to alteration of neurotransmission. This project supports these relations in rats with mild liver damage induced by CCl_4, as previously demonstrated (Leone et al., 2022). These results point out that MSC-EVs are able to reduce neuroinflammation and restore alterations in neurotransmission in the hippocampus, and improve several cognitive alterations in CCl_4-injected rats. MSCs-EVs including those derived from human adipose tissue, were reported to have beneficial effects on neurodegenerative diseases and its mechanism included reduction of neuroinflammation (Sanchez-Castillo et al., 2022; Harrell et al., 2022).

The results of this work support that rats with mild liver damage show impairment of hippocampal-dependent spatial learning and memory, and working memory, as has been previously demonstrated (Leone et al., 2022). The effects of MSCs-EVs on spatial learning and memory are greatly dependent on the task and also on the EVs content. Unmodified MSCs-EVs (C-EVs) treatment restores spatial memory measured in the NOL test but not spatial learning, working memory, or short-term memory. The analysis of hippocampal neuroinflammation showed that C-EVs also normalize microglia activation, TNF-α content, and increased IL-1β content in CA1 neurons, which was decreased in the rats with mild liver damage. Moreover, the analysis of some components of glutamatergic and GABAergic neurotransmission in the hippocampus showed that unmodified MSCs-EVs also restore the decrease in the membrane expression of GluN2B subunit and the increase of GABAA-γ2 subunit and the reduced content of isoform GAD65.

The NOL has been associated with GABAergic neurotransmission (Prut et al., 2010; Brymer et al., 2018). The reduction of the content of GAD65 could be altering GABAergic neurotransmission in rats with mild liver damage which is normalized by treatment with C-EVs. Decreased $GABA_A$-α5 (Prut et al., 2010) or $GABA_A$-β3 (Brymer et al., 2018), both impair NOL performance. Then, the decrease in membrane expression of these $GABA_A$ subunits in the hippocampus of CCl_4-treated rats must be contributing to the impairment of

NOL. However, treatment with C-EVs did not restore these alterations completely, although it improved NOL and also the decrease in membrane expression of GluN2B. It was reported that decreased expression of GluN2B in the hippocampus is associated with impairment of NOL memory test (Moraga-Amaro et al., 2016), suggesting that reversal of the decrease in membrane expression of this subunit by C-EVs must be involved in the improvement in NOL memory in CCl_4 rats (main results are summarized in Table 8).

Regarding the role of neuroinflammation, C-EVs decreased microglia activation and TNF-α levels in the hippocampus, although no astrocyte activation. In hyperammonemic rats MSCs-EVs (C-EVs) also reduce neuroinflammation, both microglia and astrocyte activation, in the hippocampus and improves cognitive function (Izquierdo-Altarejos et al., 2023). This difference in the C-EVs effect suggests that the mechanisms inducing astrocytes activation must be different in the different animal models, hyperammonemia or mild liver damage induced by CCl_4. Other treatments in hyperammonemic rats also improve neuroinflammation affecting only one type of glial cells. Bicuculline, antagonist of $GABA_A$ receptors, improves astrocytic activation but not microglia, in the hippocampus (Malaguarnera et al., 2019).

Elevated TNF-α levels in the hippocampus have also been associated with impaired NOL test (Brymer et al., 2018). Then, the decrease of TNF-α levels by C-EVs treatment must also contribute to the improvement of spatial memory in this task. In CA1 neurons, IL-1β content was decreased in CCl_4 rats, and levels were restored in CCl_4 rats treated with C-EVs. This reduced content of IL-1β in CA1 was previously observed after two weeks of CCl_4 administration and content of IL-1β increased thereafter, at four weeks of CCl_4, indicating same effect but a slightly different progression of the alterations between different experiments (Leone, 2020). The decrease of IL-1β has been reported in other situations, which also show neuroinflammation, with an increase in other pro-inflammatory cytokines but a decrease in IL-1β. For example, Terasaki and Schwarz (2017) reported a decrease in IL-1β in parallel with an increase in IL-6 levels in the hippocampus after alcohol administration and impairment of recognition memory (Terasaki & Schwarz, 2017). The decrease in IL-1β could also contribute to impaired hippocampal function, as a physiological role of IL-1β in the hippocampus on learning and memory was reported, as reduced signalling, decreased levels, or lack of this interleukin in the hippocampus decreases long-term potentiation and impairs hippocampal-dependent learning and memory (Avital et al., 2003; Goshen et al., 2007).

Levels of IL-1β modulate GABA and glutamate receptor subunits' expression and function. An increase in IL-1β induces an increase in the GluN2B receptor (Bertani et al., 2017), an increase in $GABA_A$-α5 membrane expression and function (Wang, Dian-Shi et al., 2012) and the increase in IL-1β in CA1 increases GABA receptor currents (Hellstrom et al., 2005). Then, we can suggest that the decrease in IL-1β in CA1 must contribute to the decrease in GluN2B and $GABA_A$-α5 subunits. Treatment with C-EVs restores IL-1β content and membrane expression of GluN2B and $GABA_A$-α5 subunits, supporting that these alterations are related to each other and involved in the NOL memory improvement. We have also observed that treatment with T-EVs in control rats induce NOL memory impairment and a significant decrease in GluN2B membrane expression, which supports the association between both effects. Since T-EVs also restore alterations in GABA receptors and in GAD65, but not microglia activation, IL-1β content in CA1 neurons and GluN2B membrane expression, these last should be the main mechanisms leading to NOL impairment, as T-EVs does not improve NOL test. This also suggests that TGF-β expression must mediate or modulate these events, given that lack of this cytokine eliminates these effects of EVs (Figure 47).

In contrast to the NOL memory test, both spatial learning and working memory in the radial maze that are impaired in rats with mild liver damage were improved by treatment with MSCs-EVs after silencing TGF-β (T-EVs), but not by C-EVs. In addition to $GABA_A$-α5 and γ2 subunits, also reversed by C-EVs, treatment with T-EVs restored the increased membrane expression of the $GABA_A$-α2 subunit and NKCC1, the decreased membrane expression of glutamate receptor subunit GluA2, astrocyte activation and the number of IL-$1\beta^+$ cells in the hippocampus parenchyma (not in CA1), which were not affected by C-EVs. This suggest that astrocytic activation can be responsible of the increase in IL-1β, which could induce the increase in membrane expression of the $GABA_A$-α2 subunit and the decrease in GluA2 membrane expression, that in turn, can induce impairment in learning and working memory in the radial maze (Figure 47). This sequence of events is all normalized by T-EVs treatment but not by C-EVs, indicating that TGF-β expression and the change in the cargo induced by it, leads to different and more positive effects than unmodified EVs, leading to improvement of learning and working memory. T-EVs did not reduce microglia activation, but only astrocytes activation (as occurs with bicuculline in hyperammonemic rats (Malaguarnera et al., 2019)). These results also suggest that the effects of EVs on the activation mechanisms of microglia and astrocytes depend on the presence or not of TGF-β in EVs, working in an opposite way,

and that they must be controlled by different mechanisms, and not as consequence one of the other.

It should also be noted that the assessment of these cognitive parameters in the radial maze were performed at more advanced state of liver damage (after eight weeks of CCl_4 administration) and this can also affect the effect of EVs, as neuroinflammation evolves as liver damage worsens (Leone, 2020).

The decrease in synaptic GluA2 has been related to impairment in spatial short- and long-term learning and memory (Sebastian et al., 2013). TNF-α-induced decrease in GluA2 membrane expression has been also reported in the hippocampus of hyperammonemic rats and this was associated with spatial memory impairment in the radial maze (Cabrera-Pastor et al., 2016). Impairment of spatial learning and working memory has been previously associated with astrocyte activation (Avila et al., 2018). Izquierdo-Altarejos et al (2023) showed that the reduction of neuroinflammation in the hippocampus and normalization of the associated alteration in glutamate neurotransmission by MSCs-EVs in ex-vivo hippocampal slices from hyperammonemic rats was dependent on TGF-β, as MSCs-EVs derived from MSCs with silenced TGF-β were not able to significantly reduce neuroinflammation. However, the results of this project show that this type of EVs are able to reduce astrocytic activation (but not microglial activation) produced by mild liver damage, after in vivo EVs administration, suggesting that peripheral effects can be involved in these effects of T-EVs, as discussed later.

Cl^- cotransporters also control GABAergic neurotransmission by maintaining Cl^- transmembrane gradient, which modulate the activity of $GABA_A$ receptors. The increased expression in the membrane of NKCC1 in the hippocampus has been related to repeated stress and cognitive and social behaviour alterations (Tsukahara et al., 2015).

Spatial short-term memory was impaired by both types of EVs in controls and in CCl_4-injected rats, no improving effect was found. Membrane expression of the $GABA_A$-β3 subunit was also reduced by all treatments. Several studies in knock-out mice for this subunit reported that it is required for inhibitory transmission in CA1 (Nguyen & Nicoll, 2018). The $GABA_A$-β3 subunit mediates the amnesic effects of propofol by alteration of the cortico-hippocampal interactions (Kreuzer et al., 2020) and the tonic inhibition induced by anaesthetics (Rau et al., 2011; Zarnowska et al., 2015). Then, decreased $GABA_A$-β3 subunit could be associated with

improvement of memory but impairment of inhibitory transmission in the hippocampus. Impairment of short-term memory has been related to increased inhibitory transmission (Daswani et al., 2022). However, we here found impairment of short-term memory and decrease of inhibitory transmission mediated by the reduction of $GABA_A$-β3 subunit, suggesting that other mechanism must mediate the impaired short-term memory. The reduction of GAD67 in the hippocampus was associated with the alteration of short-term memory in a rat model of schizophrenia (Riordan et al., 2018). Then, reduction of the content of GAD67, which was not improved by any of the EVs treatment, could contribute to impaired spatial short-term memory in rats with mild liver damage. The impairment of short-term memory in control rats treated with EVs can be induced by astrocytic activation, that can lead to release of other inflammatory or neurotoxic factors to induce this impairment in short-term memory. The mild reduction of astrocytes activation induced by T-EVs in CCl_4 rats is not able to improve short-term memory. Another possibility is that other mechanisms independent of astrocyte activation are involved in the impairment of short-term memory in rats with mild liver damage. Further studies may be necessary to clarify this.

Some alterations in GABAergic components were found (decrease of GAD65 and membrane expression of the α5 subunit and increase of membrane expression of α2 subunit of $GABA_A$ receptors) that were reversed by treatment with any type of EVs, C-EVs and T-EVs, suggesting that EV expression of TGF-β is not important for normalization of these alterations by EVs (Figure 47).

Our results suggest that EVs effects are highly dependent on their cargo and that TGF-β (Figure 47) has an important role in controlling their effects on the hippocampal alterations and on cognitive impairment produced by mild liver damage induced by CCl_4 administration in rats.

Table 8. *Main alterations found in the hippocampus and cognitive function.*

	ALTERATION	CCl_4	Improved by C-EVs	Improved by T-EVs
Behavior	Learning index and working memory	↓		⇧
	NOL	↓	↑	
	Short-term memory	↓		
Neurotransmission	GluN2B (NMDA)	↓	↑	
	GluA2 (AMPA)	↓		↑
	GAD65	↓	↑	↑
	GAD67	↓		
	$GABA_A$-α2	↑		↓
	$GABA_A$-α5	↓	⇧	↑
	$GABA_A$-γ2	↑	↓	↓
	$GABA_A$-β3	↓		
	NKCC1	↑		↓
Neuroinflammation	Microglia activation	↑	↓	
	Astrocytes activation	↑		↓
	IL-1β in CA1	↓	↑	
	IL-1β^+ cells	↑		↓
	TNF-α	↑	↓	

Red arrows indicate alterations produced by CCl_4, green arrows indicate normalization by C-EVs, orange arrows indicate normalization by T-EVs. Empty arrows indicate normalization without significant difference with CC and CV groups.

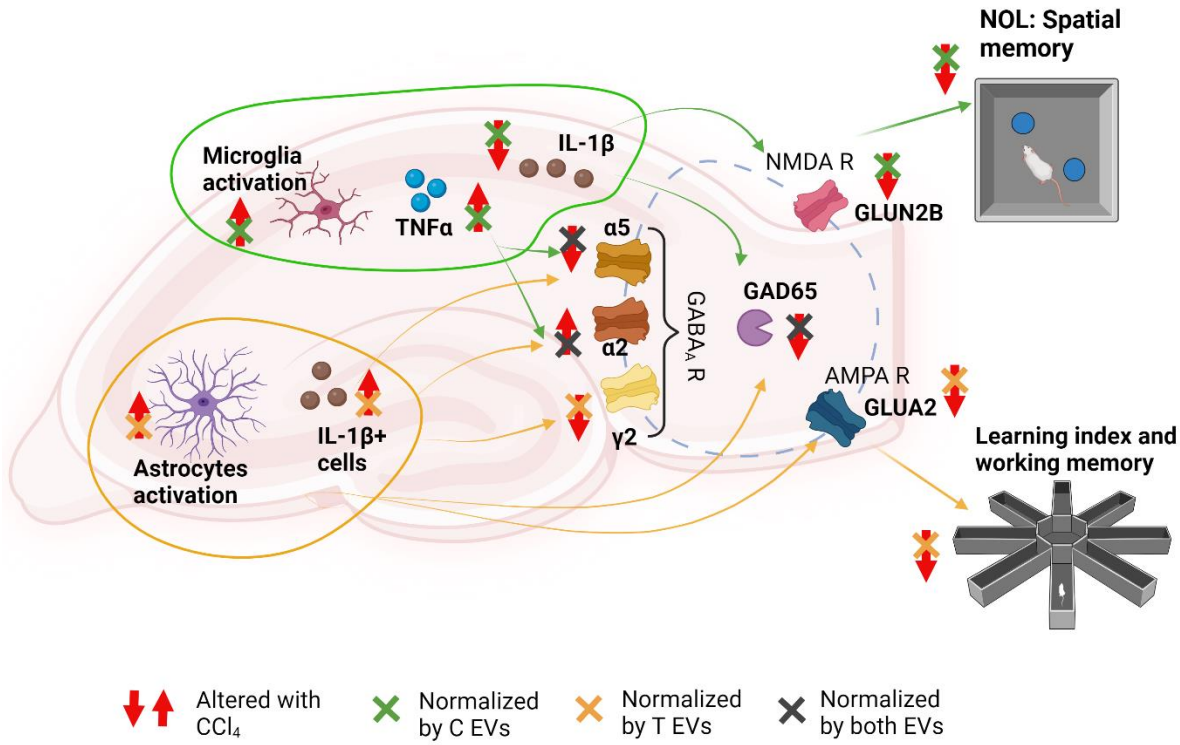

Figure 47. *Schematic representation of the neuroinflammatory and neurotransmission factors altered in the hippocampus of rats with mild liver damage, effects of MSC-EVs on those alterations and relation with different types of cognitive impairment and its modulating proposed pathways. Neuroinflammation mediated by activation of microglia, increase of TNF-α and decrease if IL-1β in CA1 would decrease membrane expression of GluN2B subunit that would impair spatial memory measured in the NOL. This mechanism is normalized by C-EVs. Neuroinflammation mediated by activation of astrocytes and increased number of IL-1β$^+$ cells would decrease membrane expression of GluA2 subunit leading to reduced learning index and working memory by a mechanism reversed by treatment with T-EVs. GABAergic neurotransmission is also altered in the hippocampus with altered membrane expression of α5 and α2 GABAA receptor subunits and GAD65 that are normalized by both types of EVs and γ2 subunit decreased membrane expression, only normalized by T-EVs. Created with Biorender.com*

2. MSC-EVs improve motor function and alterations in cerebellum in rats with mild liver damage

Regarding motor function, our results show that mild liver damage induces alterations both in motor coordination and locomotor gait in rats. Motor coordination was previously

demonstrated to be altered in rats injected with CCl_4 measured in the Rotarod and Beam walking tests (Balzano et al., 2021).

The present study shows that motor coordination in rats with mild liver damage is also altered when measured in the MotoRater. It should be considered that different coordination tests do not assess motor function in the exactly same manner. Motor assessment depends on several aspects for instance the brain area involved in each test. Moreover, some behavioural tests detect alterations equivalent to humans but there are behavioural tests designed for detecting motor dysfunction in rodent models, which do not have clinical equivalent in humans. In fact, conducting several motor tests is advantageous to assess the whole motor function with all the brain areas and pathways involved and considering the possibility that other factors could affect some of the tests (for instance anxiety). For instance, in the case of the rotarod, deficits are clear in animals with altered cerebellar or spinal cord function, although fatigue can influence the results in the accelerating rod (Mann & Chesselet, 2015).

We also analysed in this project the locomotor gait with the CatWalk XT system and several alterations in rats with mild liver damage were found. The analysis of the locomotor gait in the CatWalk XT system has been used mainly for Parkinson's disease animal models (Vandeputte et al., 2010; Chuang et al., 2010; Xiao et al., 2017; Boix et al., 2018; Tsang et al., 2019; Narbute et al., 2019). Hyperammonemic rats also show alterations in locomotor gait measured in the CatWalk that are normalized by treatment with a blocking agent of S1PR2, modulating neuroinflammation in the cerebellum (Arenas et al., 2022a); or with golexanolone, which acts on $GABA_A$ receptors and also modulates neuroinflammation, as there is a crosstalk between GABAergic neurotransmission and neuroinflammation, modulating and impairing motor function (Agusti et al., 2017; Malaguarnera et al., 2019; Abg Abd Wahab et al., 2019; Malaguarnera et al., 2021; Mincheva et al., 2022).

The regularity index has been related to interlimb coordination (Chuang et al., 2010). As mentioned before, motor incoordination is common in HE and MHE patients (Felipo, 2013). Besides, reduced stride length and increased dual stance are characteristic alterations in locomotor gait observed in cerebellar ataxia (Buckley et al., 2018). Parameters such as increased initial and terminal dual stance, reduced stride length, and increased stand index and step cycle have been related to bradykinesia and action-start hesitation symptoms in Parkinson's disease patients (Xiao et al., 2017; Boix et al., 2018). Bradykinesia and impairment

of movement initiation are also common symptoms in patients with MHE (Joebges et al., 2003; San Martin-Valenzuela et al., 2020).

These alterations in motor function were normalized by treatment with MSC-EVs. We observed a stronger effect of C-EVs, although both types were able to improve the most of the impaired motor parameters, including motor coordination. Only two gait parameters, dual stance and step cycle, were normalized by C-EVs but not by T-EVs, suggesting a role of TGF-β expression on the beneficial effects of EVs on them. These two parameters are related to freezing of gait, a typical symptom in Parkinson patients (Xiao et al., 2017). Similar improvement in motor coordination and CatWalk parameters has been shown after intranasal administration of EVs derived from human teeth stem cells in a rat model of Parkinson's disease (Narbute et al., 2019).

Motor alterations are related to alterations in GABAergic neurotransmission in the cerebellum (Hanchar et al., 2005) and this has been widely studied in other models (PCS and hyperammonemic rats) of HE (Hernandez-Rabaza et al., 2016a; Cabrera-Pastor et al., 2018). CCl_4-injected rats with impaired motor coordination also show altered GABAergic neurotransmission in the cerebellum (Balzano et al., 2021) which is supported by our results.

MSC-EVs improve most alterations in motor function, independently of TGF-β silencing; however, the mechanisms by which they induce the improvement seem to be different depending on the type of EV, and thus, depending on the expression or not of TGF-β. Although both types of EVs normalized GABA levels in Purkinje neurons, only C-EVs normalized GAT-1 membrane expression and $GABA_A$-β3 subunit while T-EVs normalized GAD67 in Purkinje neurons. Similarly to their effects in the hippocampus, EVs seem to have different effects depending on their composition.

GAT-1 membrane expression has already been shown to be reduced in rats with mild liver damage and other animal models of HE and induces increased levels of extracellular GABA and motor incoordination (Agusti et al., 2017; Cabrera-Pastor et al., 2019; Balzano et al., 2021). The alterations observed in GAT-1 and $GABA_A$-β3 membrane expression, and GABA levels in Purkinje neurons were normalized by C-EVs. These alterations in neurotransmission are produced by neuroinflammation in the cerebellum, since reducing neuroinflammation normalize membrane expression of GABA receptors and transporters and GAD expression in

Purkinje neurons, reducing GABAergic tone in the cerebellum (Agusti et al., 2017; Cabrera-Pastor et al., 2019; Balzano et al., 2021; Arenas et al., 2022b). Therefore, since C-EVs reduced astrocytes and microglia activation in the cerebellum, this normalizes GABAergic tone by increasing GAT-1 membrane expression and reduces GABA content in Purkinje neurons, that leads to improvement of motor incoordination (main results are shown in Table 9).

T-EVs did not improve astrocytes or microglia activation in the cerebellum, but they did normalize GABA and GAD67 levels in Purkinje neurons and most of the alterations observed in motor function. This would mean that T-EVs normalize part of the alterations produced in neurotransmission and improve motor impairment by a different mechanism than C-EVs. T-EVs promote microglia polarization towards M2 (anti-inflammatory) which is reduced in rats with mild liver damage. This change in the phenotype of microglia, from pro-inflammatory to anti-inflammatory, produced by T-EVs, must contribute to reduce neuroinflammation and to induce the beneficial effects on motor function, similarly to other effective treatments assessed in animal models of HE. Sulforaphane, sildenafil and bicuculline also promote polarization of microglia to the M2 phenotype, increasing YM-1 in the cerebellum of PCS or hyperammonemic rats, which is associated with normalization of extracellular GABA, and improvement of learning and motor coordination (Hernandez-Rabaza et al., 2016a; Agusti et al., 2017; Malaguarnera et al., 2021). Neuroinflammation depends on many other factors for instance levels of proinflammatory cytokines such as IL-1β and TNF-α (Abg Abd Wahab et al., 2019). In fact, levels of TNF-α are increased in the cerebellum by CCl_4 administration, as previously reported (Balzano et al., 2021). The increase in TNF-α in the cerebellum is reduced by both types of EVs, C-EVs and T-EVs.

In the cerebellum, there are some signalling pathways that explain how neuroinflammation affect neurotransmission. As explained previously, Cabrera-Pastor et al., (2018) described the TNFα-TNFR1-glutaminase-GAT3 pathway in hyperammonemic rats. In addition to TNF-α levels, glutaminase levels were also increased in the cerebellum of rats with mild liver damage, but in contrast to TNF-α (improved by both types of EVs), glutaminase levels were only normalized by T-EVs. Moreover, although GAT-3 content and membrane expression, increased in hyperammonemic rats, they were not altered by CCl_4. However, treatment with T-EVs in these rats reduced both, possibly contributing to decrease the extracellular GABA levels in rats with mild liver damage. Since C-EVs normalize TNF-α content in cerebellum but they

do not affect the increase in glutaminase, this suggests that another stimulus also induces increase in glutaminase, which is also normalized by T-EVs.

Another pathway that relates neuroinflammation to alterations in neurotransmission in the cerebellum is the one described in the Introduction (Section 7.2). The TNFα-CCL2-P2X4-BDNF-TrkB pathway was also altered in rats with mild liver damage in a similar way to the alterations observed in hyperammonemic rats (Arenas et al., 2022a). Mostly all analysed key points of this pathway were normalized by treatment with both types of EVs indicating that the pathway is also normalized, independently of the expression of TGF-β. Although CCL2 levels were only normalized by T-EVs, C-EVs normalize the downstream steps of the pathway, leading to the same final effect. C-EVs must reduce microglia activation (with reduction of P2X4 membrane expression) by other unknown mechanism, countering the induction of microglia activation by CCL2.

Considering overall effects on the neuroinflammation-induced signalling pathways and GABA system components, T-EVs show more normalizing effects than C-EVs, suggesting an interference of TGF-β on the effects of EVs on these pathways. However, C-EVs improve all motor impaired parameters, whereas T-EVs did not improved all altered gait parameters, suggesting that more pathways control these parameters.

Table 9. *Main alterations found in the cerebellum and motor function in rats with mild liver damage and effect of MSC- EVs.*

	ALTERATION	CCl_4	Improved by C-EVs	Improved by T-EVs
Behaviour	Rotarod (coordination)	↓	↑	
	Beam walking (coordination)	↓	↑	⇧
	MotoRater (coordination)	↓	↑	↑
	Regularity index (CatWalk, coordination)	↓	↑	↑
	Dual Stance (CatWalk)	↑	↓	
	Stride length (CatWalk)	↓	↑	↑
	Stand index (CatWalk)	↑		
	Step cycle (CatWalk)	↑	↓	
Neurotransmission	GAT-1 ME	↓	↑	
	$GABA_A$-β3 ME	↓	⇧	
	GABA PURKINJE	↑	↓	↓
	GAD67 PURKINJE	↑		↓
Neuroinflammation	Microglia activation	↑	↓	
	M2 microglia	↓		↑
	Astrocytes activation	↑	↓	
	TNF-α	↑	↓	↓
Pathways that relate neuroinflammation and neurotransmission	Glutaminase	↑		↓
	CCL2	↑		↓
	P2X4	↑	↓	↓
	BDNF	↑	↓	↓
	TrkB	↑	↓	↓

Red arrows indicate alterations produced by CCl_4, green arrows indicate normalization by C-EVs, orange arrows indicate normalization by T-EVs. Empty arrows indicate normalization without significant difference with CC and CV groups.

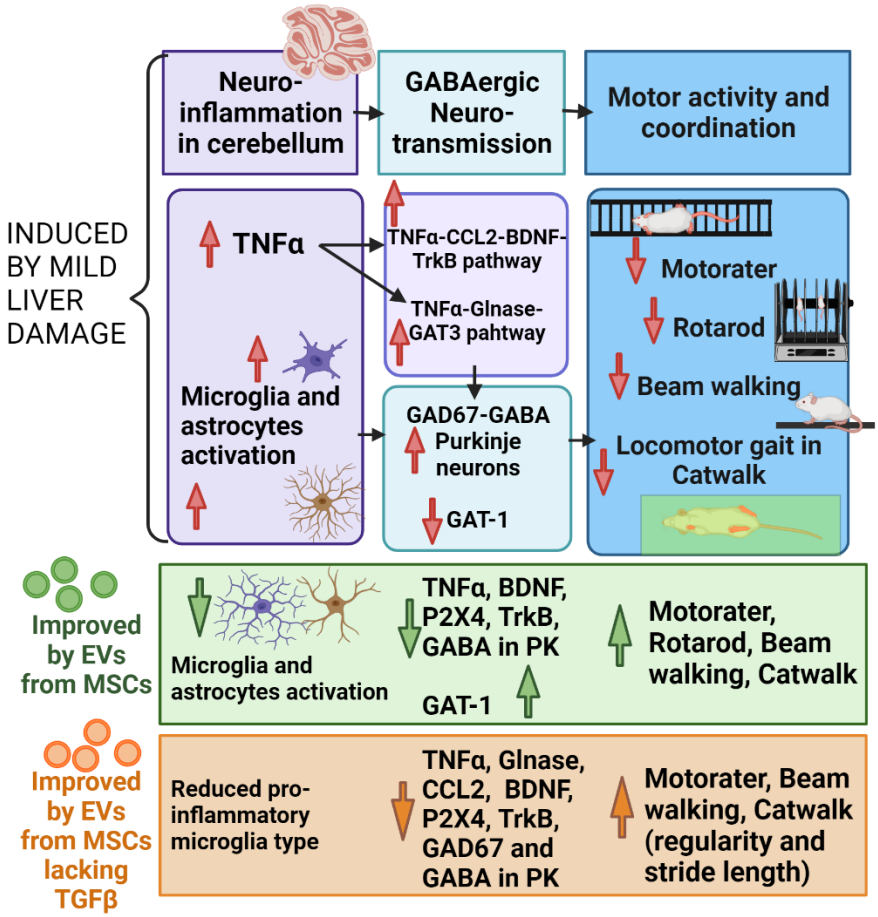

Figure 48. *Schematic representation of alterations produced in the cerebellum, related motor alterations and effects of MSC-EVs. Mile liver damage increases neuroinflammation in the cerebellum that enhances GABAergic neurotransmission and leads to motor incoordination and impair locomotor gait. Both MSC-EVs types reduce neuroinflammation, normalizing GABAergic neurotransmission and motor coordination and locomotor gait, but the mechanisms involved were slightly different depending on the TGF-β expression in the MSCs. Created with Biorender.com*

3. Effects of MSC-EVs on peripheral inflammation in rats with mild liver damage

Neuroinflammation, both in the cerebellum and the hippocampus, may have its origin in the inflammation in the periphery. Rats with mild liver damage showed alterations in cytokine levels in peripheral blood, specifically increase of proinflammatory cytokine levels, indicating presence of peripheral inflammation, as reported previously. Specifically, in rats injected with CCl_4 levels of TNF-α, IL-6, IL-15 and IL-17 increased (Balzano et al., 2021; Abdelghffar et al., 2022). The results in this work show increased levels of TNF-α, IL-6, IL-15, and HMGB1, but decreased levels of TGF-β, IL-17 and IL-21, in plasma of rats with mild liver damage after four weeks of CCl_4 administration. In a previous work in rats with mild liver damage induced by CCl_4, plasmatic levels of IL-17, IL-6 and IL-15 were increased after only one or two weeks of CCl_4 administration, returning at control levels at four weeks, indicating a rapid evolution of peripheral cytokine profile (Balzano et al., 2021). In this same previous work, a decrease in the percentage of $CD4^+$ lymphocytes ($CD4^+$ memory cells) was observed after two weeks of CCl_4 administration. This could explain the decrease in these cytokines (TGF-β, IL17 and IL-21) in plasma of our rats (Leone, 2020). Although, after four weeks of CCl_4 administration, the grade of liver damage was supposed to be the same, it could be slightly different; and, since peripheral inflammation evolved quickly in time, it could be also at a different stage, explaining the differences found between both works. The increase of IL-17 and IL-21 is classically related to progression of liver damage (Zhang, J. et al., 2010; Zhang, Y. et al., 2015; Xuan et al., 2017), supporting that liver damage is not yet sufficient in our rats to induce increase in these cytokines. IL-17 levels in plasma is increased in rats injected with CCl_4 for 12 weeks, which are mostly rats with liver cirrhosis, as in cirrhotic patients (Mangas-Losada et al., 2017; Leone, 2020). Another HE animal model with liver failure, PCS rats, also show increased plasma levels of these cytokines (Dadsetan et al., 2016b). Even hyperammonemic rats, without liver failure, show peripheral inflammation (Balzano et al., 2021). But since the rat model used in the present work did not show hyperammonemia yet, the blood cytokine profile that characterizes peripheral inflammation may be different in the different animal models of HE, since the origin is different. HMBG1 is related to liver damage which is strongly correlated with peripheral inflammation and fibrosis in children with NAFLD (Alisi et al., 2014).

Both types of EVs normalized levels of TNF-α, TGF-β, IL-17 and IL-21; IL-6 was normalized by T-EVs and partially by C-EVs and HMGB1 was only normalized by C-EVs. This can be related to the different origin and type of this pro-inflammatory molecule and it seems that the effect of EVs on HMGB1 content in plasma is dependent on TGF-β expression in MSCs. In contrast, any of the EVs were able to normalize IL-15 levels. MSC-EVs have been shown to reduce peripheral inflammation in diseases related to inflammation (Borger et al., 2017; Izquierdo-Altarejos et al., 2024). MSC-EVs administered to a patient suffering from steroid refractory graft-versus-host disease reduced peripheral blood cells, which secrete pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ (Kordelas et al., 2014) MSC-EVs administration to patients with chronic kidney disease increased anti-inflammatory cytokines TGF-β and IL-10 in peripheral blood while decreased pro-inflammatory TNF-α (Nassar et al., 2016).

4. Effects of MSC-EVs on liver damage induced by CCl_4. Role of TGF-β

Analysis of liver damage parameters and effects of MSC-EVs on these are reviewed in Table 10. Rats with mild liver damage induced by CCl_4 at 4 weeks of administration have higher grade of steatosis, lobular inflammation and fibrosis than control rats, with similar levels as the previously reported by Balzano et al., (2021). These parameters were also measured after 12 weeks of CCl_4 administration, and grade of steatosis, lobular inflammation and fibrosis was higher than at 4 weeks, as expected. Treatment with MSC-EVs was not able to significantly reduce liver damage histologically analysed. Only steatosis at 4 weeks was significantly reduced by T-EVs, although it did not eliminate it completely and did not reach similar results to the control rats.

The content of fibrosis markers, collagen I and α-SMA, were also increased in liver due to administration of CCl_4 for 4 weeks, which was also previously reported in seven weeks CCl_4-injected rats (Abe et al., 2007). Collagen I levels were reduced by T-EVs while α-SMA levels were reduced by both types of EVs, suggesting that EVs, mainly when TGF-β was silenced, are able to reduce the content of fibrotic characteristic molecules, although the effect is not sufficient to see an evident effect on hepatic fibrosis histologically analysed. Bone-marrow MSC-EVs have been reported to normalize collagen levels in liver but only reduce TGF-β

levels in rats with CCl_4 induced advanced fibrosis (Sabry et al., 2019). It was not observed an increase in TFG-β in liver of rats with mild liver damage by western blot. This could be due to the mild level of liver damage induced in the rats after only 4 weeks of CCl_4 injections. When liver damage is too mild HSC are not yet activated and the process of fibrosis is not initiated. According with our results, TGF-β seems to be increased in more severe stages of liver damage than collagen I, which content is already increased in our rats with mild liver damage.

Activity of hepatic enzymes ALT and alkaline phosphatase in serum as well as bile acids content were increased in serum of rats injected with CCl_4, although bilirubin levels were not altered and AST levels were only altered in CCl_4-injected rats with C-EVs treatment. Increased levels in serum of these markers indicate liver damage, and moreover bile acids elevated serum levels are associated with liver inflammation (Giannini et al., 2005; Li, M. et al., 2017). The increase only in some of these factors could be explained by the early stage of liver disease of the rats, that only present mild liver damage. Other models of NASH such as high-fat, high-cholesterol fed rats show increased levels in plasma of ALT and AST but not bilirubin and liver in these cases also presented steatosis (Higarza et al., 2019). Treatment with C-EVs were not able to reduce the elevated markers, however, T-EVs reduced ALT activity and induced a partial decrease in bile acids content. Sabry et al, (2019) reported that MSC-EVs reduce but not normalize ALT serum levels in rats with liver fibrosis induced by CCl_4 administration for ten weeks.

Livers of rats with mild liver damage showed increased levels of TNF-α. This indicates presence of liver inflammation which was normalized by C-EVs but not T-EVs, suggesting that TGF-β is important to this anti-inflammatory effect in the liver. However, its presence can interfere with the anti-fibrotic effect of EVs since only T-EVs were able to decrease collagen content, according to the pro-fibrotic action of TGF-β in the liver.

TGF-β is a multifunctional main cytokine with multiple and different functions in different tissues and pathological situations (Fabregat et al., 2020). TGF-β is essential in the induction of hepatic fibrosis, at same time that has general anti-inflammatory properties, being released by regulatory T-lymphocytes and anti-inflammatory M2 phenotype of microglia and macrophages (Wells, 2000). Reduction of chronic neuroinflammation by TGF-β was also reported (Chen, J. et al., 2015). Due to its multiple functions it is possible that TGF-β has more beneficial effects in other areas of the organism such as the cerebellum, were the anti-

inflammatory effect should prevail, but it can be harmful in the liver due to its profibrotic effect and this would explain why T-EVs, lacking TFG-β, seem to have more beneficial effects in liver fibrosis.

Any of the MSC-EVs were able to fully reverse the alterations observed in liver at this stage, however, they had some beneficial effects. T-EVs show beneficial effects on fibrosis markers while C-EVs improved liver inflammation. Several studies have reported that MSCs-EVs have beneficial effects on chronic hepatic disease (Tang et al., 2022; Hazrati et al., 2022) and mainly in hepatic fibrosis (Liu, Y. et al., 2023) and cirrhosis. Mechanisms of the immunomodulatory and anti-fibrotic effects of MSCs-EVs have been studied in animal models of hepatic disease (Chiabotto et al., 2020), including rats with liver fibrosis induced by CCl_4 (Ohara et al., 2018). The therapeutic efficacy of EVs derived from adipose tissue in metabolic diseases involving multi-organ crosstalk, including chronic liver disease, has been also reported (Huang & Xu, 2021).

***Table 10**. Summary of liver damage produced by CCl_4 in the liver after 4 weeks of administration and effects of MSC-EVs*

ALTERATION	**CCl_4**	**Improved by C-EVs**	**Improved by T-EVs**
Steatosis, lobular inflammation and fibrosis after 4W of CCl_4	↑		Steatosis is reduced but not to control levels
Collagen I (Western blot)	↑		↓
α-SMA (Western blot)	↑	⇩	↓
α-SMA (Immunohistochemistry)	↑	↓	↓
ALT	↑		⇩
AST		Increased in CCl_4 + C-EVs	
Alkaline phosphatase	↑		
Bile acids	↑		⇩
TNF-α	↑	↓	

Red arrows indicate alterations produced by CCl_4, green arrows indicate normalization by C-EVs, orange arrows indicate normalization by T-EVs. Empty arrows indicate normalization without significant difference with CC and CV groups.

5. MSC-EVs improve gut dysbiosis in rats with mild liver damage

The study of gut microbiota in faeces of our rats with mild liver damage and treated or not with both types of MSC-EVs revealed that there are not important shifts across the experimental groups in the alpha diversity of the samples. However, the beta diversity analysis showed that there was a significant different composition dependent on the treatment of the group. The results reveal several OUT differences between the control group and the rats with mild liver damage. Higarza et al., (2019) previously reported dysbiosis in the gut microbiota of a diet-induced NASH rat model with reduced levels of Firmicutes, and increased levels of Bacteroidetes and Proteobacteria. The taxonomic identification of the OTUs of interest of our results showed that many of them belong to the genus *Bacteroides* (Gram-negative, anaerobic bacteria) and were more abundant in rats with mild liver damage induced by CCl_4. This agrees with what was described by Higarza et al., (2019) in another model of mild liver damage. Thioacetamide-induced cirrhosis predisposes rat liver to bacterial colonization with species from the genus *Escherichia, Enterobacter* and *Bacteroides* (al-Bader et al., 1998). Moreover, *Bacteroides* abundance has been observed to be increased in NASH patients (Boursier et al., 2016). Therefore, an increase in *Bacteroides* seems to be a general effect in liver injury.

Among the species identified as different in the CCl_4-injected group, *B. cellulosilyticus, B.caccae, B.eggerthii,* and *Anaerostipes faecis* were found. Treatment with C-EVs reduced *B. cellulosilyticus, B.caccae* and *B.eggerthii* towards abundance of the control group while T-EVs normalized *A. faecis.* These species could have a role in the consequences of the liver disease as they produce metabolites such as SCFAs, fermentation products and cytokines that interfere in the presence of inflammation.

Interestingly, abundances of *B.caccae* and *B.eggerthii* correlated with the results of locomotor gait obtained in the CatWalk. Both correlated positively with dual stance, *B.caccae* correlated negatively with stride length, and *B.eggerthii* correlated positively with step cycle. These parametres are related to bradykinesia and action-start hesitation symptoms in Parkinson's disease patients (Xiao et al., 2017; Boix et al., 2018). Alterations in gut microbiota in Parkinson's diseases patients have been related to gait disturbances (Scheperjans et al., 2015;

Barichella et al., 2019). Changes of gut microbiota in different liver disease stages has already been associated with changes in cognitive function (Higarza et al., 2022).

6. Effects of MSC-EVs on control rats

MSC-EVs administration produced some alterations in the control rats (Tables 11 and 12). Learning index and working memory was worsened by C-EVs, that in fact, were not able to normalize these parameters in the rats with mild liver damage. In contrast, T-EVs reduced the performance of control rats in the NOL test, and were not able to improve the reduction that was observed in rats with mild liver damage. In both cases, the type of EV that is not able to improve the alteration in rats with mild liver damage induces it in the control rats. The short-term memory measured in the Y-maze supports this idea given that, any of the EVs were able to improve the alteration produced by CCl_4 and both reduced the short-term memory. These observations suggest that the effects of EVs on these behavioural parameters are mediated by interfering with the general regulatory mechanisms and not only by reduction of the altered mechanisms in rats with mild liver damage. MSC-EVs have more effects on cognitive function than in the motor function in which only the MotoRater (T-EVs) and the stride length (C-EVs) were impaired (Table 11).

In the brain, generally T-EVs produced more molecular alterations in control rats than C-EVs, both in the hippocampus and the cerebellum (Table 11). Neurotransmission was affected by both types of EVs in control rats. The expression of $GABA_A$ receptor subunits in the cell membrane was specially affected. In the hippocampus, T-EVs reduced the expression in the cell membrane of $GABA_A$ subunits α1, β3 (also C-EVs), and γ2; in the cerebellum, C-EVs had more effects affecting α2, β3, and γ2 while T-EVs affected δ subunit membrane expression. A possible general effect of EVs on membrane expression of proteins, interfering with general mechanisms of membrane insertion and internalization is suggested and further studies on this can be interesting to advance in the knowledge of EVs functions. Interestingly, T-EVs did not restore the alterations produced in GluN2B subunit in the hippocampus nor the NOL test and they induced the alteration of these parameters in the control rats. T-EVs clearly interfere in the mechanism that involves modulation of spatial memory measured in the NOL by GluN2B subunit in the hippocampus.

Neuroinflammation, and specifically astrocytes activation, was increased by both types of EVs in the hippocampus and the cerebellum of control rats. Astrocytes seem to be more susceptible to the effects of the MSC-EVs in the control rats that microglia that was only affected by a reduced M2 polarization in the cerebellum induced by treatment with T-EVs. This result is also interesting because T-EVs are the ones increasing the reduction of M2 microglia produced by CCl_4; T-EVs induce opposed effects in microglia polarization in controls or rats with mild liver damage. Besides, TNF-α levels are also increased by both types of EVs in the hippocampus and by T-EVs in the cerebellum. Then, EVs external administration in healthy conditions can induce some grade of neuroinflammation, mainly by astrocytes activation and increase in TNF-α, and more evident in the case of T-EVs than C-EVs.

T-EVs seem to have a specific effect on the TNF-α-CCL2-P2X4-BDNF-TrkB and the TNF-α-TNFR1-glutaminase-GAT3 pathways. They normalize the alterations induced in rats with mild liver damage and also reduce content of TNF-α, CCL2 and glutaminase in the cerebellum of control rats. T-EVs reduce the content of pathway components in controls and rats with mild liver damage, supporting the possible interference with basic regulatory mechanisms.

In contrast to the brain, in the periphery (liver and blood, Table 12), C-EVs tent to induce more alterations. This supports the idea that TGF-β has different effects in the CNS than in the periphery, according to its multiple and different functions (Wells, 2000; Fabregat et al., 2020). In the liver, C-EVs had a more anti-inflammatory effect that T-EVs in the alterations induced by CCl_4, while T-EVs improved liver steatosis and fibrosis better than C-EVs. This is supported by the effects on control rats, in which C-EVs (that contain TGF-β) induced liver damage (increase in α-SMA stained area and alkaline phosphatase activity) while T-EVs lacking TGF-β did not induce liver damage. TGF-β is essential for the induction of hepatic fibrosis (Wells, 2000). Moreover, regulation of expression and activity of alkaline phosphatase by TGF-β was reported (Shirakawa et al., 1994). All these effects observed in controls also are dependent on the EVs cargo and expression of TGF-β, as observed for the effects on the alterations induced by mild liver damage.

Table 11. *Alterations produced by MSC-EVs in the brain of control rats.*

	ALTERATION	Altered by C-EVs	Altered by T-EVs
Behaviour (cognitive function)	Learning index and working memory	↓	
	NOL		↓
	Short-term memory	↓	↓
Neurotransmission in the hippocampus	GluN2B ME		↓
	$GABA_A$-α1 ME		↓
	$GABA_A$-β3 ME	↓	↓
	$GABA_A$-γ2 ME		↓
Neuroinflammation in the hippocampus	Astrocytes activation	↑	↑
	TNF-α	↑	↑
Behavior (motor function)	MotoRater (coordination)		↓
	Stride length (CatWalk)	↑	
Neurotransmission in the cerebellum	GAD65 PURKINJE		↑
	$GABA_A$- α2 ME	↓	
	$GABA_A$-β3 ME	↓	
	$GABA_A$-δ ME		↓
	$GABA_A$-γ2 ME	↓	
Neuroinflammation in the cerebellum	Astrocytes activation	↑	↑
	M2 microglia		↓
	TNF-α		↑
Pathways in cerebellum	Glutaminase		↑
	CCL2		↑

Blue arrows indicate alteration produced by C-EVs, pink arrows indicate alteration by T-EVs.

Table 12. *Alterations produced by MSC-EVs in blood and liver of control rats.*

	ALTERATION	Altered by C-EVs	Altered by T-EVs
Peripheral inflammation	IL-15		⇧
	IL-17	⇩	
	HMGB1	⇩	
Liver damage	α-SMA (immunohistochemistry)	⇧	
	Alkaline phosphatase	⇧	

Blue arrows indicate alteration produced by C-EVs, pink arrows indicate alteration by T-EVs.

As shown, overall, MSC-EVs are able to reduce alterations induced by CCl_4-induced liver damage in rats, improving cognitive and motor functions, reducing neuroinflammation in the cerebellum and hippocampus, and normalizing alterations in glutamatergic and GABAergic neurotransmission in both areas. MSC-EVs also reduce peripheral inflammation, liver steatosis and fibrosis, and liver inflammation and are able to counteract some consequences of gut dysbiosis. MSC-EVs could be a possible treatment to improve the neurological impairments, as mild cognitive impairment, produced in patients in the early stages of liver disease, such as NASH or NAFLD, also improving liver damage and the associated peripheral inflammation.

The effects of MSC-EVs are dependent on their cargo composition. Our results have shown that TGF-β has an important role in the effects of MSCs-EVs treatment. There are mechanisms and pathways affected by TGF-β involved in the effects of EVs, having a key role modulating alterations induced by CCl_4 injection in rats. This information could be relevant for treatment in patients in which EVs cargo composition can be controlled to maximize beneficial effects. However, it is important to consider that MSC-EVs content depends not only on their cell of origin but also in the methodology used for their extraction (Courageux et al., 2022). Therapeutic differences between independent MSC-EV preparations produced in a standardized manner have been found (Madel et al., 2023). Differences in therapeutic efficiency between MSC-EVs from different donors also have been reported (Van Hoecke et al., 2021). These differences should be considered as a limitation in the use of MSC-EVs as treatment in patients because their therapeutic effects are unpredictable. Moreover, there is a

lack of information of the possible effects that MSC-EVs have on control animals or patients, on general physiological mechanisms, and as shown in the present work, these could be several. This project highlights the importance of testing and publishing of results on controls and possible side effects of MSC-EVs.

CONCLUSIONS

1. MSC-EVs improve cognitive impairment in rats with mild liver damage induced by CCl_4. EVs from unmodified MSC improve spatial memory while EVS from MSC lacking TGF-β improve learning index and working memory.
2. Cognitive impairment in rats with mild liver damage is produced by neuroinflammation-induced alterations in glutamatergic and GABAergic neurotransmission in the hippocampus. Both, EVs from unmodified MSC and MSC lacking TGF-β reduce neuroinflammation and normalize some alterations in glutamatergic and GABAergic neurotransmission in the hippocampus, but these effects are mediated by different mechanisms.
3. Mild liver damage induces neuroinflammation in the cerebellum, which enhances the activation of the TNFα-TNFR1-glutaminase-GAT3 and the TNFα-CCL2-P2X4-BDNF-TrkB pathways, leading to enhanced GABAergic neurotransmission which impairs motor coordination and locomotor gait. MSC-EVs reverse neuroinflammation and normalize activation of these pathways, GABAergic neurotransmission and motor coordination and gait.
4. MSC-EVs reduce peripheral inflammation in plasma of rats with mild liver damage, which would contribute to reduction of neuroinflammation.
5. MSC-EVs do not completely reverse liver damage. However, EVs from MSC lacking TGF-β reduce liver damage, reducing steatosis, fibrosis markers and serum markers of liver damage.
6. Mild liver damage induces gut microbiota dysbiosis in rats that is ameliorated by treatment with MSC-EVs. CCl_4-injected rats show increased levels of *Bacteroides* that are reduced by treatment with MSC-EVs and correlate with the impairment of locomotor gait.
7. Both types of MSC-EVs increase neuroinflammation and alter neurotransmission in the hippocampus and the cerebellum, and impair cognitive and motor function in control rats. EVs from unmodified MSC increase liver damage in normal rats.
8. MSC-EVs could be a promising therapy for MHE and HE, and also for other diseases involving similar pathological mechanisms, such as diseases associated with sustained peripheral inflammation and neuroinflammation.

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ANNEX

RESUMEN

INTRODUCCIÓN

El hígado es un órgano importante presente en los vertebrados que está relacionado con muchos procesos fisiológicos, como la desintoxicación de sustancias tóxicas para el organismo, el metabolismo de macronutrientes, el almacenamiento de glucosa en forma de glucógeno, la homeostasis de lípidos y colesterol, el metabolismo de proteínas y aminoácidos, la regulación del volumen sanguíneo, el apoyo al sistema inmunitario, el control endocrino de las vías de señalización del crecimiento y la descomposición de compuestos, incluidos muchos fármacos actuales (Trefts et al., 2017).

El hígado empieza a acumular una cantidad excesiva de triacilglicéridos como respuesta habitual a lesiones o enfermedades. Esta afección se conoce como esteatosis y puede estar relacionada con el abuso de ciertas sustancias como el alcohol o las drogas ilegales, pero también con otros factores como la obesidad, la diabetes tipo 2 o algunos medicamentos. En muchos de estos casos, cuando la enfermedad no está relacionada con el abuso de alcohol, se conoce como enfermedad del hígado graso no alcohólico (NAFLD).

La esteatosis en fases tempranas y leves es reversible y permite llevar una vida relativamente normal. No obstante, la esteatosis puede empeorar de forma significativa. La acumulación excesiva de lípidos en los hepatocitos puede alterar los constituyentes celulares y provocar la apoptosis o el estallido. Con la muerte de los hepatocitos, se liberan triacilglicéridos y ácidos grasos tóxicos en el hígado, que lo dañan aún más y producen inflamación, convirtiéndose así en esteatohepatitis (Wang et al., 2013). Dependiendo de la presencia o ausencia de consumo de alcohol relacionado con la enfermedad hepática, se pueden diferenciar la esteatohepatitis alcohólica (ASH) y la esteatohepatitis no alcohólica (NASH). La esteatohepatitis aumenta el riesgo de avanzar hacia las siguientes etapas del daño hepático, fibrosis, cirrosis y carcinoma hepático (Schuppan y Kim, 2013) (Figura 1).

La fibrosis hepática consiste en una acumulación de proteínas de la matriz extracelular, como el colágeno, en el hígado (Tacke y Weiskirchen, 2012). Esto es común en la mayoría de los tipos de enfermedades hepáticas crónicas. Las células estrelladas hepáticas activadas desempeñan el papel principal en la fibrosis hepática como principales productoras de colágeno. En consecuencia, se altera la arquitectura del hígado y conduce a la formación de una cicatriz fibrosa. Además, la lesión hepática y la apoptosis celular estimulan la regeneración hepática, lo que contribuye a la distorsión de la arquitectura y a la formación de nódulos hepáticos. Este entorno conduce a la cirrosis, un proceso caracterizado por una fibrogénesis agresiva y el posterior desarrollo de una estructura anormal de los nódulos. (Pinzani et al., 2011). La cirrosis produce disfunción celular hepática y complicaciones en el flujo sanguíneo en el hígado, lo que conduce a insuficiencia hepática e hipertensión portal (Bataller & Brenner, 2005). De hecho, la hipertensión portal es el principal mecanismo que conduce a la muerte de los pacientes cirróticos (Pinzani et al., 2011).

La insuficiencia hepática provoca alteraciones en muchas funciones, como la desintoxicación de compuestos tóxicos de la sangre. Estos compuestos, como el amoníaco, empiezan a acumularse y pueden llegar al cerebro y afectar a su función. Las alteraciones de la función cerebral debidas a la insuficiencia hepática causan un complejo síndrome neuropsiquiátrico conocido como encefalopatía hepática (HE). La hiperamonemia crónica, aumento de los niveles de amoníaco en sangre, es una de las principales causas de la HE. La EH incluye alteraciones de la función motora como bradicinesia y asterixis, disfunción de las capacidades cognitivas, alteración de los ritmos circadianos y cambios de personalidad y conciencia que en fases avanzadas pueden conducir al coma o incluso a la muerte.

Los pacientes con daño hepático crónico pueden no mostrar alteraciones neurológicas evidentes en las primeras fases de la EH crónica, pero pueden mostrar algunas alteraciones leves a nivel cognitivo y motor como alteraciones en la velocidad de movimiento, coordinación bimanual, atención, concentración y percepción visuoespacial. La presencia de estas alteraciones en pacientes cirróticos se define como encefalopatía hepática mínima (EHM), considerada un tipo subclínico de EH (actualmente también denominada EH encubierta). Las alteraciones neurológicas relacionadas con la EHM no suelen ser graves, pero sí suficientes para impedir o complicar las actividades de la vida diaria, por ejemplo, conducir. La EHM también aumenta el riesgo de accidentes domésticos y laborales, reduce la calidad de vida y la esperanza de vida de los pacientes y aumenta la posibilidad de padecer EH clínica (Felipo, 2013). Para el diagnóstico de la EMH, existe consenso en utilizar la puntuación psicométrica de la encefalopatía hepática (PHES). Esta prueba incluye cinco tareas con puntuación psicométrica con las que se puede identificar la presencia de EHM en pacientes cirróticos.

Las alteraciones neurológicas observadas en la EH se producen principalmente por la acción sinérgica de dos factores principales: la hiperamonemia y la inflamación. La interacción entre ambos factores induce neuroinflamación que conduce a alteraciones en la neurotransmisión y éstas a las alteraciones cognitivas y motoras de la EH (Shawcross. et al., 2004; Shawcross. et al., 2007; Montoliu et al., 2009; Felipo, et al., 2012; Felipo, 2013). Tras una insuficiencia hepática, la desintoxicación del amonio no se lleva a cabo correctamente y, por tanto, tiende a acumularse. La alteración de la microbiota, disbiosis intestinal, frecuentemente asociada al daño hepático, conduce a menudo a un aumento de bacterias con ureasa que contribuye a aumentar la producción de amonio. Además, el aumento de la permeabilidad de la barrera intestinal asociado a la lesión hepática provoca un aumento de la permeabilidad del amonio en la circulación sanguínea. La hiperamonemia se refiere al aumento de los niveles de amonio en sangre. La hiperamonemia se consideró durante mucho tiempo el principal factor responsable de la EH. De hecho, la gravedad de la enfermedad se correlaciona con la concentración de amoníaco en sangre (Clemmesen et al., 1999). Además, actualmente todos los tratamientos eficaces de la EH se basan en la reducción de los niveles de amonio. En las últimas décadas, la inflamación también se ha relacionado con la aparición de la EH. La inflamación periférica se manifiesta por la alteración de los niveles de citoquinas proinflamatorias y antiinflamatorias en la sangre, asociado a un cambio del inmunofenotipo hacia un perfil pro-inflamatorio, con activación de linfocitos y monocitos.

En algunas enfermedades crónicas como la obesidad, la diabetes y la artritis reumatoide, la presencia de inflamación crónica conduce a un deterioro cognitivo y motor. Shawcross et al. (2004) demostraron que la hiperamonemia provoca un empeoramiento de las puntuaciones neuropsicológicas de los pacientes si también está presente la inflamación sistémica. La cirrosis hepática induce una inflamación periférica que suele exacerbar las alteraciones neuropsicológicas inducidas por la hiperamonemia. Además, en pacientes con enfermedades que cursan con alteración de los niveles de amonio e inflamación, puede aparecer deterioro cognitivo incluso sin enfermedad hepática, si la hiperamonemia y la inflamación son suficientemente elevadas (Felipo et al., 2012b). De hecho, la concentración de algunos mediadores inflamatorios como la IL-6 y la IL-18 podría ser un posible marcador de EHM en pacientes cirróticos (Montoliu et al., 2009). Se ha comprobado recientemente que un cambio hacia un perfil proinflamatorio del inmunofenotipo está estrechamente correlacionado con la presencia de EHM en pacientes cirróticos (Mangas-Losada et al, 2017).

Los mecanismos patológicos de la EH se han estudiado en modelos animales. Entre ellos los más utilizados son las ratas con ligadura biliar, que produce cirrosis biliar primaria y alteraciones neurológicas que reproducen aquellas encontradas en pacientes con EH; las ratas con anastomosis porta-cava, que evita la circulación sanguínea a través del hígado y produce insuficiencia hepática, presentando también alteraciones cognitivas y motoras típicas de la EH, neuroinflamación, alteraciones en la neurotransmisión e inflamación e hiperamonemia; y un modelo basado en una dieta hiperamonémica, que produce hiperamonemia pero también inflamación periférica y neuroinflamación (Balzano et al, 2020, Rodrigo et al, 2010). Además, algunos modelos están basados en producir daño hepático progresivo mediante administración de hepatotoxinas como la tioacetamida o el tetracloruro de carbono (CCl_4). El modelo de administración crónica de CCl_4 reproduce diferentes fases de daño hepático, incluyendo esteatosis, esteatohepatitis, fibrosis y cirrosis. Se ha descrito recientemente que en este modelo, en fases tempranas de daño hepático, esteatohepatitis leve, las ratas ya muestran deterioro cognitivo y motor debido a alteraciones en la neurotransmisión glutamatérgica y GABAérgica en hipocampo y cerebelo, asociadas a neuroinflamación, la cual está mediada en parte por infiltración de linfocitos y monocitos inducida por la inflamación periférica, presente en sangre e hígado en este modelo de daño hepático leve (Balzano et al, 2021, Leone et al, 2022).

La inflamación periférica se transduce al cerebro por distintas vías en situaciones patológicas crónicas causando neuroinflamación (Sun et al., 2022). La inflamación periférica puede transmitirse al sistema nervioso central (SNC) debido a alteraciones en la barrera hematoencefálica (BHE). La BHE está compuesta por células endoteliales, pericitos y astrocitos, y su función es evitar que las señales periféricas lleguen al SNC. Sin embargo, si alguno de estos componentes se ve afectado la permeabilidad de la BHE aumenta. Cuando la BHE se vuelve permeable, se permite la entrada de citoquinas inflamatorias, iones e infiltración de células inmunitarias al SNC produciendo neuroinflamación. El eje microbiota-intestino-cerebro también interviene en la relación entre la inflamación periférica y la función cerebral. La comunicación bidireccional de señales inflamatorias a través de este eje es clave en la patología de enfermedades neurológicas que se asocian a inflamación crónica, ya que ésta está modulada e incluso inducida por cambios en la microbiota intestinal. Los intercambios de

señales inflamatorias se producen a través de tres vías principales: sistémica humoral, inmunitaria celular y neuronal, sobre todo a través del nervio vago.
Las vesículas extracelulares (EVs) son pequeñas vesículas de membrana secretadas por diversas células que también desempeñan un papel crucial en la comunicación entre la periferia y el cerebro. Tienen un papel importante en la comunicación intercelular al transferir información a través de su contenido, que incluye proteínas, lípidos, ácidos nucleicos, azúcares, entre otros (Yáñez-Mó et al., 2015). Las EVs tienen la capacidad de modular la respuesta inmune al participar en la presentación de antígenos y estimular la liberación de mediadores pro o antiinflamatorios (Bhatnagar y Schorey, 2007). Estudios de Izquierdo-Altarejos et al. (2020 y 2023) demostraron que las EVs están involucradas en la transmisión de alteraciones periféricas al cerebro en ratas hiperamonémicas. La inyección de EVs plasmáticas de ratas hiperamonémicas en ratas normales indujo neuroinflamación, incoordinación motora, y alteraciones en el aprendizaje y la memoria en estas últimas.

La respuesta inflamatoria que se produce en el SNC debido a infecciones, lesiones traumáticas, metabolitos tóxicos o autoinmunidad se denomina neuroinflamación. La neuroinflamación está mediada por la producción y liberación de citocinas, quimiocinas, especies reactivas del oxígeno (ROS) y mensajeros secundarios por parte de la glía residente en el SNC, microglía y astrocitos, cuando son activados, las células inmunitarias periféricas y las células endoteliales e incluso por neuronas (DiSabato et al., 2016).
La presencia de neuroinflamación altera la neurotransmisión en el cerebro. La alteración de la neurotransmisión glutamatérgica y GABAérgica en el hipocampo conduce a alteraciones de la función cognitiva incluyendo alteraciones del aprendizaje y la memoria espaciales, la memoria a corto plazo y la memoria de trabajo. Por otra parte, el aumento de la neurotransmisión GABAérgica en el cerebelo produce alteraciones de la función motora incluyendo la coordinación motora y la alteración de la marcha.

Cabrera-Pastor et al 2018 describieron una vía por la que la neuroinflamación aumentaría el GABA extracelular en cerebelo y deterioraría la coordinación motora en ratas hiperamonémicas. La hiperamonemia induce neuroinflamación con activación de microglía y astrocitos y aumento de los niveles de TNF-α y de la cantidad de su receptor (TNFR1) en la membrana. Esto aumenta la activación de TNFR1 y conduce a la translocación de la subunidad p50 de NF-κB al núcleo. Así, aumenta la expresión de la glutaminasa, lo que conduce a un aumento de los niveles de glutamato que incrementa la activación de sus transportadores (GLT-1 y GLAST). Los transportadores de glutamato también introducen Na^+ en las células mientras transportan glutamato, por lo que su mayor activación también aumenta los niveles de Na^+ intracelular, lo que invertiría la función de GAT-3 en los astrocitos activados, dando lugar a la liberación y el aumento de la concentración extracelular de GABA. Esto a su vez conduce a la incoordinación motora.

Arenas et al 2022 describieron otra vía por la que la neurotransmisión GABAérgica se vería afectada por la neuroinflamación en el cerebelo, la vía TNFR1-S1PR2-CCR2-TrkB-KCC2. El contenido en la membrana de TNFR1 y S1PR2 y, por tanto, su activación están aumentados en las neuronas de Purkinje de ratas hiperamonémicas, lo que conduce a una mayor producción

de CCL2 por parte de las neuronas de Purkinje. El CCL2 se libera y activa su receptor CCR2 en la microglía, lo que conduce a la activación microglial y a una mayor expresión en la membrana de los receptores CCR2 y P2X4, así como a una mayor formación de BDNF en la microglía. El BDNF liberado activa las señales mediadas por TrkB en las neuronas, lo que conduce a un mayor contenido del cotransportador KCC2 en la membrana celular, que altera el gradiente transmembrana de cloruro (Cl^-), lo cual altera la función de los receptores ionotrópicos $GABA_A$.

Actualmente los tratamientos de la EH son muy limitados y se basan en disminuir los niveles de amonio y son disacáridos no absorbibles o antibióticos como la rifaximina, que producen mejoras de la EH en algunos pacientes, pero no en todos. Sigue siendo imprescindible proponer y testar nuevos tratamientos basados en las dianas terapéuticas que se postulan tras el estudio de los mecanismos que median la aparición de EH para retrasar o prevenir la aparición de EHM y EH.

Las células madre mesenquimales (MSC) son células que se encuentran en diversos tejidos humanos y son capaces de diferenciarse en distintos tipos celulares. Para ser clasificadas como MSC, deben cumplir criterios como adherencia al plástico en cultivo, marcadores de superficie específicos (CD105, CD73, CD90) y potencial de diferenciación en los tres linajes celulares. Poseen propiedades inmunomoduladoras, antiinflamatorias y regenerativas, lo que las hace atractivas para terapias. Sus factores paracrinos, incluidas las citocinas y los factores de crecimiento, contribuyen a sus efectos sobre las células inmunitarias. Los ensayos clínicos han demostrado los beneficios de la terapia con MSC para diversas enfermedades, entre ellas la cirrosis hepática. Recientemente se ha comprobado que su acción paracrina está mediada en gran parte por la segregación de EVs, lo que ofrece posibles beneficios terapéuticos. Las EVs tienen una composición y unas propiedades que dependen de su origen. Las MSC-EVs se parecen a sus células progenitoras y expresan marcadores específicos. Contienen proteínas, ARNm y miARNs con diversas funciones. Las MSC-EVs de tejido adiposo tienen potencial en el tratamiento de enfermedades neurodegenerativas, con beneficios en esclerosis múltiple, hemorragia intracerebral, recuperación de ictus, fibrosis hepática y enfermedad de Alzheimer en modelos animales. Las MSC-EVs ofrecen ventajas como un menor riesgo de efectos secundarios y una penetración más fácil en los tejidos, lo que las convierte en una prometedora terapia sin células. Las MSC se consideran para el tratamiento de enfermedades hepáticas, con beneficios demostrados en la fibrosis hepática. Las MSC pueden diferenciarse en hepatocitos y tener efectos inmunomoduladores, pero también pueden contribuir a la fibrosis en determinadas condiciones. Los factores liberados por las MSC, incluidas las citocinas, los factores de crecimiento y las EVs, pueden reducir el daño y la inflamación hepáticos. Las EVs de diversas fuentes han demostrado su eficacia para reducir la inflamación y la fibrogénesis en modelos animales. Los estudios sugieren posibles mejoras de los síntomas neurológicos en la encefalopatía hepática con el tratamiento con MSC. Izquierdo-Altarejos et al. (2023) encontraron una reducción de la neuroinflamación y una mejora de la función cognitiva en ratas hiperamonémicas tras el tratamiento con MSC-EV.

HÍPOTESIS Y OBJETIVOS

Los pacientes con cirrosis hepática pueden desarrollar MHE con alteraciones cognitivas y motoras siendo la hiperamonemia y la inflamación periférica los principales factores que contribuyen al desarrollo de la enfermedad. Además, en pacientes con enfermedades hepáticas crónicas, como la enfermedad del hígado graso no alcohólica (NAFLD, en inglés), la hiperamonemia y la inflamación periférica pueden inducir alteraciones a nivel cognitivo y motor incluso sin llegar a la cirrosis. En ratas con daño hepático leve, el entorno proinflamatorio en sangre periférica induce la infiltración de células inmunitarias en cerebelo e hipocampo, lo que induce neuroinflamación. La neuroinflamación altera la neurotransmisión glutamatérgica y GABAérgica en el cerebelo y el hipocampo, lo que provoca trastornos cognitivos y motores.

Numerosos estudios demuestran que las EVs desempeñan un papel principal en la comunicación intercelular, estando relacionadas con muchos procesos inflamatorios e inmunitarios. Muchos estudios señalan el potencial terapéutico de las MSC-EVs, principalmente en trastornos relacionados con la inflamación. La inyección de MSC-EVs en ratas hiperamonémicas, un modelo de ES sin insuficiencia hepática, reduce la neuroinflamación en cerebelo e hipocampo y mejora la coordinación motora y la función cognitiva. Aunque los mecanismos por los que las MSC-EVs ejercen sus funciones no están claros, el TGFβ parece tener un papel importante en las EVs mediadoras de sus efectos beneficiosos.

Nuestra hipótesis principal es que el tratamiento con MSCs-EVs puede ser útil para reducir la inflamación periférica, el daño hepático, la neuroinflamación y las alteraciones cognitivas y motoras encontradas en ratas con daño hepático leve inducido por tetracloruro de carbono (CCl_4), lo que puede apoyar el uso terapéutico de MSCs-EVs para NAFLD para prevenir la progresión del daño hepático y neurológico. Esta hipótesis podría dividirse en:

1. El tratamiento con MSC-EVs puede normalizar las alteraciones cognitivas y motoras observadas en ratas con daño hepático leve.
2. El tratamiento con MSC-EVs puede reducir la neuroinflamación en cerebelo e hipocampo y normalizar las alteraciones en la neurotransmisión glutamatérgica y GABAérgica observadas en ratas con daño hepático leve.
3. El tratamiento con MSC-EVs puede reducir la inflamación periférica y la lesión hepática producida por el daño hepático leve inducido por CCl_4 en ratas.
4. Los efectos de las MSC-EVs en el daño hepático leve, la inflamación periférica, la neuroinflamación en cerebelo e hipocampo y el deterioro cognitivo y motor pueden depender de su contenido en TGF-β

Para evaluar esta hipótesis, nos planteamos los siguientes objetivos:

1. Evaluar si el tratamiento con MSC-EVs puede reducir las alteraciones observadas en ratas con daño hepático leve en funciones cognitivas y motoras, neuroinflamación y

neurotransmisión glutamatérgica y GABAérgica en cerebelo e hipocampo, inflamación periférica y estructura e inflamación hepática.

2. Analizar si el TGF-β tiene un papel importante en los efectos que las MSC-EVs pueden tener en las alteraciones producidas por el daño hepático leve en ratas.

3. Ampliar el conocimiento sobre los mecanismos subyacentes por los que las MSC-EVs ejercen su efecto terapéutico sobre el daño hepático leve en ratas.

MATERIALES Y MÉTODOS

Modelo y tratamiento

Se utilizaron ratas macho Wistar (Charles River) de 150-180 g de peso. Las ratas fueron mantenidas en condiciones estándar de temperatura (22ºC) y humedad (40-60%) y ciclo luz-oscuridad de 12:12 h, con dieta estándar y comida y bebida *ab libutum*. Para inducir el daño hepático, la mitad de las ratas fueron inyectadas por vía intraperitoneal 3 veces/semana durante 4 semanas con 1 mL/kg de peso corporal con CCl_4 (1:10 (v:v) en aceite de maíz). A las ratas de control se les inyectó por vía intraperitoneal 1 mL/kg de aceite de maíz.

Las ratas fueron tratadas con vesículas extracelulares (EVs) de células madre mesenquimales sin modificar (C-EVs), con EV de células madre con el gen de TGF-β silenciado (T-EVs) o con solución salina tamponada con fosfato (PBS). Las EVs se aislaron del medio de cultivo de células madre mesenquimales derivadas de adipocitos humanos subconfluentes (sin modificar y con TGF-β silenciado) mediante centrifugaciones, como se detalla en Castro et al. (2019). Se administraron a las 2 y 3 semanas de tratamiento con CCl_4 por inyección intravenosa a 50 μg en 300 μL de PBS (o solo 300 μL de PBS como vehículo). El experimento consistió en 6 grupos de ratas: Control-vehículo (CV); daño hepático inducido por CCl_4 (CC); ratas control-tratadas con C-EVs (CE); ratas control tratadas con T-EVs (CE-T); ratas CCl_4 tratadas con C-EVs (CCE) y ratas inyectadas con CCl_4 con T-EVs (CCE-T). Los experimentos fueron aprobados por el Comité de Experimentación y Bienestar Animal del Centro de Investigación Príncipe Felipe (Valencia, España) y la Conselleria de Agricultura de la Generalitat Valenciana (Comunidad Valenciana, España) y se realizaron de acuerdo con las directrices de la Directiva de la Comisión Europea (2010/63/UE) para el cuidado y manejo de animales de experimentación. Todos los experimentos se realizaron de acuerdo con las directrices ARRIBE para la experimentación animal.

Análisis de la función cognitiva y motora

La coordinación motora se analizó por medio de los tests de comportamiento Rotarod (cilindro giratorio con aceleración), Beam walking (listón de madera elevado) y MotoRater (escalera horizontal). La marcha se analizó en el aparato CatWalk y se cuantificó en su propio software. La memoria espacial se midió por medio del test de localización nueva de objeto (NOL). LA memoria a corto plazo se analizó con el laberinto en Y. El índice de aprendizaje y la memoria espacial se midieron en el laberinto radial de 8 brazos.

Análisis de la neurotransmisión

La neurotransmisión glutaminérgica y GABAérgica en cerebelo y en hipocampo se analizaron cuantificando el contenido de transportadores y receptores de glutamato y GABA en la membrana celular. Para esto, se utilizó el crosslinker BS3 al preparar los homogenados de los tejidos, que se une a las proteínas de membrana y no las deja avanzar en el *Western blot*. De esta forma se calcula la expresión en membrana como la diferencia entre la muestra sin BS3 (contenido total) y la muestra con BS3 (contenido del citosol). El contenido de las isoformas GAD65 y GAD67 de la glutamato descarboxilasa, enzima productoras de GABA, se analizó por *Western blot* y por inmunohistoquímica (en las células de Purkinje de cerebelo). El contenido de GABA también se analizó por inmunohistoquímica en las células de Purkinje del cerebelo.

Análisis de la neuroinflamación

La neuroinflamación se analizó midiendo la activación de microglía y astrocitos por inmunohistoquímica con sus respectivos marcadores Iba1 y GFAP. La IL-1β también se analizó por inmunohistoquímica en hipocampo y TNF-α se cuantificó por *Western blot* en cerebelo y en hipocampo. Además, se cuantificó por *Western blot* el marcador YM-1 de microglía tipo M2 (antiinflamatoria) en el cerebelo.

Análisis de la inflamación periférica

Se midieron los niveles de TNFα y TFG-β con kits de ELISA comerciales y de otras citocinas pro y antiinflamatorias por *Western blot,* en el plasma obtenido de sangre extraída de la vena safena.

Análisis del daño hepático

El estado del hígado se midió por histología con las tinciones de hematoxilina y eosina y la tinción tricrómica de Massson. El daño hepático se clasificó utilizando un sistema de puntuación como el de (Kleiner et al., 2005). Las características histológicas consideradas fueron esteatosis (0-3), inflamación lobular (0-3) y grado de fibrosis (0-3).

Se enviaron muestras de suero a analizar al Laboratorio Dr. F. Echevarne, Analisis, S.A. para analizar los marcadores serológicos de daño hepático alanina transaminasa (GPT/ALT), aspartato transaminasa (GOT/AST), fosfatasa alcalina (ALP), bilirrubina y ácidos biliares.

También se analizó el contenido de los marcadores de fibrosis colágeno I y α-SMA en hígado por *Western blot* y por inmunohistoquímica. Algunas citocinas también se analizaron por *Western blot* en el hígado para analizar la inflamación hepática.

Análisis de la microbiota intestinal

Se analizó la microbiota intestinal a partir de las heces de las ratas. Se extrajo el ADN y se secuenció la subunidad 16s del ARN ribosomal. Se analizó la alfa y la beta diversidad por el método de selección de unidades taxonómicas operativas (UTO) que se identificaron como especies comparando con las bases de datos. También se correlacionaron estos datos con los datos de comportamiento. Este experimento se analizó por un método estadístico distinto.

Estadística

Se utilizó el software GraphPad Prism v. 9.5.0 para el análisis estadístico y el dibujo de gráficos. Los datos se expresan como media ± SEM. El análisis estadístico se realizó mediante ANOVA de una vía de cuatro grupos. Los grupos se analizaron de cuatro en cuatro para analizar los efectos de cada tipo de MSC-EV por separado. Para analizar el efecto de las C-EVs se incluyeron los grupos CV, CE, CC y CCE, mientras que los efectos de las T-EVs se analizaron con los grupos CV, CE-T, CC y CCE-T. Para las comparaciones múltiples se utilizó la prueba de Tukey y la prueba LSD no corregida de Fisher. En su caso, se utilizaron ANOVA de dos vías con medidas repetidas y la prueba de comparaciones múltiples de Bonferroni. Los datos que no superaron la prueba de normalidad (pruebas de D'Agostino y Pearson o de Kolmogorov-Smirnov) se analizaron con la prueba no paramétrica de Kruskal-Wallis, seguida de la prueba de Dunn (o la prueba de Dunn sin corregir) para comparaciones múltiples. Cuando las desviaciones estándar (SD) no eran iguales, se aplicó el ANOVA de Welch seguido de la prueba de comparaciones múltiples T3 de Dunnett o pruebas t no apareadas con corrección de Welch. Se consideró significativo un nivel de confianza del 95%.

RESULTADOS Y DISCUSIÓN

Efectos de las MSC-EVs sobre la función cognitiva y la neuroinflamación y alteraciones en la neurotransmisión en hipocampo

Los resultados de este proyecto corroboran que las ratas con daño hepático leve muestran un deterioro del aprendizaje y la memoria espaciales dependientes del hipocampo, así como de la memoria de trabajo, como se ha demostrado previamente (Leone et al., 2022).

Los efectos de las MSCs-EVs sobre el aprendizaje y la memoria espacial dependen en gran medida de la tarea y también del contenido de EVs. El tratamiento con MSCs-EVs no modificadas (C-EVs), pero no con aquellas que provienen de MSCs con TGF-β silenciado (T-EVs), restaura la memoria espacial medida en el test NOL pero no el aprendizaje espacial, la memoria de trabajo o la memoria a corto plazo. El análisis de la neuroinflamación del hipocampo mostró que las C-EVs también normalizan la activación de la microglía, el contenido de TNF-α y aumentan el contenido de IL-1β en las neuronas CA1, que disminuyó en las ratas con daño hepático leve. Además, el análisis de algunos componentes de la neurotransmisión glutamatérgica y GABAérgica en el hipocampo mostró que las MSCs-EVs no modificadas también restablecen la disminución de la expresión de membrana de la subunidad GluN2B y el aumento de la subunidad GABAA-γ2 y el contenido reducido de la isoforma GAD65.

Por el contrario, tanto el deterioro del aprendizaje espacial como de la memoria de trabajo en el laberinto radial, mejoraron con el tratamiento con T-EVs, pero no con C-EVs. Además de las subunidades GABAA-α5 y γ2, también revertidas por C-EVs, el tratamiento con T-EVs restauró el aumento de la expresión de membrana de la subunidad $GABA_A$-α2 y del intercambiador iónico NKCC1, la disminución de la expresión de membrana de la subunidad GluA2 del receptor de glutamato, la activación de astrocitos y el número de células IL-1β+ en el hipocampo, que no se vieron afectados por C-EVs. Esto sugiere que la activación astrocítica

puede ser responsable del aumento de IL-1β, que podría inducir el aumento de la expresión de membrana de la subunidad GABAA-α2 y la disminución de la expresión de membrana GluA2, que a su vez, puede inducir el deterioro en el aprendizaje y la memoria de trabajo en el laberinto radial. Toda esta secuencia de acontecimientos se normaliza con el tratamiento con T-EVs, pero no con C-EVs, lo que indica que la expresión de TGF-β y el cambio en la carga inducido por ella, conduce a efectos diferentes y más positivos que las EVs no modificadas, mejorando el índice de aprendizaje y la memoria de trabajo.

Estos resultados demuestran el efecto beneficioso del tratamiento con MSC-EVs sobre el deterioro cognitivo en ratas con daño hepático leve y que los mecanismos implicados incluyen reducción de la neuroinflamación y normalización de algunas alteraciones que afectan a la neurotransmisión glutamatérgica y GABAérgica. Nuestros resultados también sugieren que los efectos de las EVs dependen en gran medida de su carga y que el TGF-β desempeña un papel importante en el control de sus efectos sobre las alteraciones producidas por el daño hepático leve inducido por la administración de CCl_4 en ratas.

Efectos de las MSC-EVs sobre la función motora y la neuroinflamación y alteración de la neurotransmisión GABAérgica en cerebelo

Nuestros resultados muestran que el daño hepático leve induce alteraciones tanto en la coordinación motora como en la marcha locomotora en ratas. Anteriormente se había demostrado que la coordinación motora estaba alterada en ratas inyectadas con CCl_4 medida en las pruebas Rotarod y Beam walking (Balzano et al., 2021). El presente estudio muestra que la coordinación motora en ratas con daño hepático leve también está alterada cuando se mide en el MotoRater. También analizamos la marcha locomotora con el sistema CatWalk XT y se encontraron varias alteraciones en ratas con lesiones hepáticas leves. El análisis de la marcha locomotora en el sistema CatWalk XT se ha utilizado principalmente para modelos animales de la enfermedad de Parkinson (Vandeputte et al., 2010; Chuang et al., 2010; Xiao et al., 2017; Boix et al., 2018; Tsang et al., 2019; Narbute et al., 2019). Las ratas hiperamonémicas también muestran alteraciones en la marcha locomotora (Arenas et al., 2022; Mincheva et al., 2022). Estas alteraciones reflejan la akinesia, bradikinesia y síntomas Parkinsonianos que presentan pacientes con EH.
Estas alteraciones de la función motora se normalizaron con el tratamiento con MSC-EVs. Observamos un mayor efecto de las C-EV, aunque ambos tipos fueron capaces de mejorar la mayoría de los parámetros motores alterados, incluida la coordinación motora. Sólo dos parámetros de la marcha, la postura dual y el ciclo de paso, se normalizaron con las C-EVs pero no con las T-EVs, lo que sugiere un papel de la expresión de TGF-β en los efectos beneficiosos de las EVs sobre ellos. Sin embargo, los mecanismos por los que inducen la mejoría parecen depender de la expresión o no de TGF-β. Aunque ambos tipos de EVs normalizaron los niveles de GABA en las neuronas de Purkinje, sólo las C-EVs normalizaron la expresión en membrana de GAT-TNF1 y de la subunidad GABAA-β3, mientras que las T-EVs normalizaron el contenido de GAD67 en las neuronas de Purkinje. Estas alteraciones en la neurotransmisión se producen por la neuroinflamación en el cerebelo y las C-EV también

redujeron la activación de astrocitos y microglía en el cerebelo. Las T-EV no mejoraron la activación de los astrocitos ni de la microglía en el cerebelo, pero promueven la polarización de la microglía hacia el tipo M2 (antiinflamatorio), en ratas con daño hepático leve. Además, los niveles de TNF-α aumentan en el cerebelo con la administración de CCl_4, como ya se había descrito (Balzano et al., 2021), y se reducen con ambos tipos de EVs.

En el cerebelo, existen algunas vías de señalización que explican cómo la neuroinflamación afecta a la neurotransmisión. Cabrera-Pastor et al., (2018) describieron la vía TNFα-TNFR1-glutaminasa-GAT3 en ratas hiperamonémicas. Además de los niveles de TNF-α, los niveles de glutaminasa también estaban aumentados en el cerebelo de ratas con daño hepático leve, pero a diferencia del TNF-α, los niveles de glutaminasa solo se normalizaron con las T-EVs. Además, aunque el contenido de GAT-3 y la expresión de la membrana aumentaron en las ratas hiperamonémicas, no se vieron alterados por el CCl_4. Sin embargo, el tratamiento con T-EVs en estas ratas redujo ambos, contribuyendo posiblemente a disminuir los niveles extracelulares de GABA en ratas con daño hepático leve.

Otra vía que relaciona la neuroinflamación con alteraciones en la neurotransmisión en el cerebelo es la descrita por (Arenas et al., 2022). La vía TNFα-CCL2-P2X4-BDNF-TrkB también se alteró en ratas con daño hepático leve de forma similar a las alteraciones observadas en ratas hiperamonémicas (Arenas et al., 2022). La mayoría de los puntos clave analizados de esta vía se normalizaron mediante el tratamiento con ambos tipos de EVs, lo que indica que la vía también está normalizada. Sin embargo, los niveles de CCL2 sólo se normalizaron con las T-EVs.

Efectos de las MSC-EVs sobre la inflamación periférica y el grado de daño hepático

La neuroinflamación, tanto en el cerebelo como en el hipocampo, puede tener su origen en la inflamación periférica. Las ratas con daño hepático leve mostraron un aumento de los niveles de citoquinas proinflamatorias, lo que indica la presencia de inflamación periférica, tal y como se describió anteriormente. Ambos tipos de EVs normalizaron los niveles de TNF-α, TGF-β, IL-17 e IL-21; la IL-6 fue normalizada por las T-EVs y parcialmente por las C-EVs y la HMGB1 sólo fue normalizada por las C-EVs. Por el contrario, ninguna de las EVs fue capaz de normalizar los niveles de IL-15.

Las ratas con daño hepático leve inducido por CCl_4 a las 4 semanas de administración presentan esteatosis, inflamación lobular y fibrosis. Estos parámetros también se midieron tras 12 semanas de administración de CCl_4, y el grado de esteatosis, inflamación lobular y fibrosis fue mayor que a las 4 semanas. El tratamiento con MSC-EVs no fue capaz de reducir significativamente el daño hepático analizado histológicamente. Solo la esteatosis a las 4 semanas se redujo significativamente con las T-EVs, aunque no alcanzó niveles similares a los de las ratas control.

El contenido de marcadores de fibrosis, colágeno I y α-SMA, también se incrementó en el hígado debido a la administración de CCl_4 durante 4 semanas. Los niveles de colágeno I se

redujeron con las T-EVs, mientras que los niveles de α-SMA se redujeron con ambos tipos de EVs, lo que sugiere que las EVs, principalmente cuando se silenció el TGF-β, son capaces de reducir el contenido de moléculas profibróticas características. Puesto que el TGF-β es una citoquina implicada en la inducción de fibrosis cuando aumenta su producción por las células estrelladas del hígado, la falta de TGF-β en las EVs administradas debe facilitar el mayor efecto de estas, comparado con las C-EVs, sobre los niveles de marcadores profibróticos.

La actividad de las enzimas hepáticas ALT y fosfatasa alcalina en suero, así como el contenido de ácidos biliares, aumentaron en el suero de las ratas inyectadas con CCl_4, aunque los niveles de bilirrubina no se alteraron y los niveles de AST sólo se alteraron en las ratas inyectadas con CCl_4 con tratamiento con C-EVs. El tratamiento con C-EVs no fue capaz de reducir los marcadores elevados, sin embargo, los T-EVs redujeron la actividad ALT e indujeron una disminución parcial del contenido de ácidos biliares. Estos efectos estarían mediados posiblemente por el mayor efecto de las T-EVs sobre el proceso fibrótico y por ello son éstas, pero no las C-EVs, las que disminuyen los marcadores de daño hepático y por tanto mejoran su función.

Los hígados de las ratas con daño hepático leve mostraron mayores niveles de TNF-α, pero no de IL-6, y los niveles de TGF-β no se alteraron. Esto indica la presencia de un nivel leve de inflamación hepática que se normalizó con las C-EV, pero no con las T-EV. Por tanto, las T-EVs muestran efectos beneficiosos sobre los marcadores de fibrosis y la función hepática, pero las C-EVs mejoran la inflamación hepática.

Efectos de las MSC-EVs sobre la microbiota intestinal

El estudio de la microbiota intestinal reveló que no hay cambios importantes entre los grupos experimentales en la diversidad alfa de las muestras. Sin embargo, el análisis de la diversidad beta mostró que existía una composición significativamente diferente en función del tratamiento del grupo. Los resultados revelan varias diferencias de UTO entre el grupo de control y las ratas con daño hepático leve. La identificación taxonómica de las UTO de interés de nuestros resultados mostró que muchas de ellas pertenecen al género *Bacteroides* que eran más abundantes en ratas con daño hepático leve inducido por CCl_4. La cirrosis inducida por tioacetamida predispone al hígado de rata a la colonización bacteriana con especies de los géneros *Escherichia, Enterobacter y Bacteroides* (al-Bader et al., 1998). Además, se ha observado que la abundancia de Bacteroides aumenta en pacientes con NASH (Boursier et al., 2016). Entre las especies identificadas como diferentes en el grupo inyectado con CCl_4, se encontraron *B. cellulosilyticus, B.caccae, B.eggerthii* y *Anaerostipes faecis*. De hecho, el tratamiento con C-EVs redujo *B. cellulosilyticus, B.caccae, B.eggerthii*, hacia la abundancia del grupo de control mientras que las T-EVs normalizaron *A. faecis*. Curiosamente, las abundancias de *B.caccae* y *B.eggerthii* se correlacionaron con los resultados de la marcha locomotora obtenidos en el CatWalk. Los cambios de la microbiota intestinal en diferentes estadios de la enfermedad hepática ya se han asociado con cambios en la función cognitiva (Higarza et al., 2022).

Efectos de las MSC-EVs en las ratas control

La administración de MSC-EVs en ratas control indujo algunas alteraciones. Ambos tipos de MSC-EVs indujeron cierta neuroinflamación y alteraron la neurotransmisión en el hipocampo y el cerebelo, deteriorando la función cognitiva y motora en ratas control. Los dos tipos de EVs alteran parámetros distintos, sin embargo, las T-EVs producen más alteraciones en el cerebro que las C-EVs, lo cual podría estar relacionado con la ausencia de la actividad anti-inflamatoria del TGF-β, aunque en principio las ratas control no muestran neuroinflamación. Por otra parte, las C-EVs aumentan el daño hepático en ratas normales, posiblemente por su elevado contenido en la citoquina profibrótica TGF-β.

CONCLUSIONES

1. Las MSC-EVs mejoran el deterioro cognitivo en ratas con daño hepático leve inducido por CCl_4. Las EVs de MSC no modificadas mejoran la memoria espacial mientras que las EVS de MSC carentes de TGF-β mejoran el índice de aprendizaje y el trabajo.
2. El deterioro cognitivo en ratas con daño hepático leve se produce por alteraciones inducidas por neuroinflamación en la neurotransmisión glutamatérgica y GABAérgica en el hipocampo. Las EVs de MSC no modificadas y MSC carentes de TGF-β normalizan la neuroinflamación y la neurotransmisión glutamatérgica y GABAérgica y en el hipocampo por diferentes mecanismos.
3. El daño hepático leve induce neuroinflamación en el cerebelo, lo que aumenta la activación de las vías TNFα-TNFR1-glutaminasa-GAT3 y TNFα-CCL2-P2X4-BDNF-TrkB, lo que conduce a un aumento de la neurotransmisión GABAérgica que deteriora la función motora y la coordinación. Las MSC-EV revierten la neuroinflamación y normalizan la activación de estas vías, la neurotransmisión GABAérgica y la coordinación motora.
4. Las MSC-EVs reducen la inflamación periférica en plasma de ratas con daño hepático leve, lo que contribuiría a la reducción de la neuroinflamación.
5. Las MSC-EVs no revierten completamente el daño hepático. Sin embargo, las EVs de MSC carentes de TGF-β reducen el daño hepático, reduciendo los marcadores de fibrosis y los marcadores séricos de daño hepático.
6. El daño hepático leve induce disbiosis de la microbiota intestinal en ratas que se mejora con el tratamiento con MSC-EVs. Las ratas inyectadas con CCl_4 muestran niveles elevados de Bacteroides que se reducen con el tratamiento con MSC-EVs y se correlacionan con el deterioro de la microbiota intestinal.
7. Ambos tipos de MSC-EVs aumentan la neuroinflamación y alteran la neurotransmisión en el hipocampo y el cerebelo, y deterioran la función cognitiva y motora en ratas control. Las EVs de MSC no modificadas aumentan el daño hepático en ratas normales.
8. Las MSC-EVs podrían ser una terapia prometedora para la MHE y la HE, y también para otras enfermedades que implican mecanismos patológicos similares, como las enfermedades asociadas con la inflamación periférica sostenida y la neuroinflamación.