SAPIENZA
UNIVERSITÀ DI ROMA

FROM THE GUT TO THE BRAIN: EFFECTS OF BOWEL INFLAMMATION ON SYNAPTIC TRANSMISSION AND GLIAL PROPERTIES

PhD in Clinical-Experimental Neuroscience and Psychiatry

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Abstract

Inflammatory bowel diseases (IBD) affect the gastrointestinal tract and can lead to extra-intestinal manifestations associated with cerebral function, including depression and anxiety. Previous studies have shown that during acute inflammation, different brain areas, including hippocampus, undergo alterations in glial properties and glutamatergic synaptic transmission, along with impairment of blood-brain barrier (BBB) permeability, leading to immune cell infiltration and increase of cytokines levels. However, a comprehensive understanding of the mechanisms involved remains elusive. This study aims to elucidate the cellular and molecular mechanisms underlying the alterations in the central nervous system. Using a murine model of acute peripheral inflammation induced by 2,4-Dinitrobenzenesulfonic acid hydrate (DNBS), we explored the impact of the gut-brain axis on cerebral function and identified potential mediators of central dysfunction. Through transcriptomic and immunofluorescence analyses, we show that colitis induces neuroinflammation and glial reactivity. In addition, by means of electrophysiological recordings, we assessed the effects of bowel inflammation on excitatory and inhibitory synaptic transmission in the hippocampus. Finally, we investigated the molecular mechanisms underlying the hippocampal alterations induced by colitis through metabolomic analysis. Our findings reveal significant changes in the hippocampal transcriptome, glial reactivity, and synaptic transmission, as well as changes in gut and hippocampal metabolites in the DNBS model. In conclusion, our study demonstrates that bowel inflammation and dysbiosis are associated with hippocampal dysfunctions, providing new perspectives for future investigations.

Index

Abbreviations

ACTH = AdrenoCorticoTropic Hormone

AhR = Aryl Hydrocarbon Receptor

Ala = Alanine

Asp = Aspartate

BBB = Blood-Brain Barrier

BSA = Bovine Serum Albumin

Cacna2d1 = Calcium voltage-gated channel subunit alpha2delta 1

CNS = Central Nervous System

DAI = Disease Activity Index

DEGs = Differentially Expressed Genes

DGE = Differential Gene Expression

DNBS = 2,4-Dinitrobenzenesulfonic acid hydrate

DSS = Dextrane Sodium Sulfate

E/I = Excitation-to-Inhibition

ENS = Enteric Nervous System

eEPSCs = evoked Excitatory PostSynaptic Currents

eIPSCs = evoked Inhibitory PostSynaptic Currents

ePSCs = evoked PostSynaptic Currents

EPSC = Excitatory PostSynaptic Currents

GABA = Gamma-AminoButyric Acid

Gabra3 = Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha3

Gad1 = Glutamate decarboxylase 1

Gfap = Glial fibrillary acidic protein

Gria1 = Glutamate receptor ionotropic AMPA Type Subunit 1

Gria2 = Glutamate receptor ionotropic AMPA Type Subunit 2

Hadh = Hydroxyacyl-CoA Dehydrogenase

H&E = Hematoxylin and Eosin

HMBC = Heteronuclear Multiple Bond Correlation

HMDSO = HexaMethylDiSilOxane

HPA = Hypothalamic–Pituitary–Adrenal

HSQC = Heteronuclear Single Quantum Correlation

IBD = Inflammatory Bowel Diseases

IEI = Inter-Event Interval

IL-1 = Interleukin-1

IL-2 = Interleukin-2

IL-6 = Interleukin-6

IL-10 = Interleukin-10

I/O = Input/Output

IPSC = Inhibitory PostSynaptic Currents

IS = Internal Standard

ISI = Inter-Stimulus Intervals

Jak2 = Janus Kinase 2

Lair1 = Leukocyte-associated immunoglobulin-like receptor 1

LTD = Long-Term Depression

LTP = Long-Term Potentiation

LY6Chi = Lymphocyte Antigen 6C-high

mEPSC = miniature Excitatory PostSynaptic Currents

mGluR5 = metabotropic Glutamate Receptor 5

Mmut = Mut Methylmalonyl-CoA Mutase

MRI = Magnetic Resonance Imaging

MSEA = Metabolite Set Enrichment Analysis

NMR = Nuclear Magnetic Resonance

PB = Phosphate Buffer

PCA = Principal Component Analysis

PFA = Paraformaldehyde

Prkcq = Protein Kinase C Theta

PLS-DA = Partial Least-Squares Discriminant Analysis

PPR = Paired-Pulse Ratio

PSCs = PostSynaptic Currents

RFU = Relative Fluorescence Units

Rims1 = Regulating Synaptic Membrane Exocytosis 1

ROI = Region Of Interest

RT = Room Temperature

SCFAs = Short-Chain Fatty Acids

sEPSCs = spontaneous Excitatory PostSynaptic Currents

sIPSCs = spontaneous Inhibitory PostSynaptic Currents

TGF-**β** = Transforming Growth Factor-**β**

Tjp1 = Tight junction protein 1

TNF **α** = Tumor Necrosis Factor **α**

TOCSY = Total Correlation Spectroscopy

TSP = 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt

VIP = Variable Importance in Projection

WT = Wild Type

Zo-1 = Zonula Occludens 1

Chapter 1: Introduction

The gut-brain axis

Definition and mechanisms

The gut-brain axis is a complex bidirectional communication system that connects the gastrointestinal tract and the central nervous system (CNS). This network not only integrates and monitors the intestinal activity, but also links emotional and cognitive processes, playing a crucial role in maintaining physiological homeostasis. These functions are mediated by peripheral mechanisms that encompass neuro-immune-endocrine factors, including immune activation, gut permeability, enteric reflexes and entero-endocrine signaling (Carabotti et al., 2015; Gwak & Chang, 2021) (Figure 1).

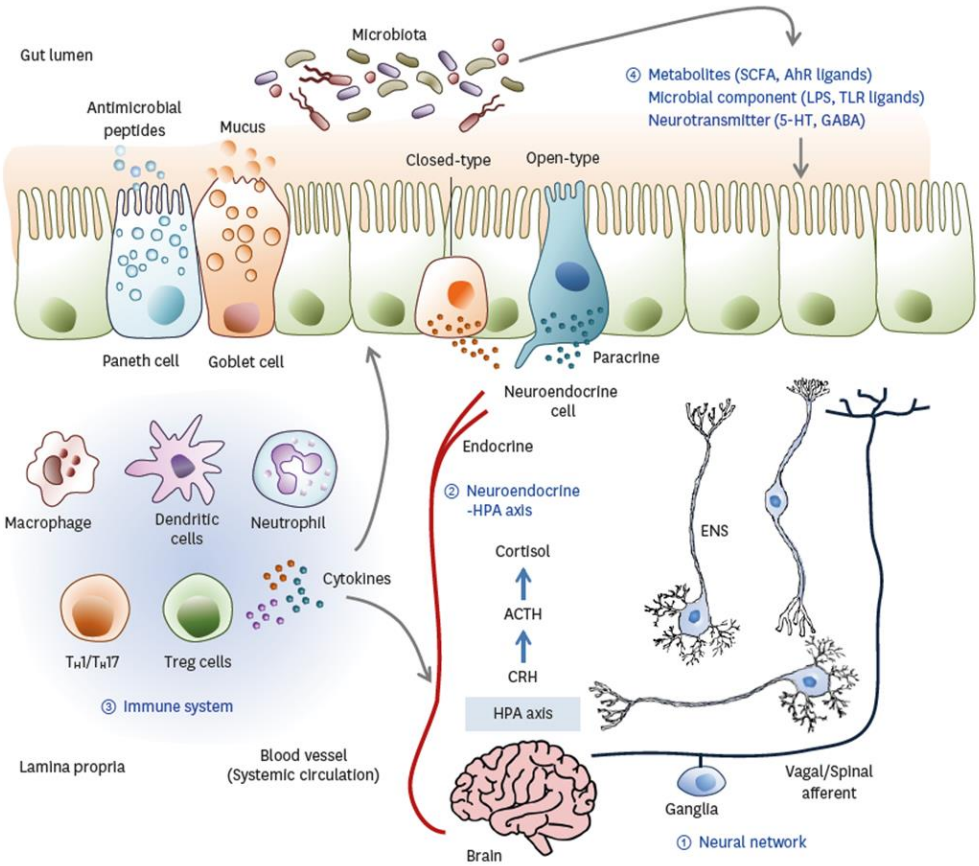


Figure 1- The gut-brain axis involves neuro-immune-endocrine pathways. (from Gwak & Chang, 2021)

Specifically, the gut-brain communication is mediated by:

- **Neuronal circuits**: We can identify two main neuroanatomic pathways that connect the gut to the brain. The first pathway is a direct route mediated by the parasympathetic and sympathetic branches of the autonomic nervous system, which regulates involuntary peripheral functions such as gut motility, fluid secretion, electrolyte transport, epithelial barrier integrity, and immune responses. A crucial component of this connection is the vagus nerve, the tenth cranial nerve, which conveys afferent signals from mechanoreceptors and chemoreceptors in the intestinal mucosa and muscularis layers to the CNS. Additionally, through its efferent fibers, the vagus nerve is involved in regulating metabolic processes and visceral immunity (Gwak & Chang, 2021). The central autonomic neuronal networks, which include the amygdala, hypothalamus, insula, hippocampus, and thalamus, are crucial for the regulation of the autonomic nervous system (Benarroch, 1993). Interestingly, a group recently found a multisynaptic neuroanatomic pathway that projects vagal gastrointestinal signals to the dorsal hippocampus, passing through medial nucleus tractus solitarius and medial septum (Suarez et al., 2018). This group hypothesized that this neuroanatomic pathway connecting vagus nerve to dorsal hippocampus mediates memory control (Suarez et al., 2018).

The second pathway is indirect and originates from the enteric nervous system (ENS), often referred to as the “second brain” due to its autonomous structure. Although the ENS was first identified in the 1700s, it was not until the mid-1990s that it was thoroughly characterized as a complex network of enteric neurons and glial cells organized into interconnected ganglia within the submucosal plexus (Meissner’s plexus) and the myenteric plexus (Auerbach's plexus) (Seguella & Gulbransen, 2021; Spencer & Hu, 2020). The ENS operates autonomously, managing digestive processes such as peristalsis and secretion (Hazart et al., 2023). Specifically, the submucosal plexus is crucial for regulating secretion and absorption, while the myenteric plexus coordinates smooth muscle contractions (Spencer & Hu, 2020).

Despite its independent functioning, the ENS is subject to regulation by the brain, as it is linked to the autonomic nervous system and the dorsal motor nuclei of the vagus nerve in the spinal cord. These pathways extend to the solitary tract nucleus in the brainstem, affecting various cortical and subcortical regions (Gwak & Chang, 2021). Enteric neurons and glia originate from the vagal and sacral neural crest during development, with the majority coming from the cervical region of the vagal crest and approximately 20% from sacral neural crest cells in the descending colon and rectum (Espinosa-Medina et al., 2017; Uesaka et al., 2015). The development of the ENS is a complex and ongoing process that continues postnatally, influenced by factors such as gut microbiota and the immune system (Hao et al., 2016; Kabouridis et al., 2015). Furthermore, enteric neurons exhibit plasticity that allows mature neurons to reinnervate the intestine with the aid of enteric glial cells (Seguella & Gulbransen, 2021). Within the myenteric ganglia, two primary types of neurons are identified: S-neurons, which function as mechanosensory and motor neurons projecting into the circular muscle layer, and AH-neurons, which serve as mechano- and chemo-sensory neurons and primarily project into the mucosa (Seguella & Gulbransen, 2021; Spencer & Hu, 2020). Nevertheless, this classification is overly simplistic in light of the functional diversity present. Recent studies identified 20 distinct classes of myenteric neurons in the human colon and 12 classes in the mouse myenteric plexus (Seguella & Gulbransen, 2021). Moreover, in the myenteric ganglia the enteric glia are crucial for maintaining homeostasis in the ENS. They can be classified into intra-ganglionic and extra-ganglionic types, with distinct subtypes within the intra-ganglionic group. The two subtypes, myenteric glia (type I-MP) and submucosal glia (type I-SMP), play different roles, where type I-MP regulates myenteric neuron activity and supports neural health, while type I-SMP modulates secretomotor neuron activity (Seguella & Gulbransen, 2021). Instead, extraganglionic glia consist of several types, including interganglionic glia, mucosal glia, enteric plexus glia, and intramuscular glia, each with distinct processes that support neuronal function and influence epithelial and immune responses (Figure 2) (Seguella & Gulbransen, 2021). Nevertheless, the exact roles of enteric plexus glia and intramuscular glia remain underexplored and require further investigation. Enteric glia have roles in the regulation of intestinal motility through gliotransmitter release, which is influenced by neurotransmitter signaling and serves to modulate the activity of associated neurons (Seguella & Gulbransen, 2021).

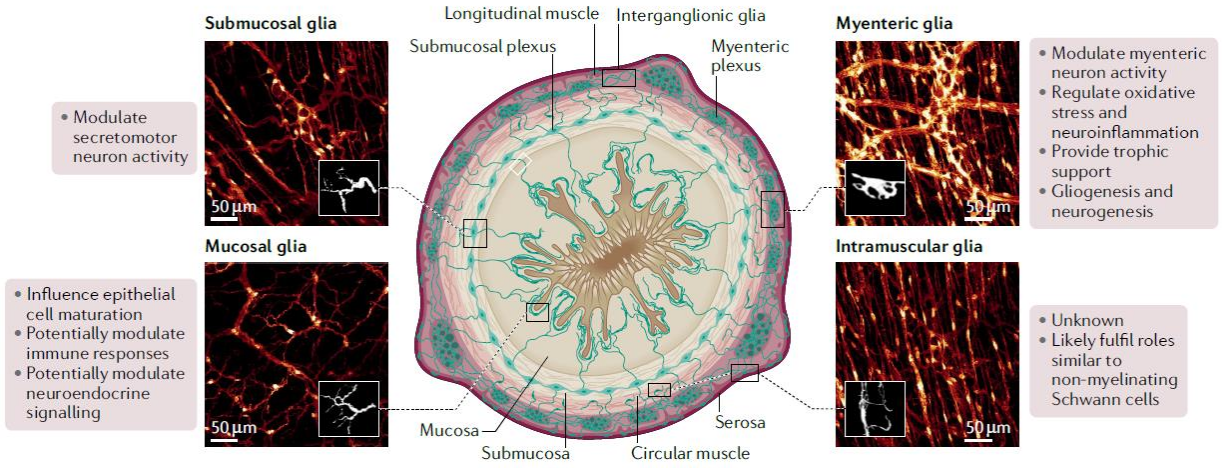


Figure 2 - Classification and main functions of the enteric glia in the ENS. (from Seguella & Gulbransen, 2021).

- **The hypothalamic–pituitary–adrenal axis (HPA axis):** It is a crucial part of the limbic system, which is essential for regulating memory, emotional responses, and coordinating adaptive reactions to stress (Carabotti et al., 2015). When the body is faced with environmental stressors and increased levels of proinflammatory cytokines in the intestine, the hypothalamus is activated. It responds by releasing corticotropin-releasing factor, which then stimulates the pituitary gland to produce adrenocorticotropic hormone (ACTH). ACTH subsequently triggers the adrenal glands to generate cortisol, the primary stress hormone, impacting various organs, including the brain (Carabotti et al., 2015).

- **Immune system**: The immune system reacts to a range of stimuli, including microbes, macromolecules, and self-derived molecules associated with autoimmune responses. It comprises a variety of immune cells such as macrophages, dendritic cells, and T cells, which contribute to innate and/or adaptive immunity (Abbas et al., 2012). Innate immunity, characterized by epithelial barriers and leukocytes, neutrophils and NK cells, is non-specific and provides immediate defense through pre-existing mechanisms (Abbas et al., 2012). In contrast, adaptive immunity targets specific antigens and can be amplified through immunological memory, facilitated by B lymphocytes that produce antibodies and T lymphocytes which respond to intracellular pathogens (Abbas et al., 2012). In this context, antigen-presenting cells are essential

for the activation and differentiation of lymphocytes into effector and memory cells by presenting antigens (Abbas et al., 2012). Key cytokines, including Tumor Necrosis Factor **α** (TNF**α**), Interleukin-1 (IL-1), and Interleukin-6 (IL-6), produced by activated macrophages and other immune cells, help regulate inflammation and can affect both local and systemic immune responses by crossing the BBB (Abbas et al., 2012).

In the gastrointestinal tract, intestinal epithelial cells play a critical role in innate immunity by producing mucins that prevent microbial adhesion and defensins for bacterial protection (Garavaglia et al., 2024). Tight junctions between epithelial cells, consisting of proteins like zonula occludens 1 (Zo-1), occludins, and claudins, further restrict pathogen entry (Garavaglia et al., 2024). The adaptive immune response in the gut is predominantly mediated by Th17 cells, with various cytokines such as Transforming growth factor-**β** (TGF-**β**), Interleukin-10 (IL-10), and Interleukin-2 (IL-2) being crucial for maintaining intestinal homeostasis (Garavaglia et al., 2024).

Role of the gastrointestinal microbiome

Although we tend to focus on a human-centered view, it is the microbial populations that have established themselves in vertebrates and a wide range of environments, creating symbiotic relationships with humans in various parts of the body. Microbiota generate metabolites that can significantly impact human physiology. Studies have connected changes in microbiota and their metabolites to a number of health issues, such as IBD, autism spectrum disorder, and Parkinson's disease (Hou et al., 2022). The core native microbiota, which are fundamental to personal health, tend to remain relatively stable throughout adulthood. However, the microbiota composition can differ significantly between individuals due to a variety of factors including enterotype, body mass index, physical activity levels, lifestyle decisions, as well as cultural and dietary habits (Rinninella et al., 2019). For this reason, personalized assessments of the microbes present in individuals could provide valuable therapeutic insights for addressing the physiopathological conditions (Eloe-Fadrosh & Rasko, 2013).

The gastrointestinal system is among the most densely microbial populated organs in vertebrates. Within the intestine, microbiota inhabit the interface of the mucosal

epithelial barrier, fulfilling essential functions in host defense, immune regulation, and nutrition. The symbiotic relationship between humans and microbiota commences at birth with maternal microbes colonizing infants (Eloe-Fadrosh & Rasko, 2013). In early childhood, a growing diversity of microorganisms forges a close bond with the host, and this symbiosis continues to evolve throughout life (Eloe-Fadrosh & Rasko, 2013). Additionally, other microorganisms, such as archaea, fungi, and viruses, also play a role in this intricate interaction. The composition of the microbiota can be affected by various factors, including antibiotics, prebiotics, probiotics, postbiotics, and environmental influences such as diet, stress, and exposure to pathogens (Eloe-Fadrosh & Rasko, 2013) (Figure 3).

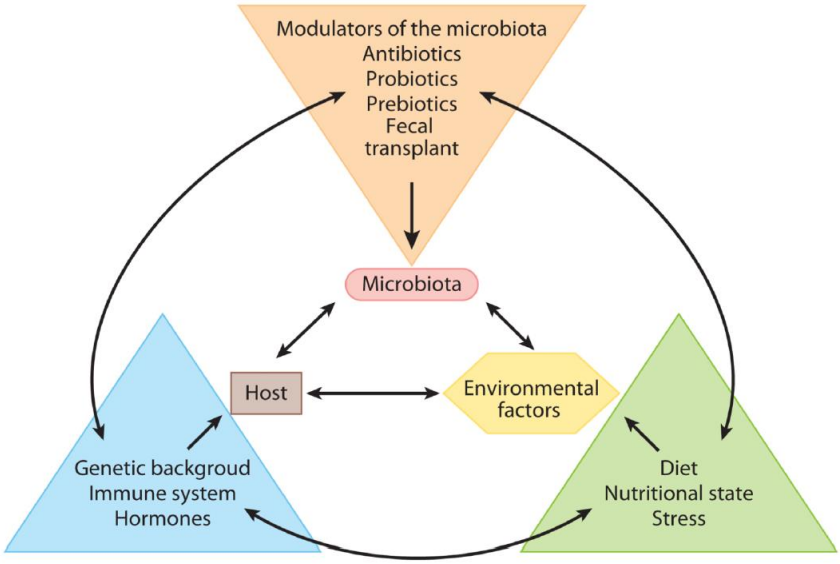


Figure 3- The relationship between microbiota and the host is influenced by host-derived and environmental factors. (from Eloe-Fadrosh & Rasko, 2013).

Most microbiota research has overlooked the genetic backgrounds of human hosts. A systematic study by Benson et al. (2010) used a mouse model with a defined genetic background to explore factors influencing gastrointestinal microbiota composition. They identified specific host genetic loci that regulate individual microbial species and related taxa, demonstrating pleiotropic effects on more distantly related organisms (Benson et al., 2010). Their findings suggest that gastrointestinal microbial diversity is a complex polygenic trait influenced by host genotype and various external factors

(Benson et al., 2010). Additionally, significant alterations in gut microbiota composition and decreased community diversity have been consistently observed in patients with IBD compared to healthy individuals (MetaHIT Consortium et al., 2010). However, the functional implications of these changes remain to be thoroughly investigated, highlighting an important area for personalized therapies.

In addition, recent researches underscore the profound influence of gut microbiota on brain function, revealing that dysbiosis is linked to a range of neurological and psychiatric conditions, including anxiety, depression, and neurodegenerative diseases (Ashique et al., 2024; Bakshi et al., 2024). The microbiota present in the intestinal lumen affect the neuronal, endocrine, and immune pathways of the gut-brain axis through interactions with antigens, such as lipopolysaccharides, and by generating metabolites like short-chain fatty acids (SCFAs) and ligands for the aryl hydrocarbon receptor (AhR). Furthermore, gut microbiota can modulate levels of neurotransmitters, including serotonin, dopamine, and GABA (Gwak & Chang, 2021; Schneider et al., 2024). Gut microbiota is essential for regulating the ENS and influencing vagal and autonomic afferent pathways. They also impact neuroendocrine cells in the intestinal epithelium, stimulating hormones that regulate the intestinal barrier, thereby facilitating the transport of neurotransmitters, stress hormones, metabolites, and immune cell factors (Gwak & Chang, 2021).

Inflammatory bowel diseases

Peripheral inflammation, caused by infections, chronic diseases, and immune responses, can lead to systemic effects that impair brain function and general health. This is primarily due to increased levels of inflammatory cytokines such as IL-1**β**, IL-6, and TNF**α**, which adversely impact cognitive abilities and contribute to neuropsychiatric symptoms. While the body has a protective inflammatory response aimed at recovery, failures to resolve prolonged inflammation can result in chronic inflammatory conditions, like IBD (Sun et al., 2022). IBD are a group of autoimmune relapsing-remitting diseases caused by an abnormal immune response in the intestinal tract. IBD affects approximately 1.5 million people in the United States and 2.2 million people in Europe, with an increasing global incidence (Jairath & Feagan, 2020). Since IBD is prone to relapse and difficult to treat, it strongly impairs the quality life of patients. In humans, the two most diffused IBD are ulcerative colitis, characterized by widespread mucosal inflammation in the colon, and Crohn's disease, which causes transmural ulcers in multiple portions of the gastrointestinal tract (Guan, 2019). The symptoms of IBD are diarrhea, vomit, abdominal pain and weight loss but the chronic persistence of gut inflammation can lead to extraintestinal effects, such as anxiety and depressive-like behaviors (Mikocka-Walus et al., 2016; Rogler et al., 2021). Although the exact etiology is not well understood, the pathogenesis of IBD derives from the complex interaction between genetic factors, environmental factors, host immune regulation, intestinal microbes and microbial metabolites (J. Jang & Jeong, 2023). Current therapeutic treatments include anti-inflammatory drugs and anti-TNF drugs helping to reduce the inflammation and induce remission (Abbas et al., 2012). While these treatments have improved patient outcomes, challenges remain, including varying responses and potential adverse effects, highlighting the need for personalized medicine approaches (Hentschel & Klaus, 2023). Recently, some studies suggest that the treatment with probiotics, prebiotics and postbiotics ameliorates not only gut inflammation, but also depression-related symptoms (Jang et al., 2019).

While the exact cause of IBD remains unclear and is thought to involve multiple factors, research suggests that alterations in defensin production and the immune response to

gut bacteria may trigger the onset of IBD (Na et al., 2019). In this context, macrophages are essential for maintaining tissue stability by responding to these microorganisms. Additionally, inflammation leads to an influx of neutrophils and monocytes, which obstructs the maturation of lymphocyte antigen 6C-high (LY6Chi) monocytes into macrophages, resulting in an excess of immature macrophages that produce more proinflammatory cytokines, exacerbating epithelial damage (Na et al., 2019). Moreover, IBD patients show increased Th17 cell activation due to macrophage interactions, which is linked to genetic factors like IL-23 receptor polymorphisms that elevate IBD risk (Na et al., 2019). In particular, Crohn's disease is characterized by Th1-driven granuloma formation, and ongoing clinical trials are exploring monoclonal antibodies that target components of IL-23 and IL-12 to attenuate immune responses (Abbas et al., 2012). Additionally, IBD is associated with weakened regulatory T cell responses, deficiencies in autophagy genes, and reduced defensin secretion from Paneth cells (Abbas et al., 2012).

In this scenario, the gut microbiome and its metabolites play a crucial role in regulating immune and inflammatory responses (C.-S. Kim, 2024; N. Y. Kim et al., 2024; Schneider et al., 2024). In physiological conditions, sealing the epithelial cells in the intestinal epithelium the tight junctions prevent the passage of antigens and bacteria into the gut wall and bloodstream. In the case of IBD, the expression of some proteins that make up the tight junctions of the intestinal epithelium, such as Zo-1 and occludin, is reduced therefore increasing intestinal permeability for antigens and bacteria with consequent activation of immune cells and their release of pro-inflammatory cytokines that induce peripheral inflammation (Chelakkot et al., 2018). Prominent among these metabolites are SCFAs and bile acids, which appear to modulate the inflammatory response and glial activity in the brain (N. Y. Kim et al., 2024; Schneider et al. 2024). SCFAs are produced through the metabolism and fermentation of dietary fibers by gut microbiota in the cecum and colon. Their concentrations diminish along the gastrointestinal tract, with propionate, butyrate, and acetate being the most prevalent in the cecum to the distal colon (Mann et al., 2024). SCFAs serve as signaling molecules for G-protein coupled receptors (GPCR) throughout the body (Mann et al., 2024). Notably, in vitro studies demonstrated that SCFAs promote neurogenesis and mitigate neuroinflammation by interacting with astrocytes (Ribeiro et al., 2020; Spichak

et al., 2021). Research involving germ-free mice indicated that administering SCFAs in drinking water improved both the morphological and functional maturation of cortical microglia, reinforcing the idea that SCFAs have a beneficial impact on brain health (Erny et al., 2015). Additionally, primary bile acids, which are generated from cholesterol metabolism in the liver, are conjugated with glycine and taurine before being secreted into the intestinal lumen in response to food intake. Of these bile acids, 95% are reabsorbed into the liver, while the remaining 5% are either excreted in feces or converted into secondary bile acids by gut microbiota, which can traverse the BBB and act as antagonists to NMDA and GABA-A receptors (Shulpekova et al., 2022). An in vitro study using brain-derived astrocytes from a mouse model of peripheral inflammation found that exposure to bile acids decreased the polarization of astrocytes towards a neurotoxic phenotype and microglia towards a pro-inflammatory state (Bhargava et al., 2020; Hurley et al., 2022). Additionally, studies suggest that a deficiency in secondary bile salts leads to dysbiosis, which in turn exacerbates intestinal inflammation (Sinha et al., 2020). Changes in bacterial composition and their metabolites in the intestinal lumen may be pivotal in the systemic adverse effects of colitis.

Gut inflammation influences the brain

IBD can also manifest extra-intestinal effects, such as cardiovascular, liver, thyroid, skin, immune and brain alterations, including stress, insomnia, anxiety and depression (Rogler et al., 2021).

The brain can be influenced by the condition of the peripheral system via both direct and indirect signals from various organs (Dantzer, 2018; Goehler et al., 2000; Pavlov et al., 2018). As described above, the gut and brain communicate bidirectionally and the information from the intestine are integrated in the central autonomic network, comprehending limbic system and striatal structures. Goodyear and colleagues, through magnetic resonance imaging (MRI), observed the increased activation and functional connectivity of these areas in both female and male patients with IBD in respect to the controls, with a sex-dependence activation (Figure 4) (Goodyear et al., 2023).

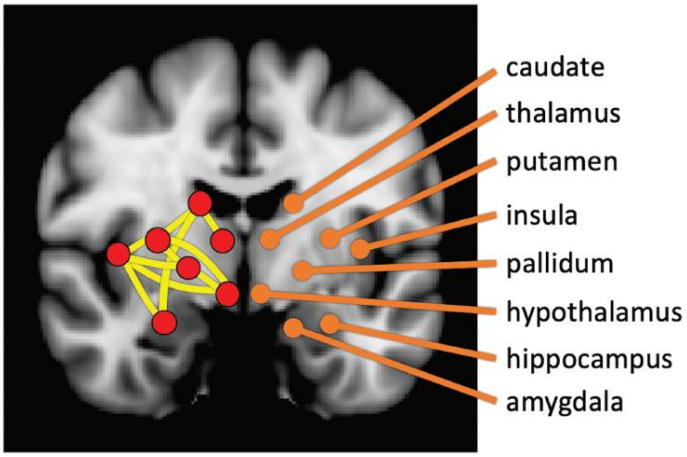


Figure 4 - Connections (in yellow) within the functional deep gray matter of some brain regions (in red) were found increased in IBD patients compared to control. (from Goodyear et al., 2023).

These central autonomic network areas manage the stress response and emotional reactions. Moreover, patients with systemic inflammation, such as IBD, have high comorbidities with anxiety and depression (Mikocka-Walus et al., 2016; Popov et al., 2021).

Other evidences have observed that some areas of the brain, including the insular cortex, the amygdala, the supplementary somatosensory cortex and the hippocampus, are more active in the presence of peripheral inflammation (Abraham, 2009; Koren et al., 2021). Specifically, in animal models of colitis and peritonitis, it is possible to induce peripheral inflammation by activating a cluster of neurons known as neuronal ensembles in the insular cortex (Koren et al., 2021). Additionally, in an acute colitis rat model, hippocampal glutamatergic synaptic transmission is hyperactive and exhibits a reduced threshold for seizures (Riazi et al., 2008, 2015).

The effects of intestinal inflammation on the brain may be potentially due to the disruption of the BBB, which in physiological condition protects the brain by preventing the entry of macromolecules greater than 400 Da, antigens and microorganisms from the bloodstream. It is shown that in the presence of peripheral inflammation the BBB is more permeable thus allowing the passage of antigens, microorganisms and immune cells into the brain resulting in neuroinflammation (X. Huang et al., 2021; Natah et al., 2005). Among the biological consequences of the leaky BBB also the

infiltration of peripheral immune cells and metabolites can potentially exacerbate the neuroinflammation in the brain. In animal models of intestinal inflammation, an increase in the number of macrophages, monocytes and pro-inflammatory cytokines (IL-1β, TNFα, IL-18) in the brain has been observed (Carloni et al., 2021; Gampierakis et al., 2021; Riazi et al., 2008; Sroor et al., 2019). Furthermore, it seems that the increase in TNFα levels may mediate the observed alterations in hippocampal glutamatergic synaptic transmission (Riazi et al., 2008, 2015). In addition, some studies have found that gut inflammation alters microglia and astrocytes (Carloni et al., 2021; Riazi et al., 2008, 2015; Sroor et al., 2019; Zhao et al., 2022). Although in several models of peripheral inflammation, such as LPS-induced inflammation, microglia have been shown to be affected by peripheral inflammation by playing a dual role in maintaining BBB integrity at different stages of peripheral inflammation, in animal models of IBD the presence of microglial reactivity following intestinal inflammation remains controversial (Carloni et al., 2021; Haruwaka et al., 2019; Riazi et al., 2008; Sroor et al., 2019). In fact, some researchers have observed increased density and activation of microglia, while one group found a reduction in the percentage of microglia in the brain (Barnes et al., 2021; Carloni et al., 2021; Gampierakis et al., 2021; Riazi et al., 2008; Sroor et al., 2019). Instead, astrocytes proliferate and undergo changes, including the release of pro- and anti-inflammatory factors, induced by systemic inflammation (Biesmans et al., 2013; Siracusa et al., 2019; Zamanian et al., 2012).

A recent study explored the mechanisms linking inflammatory pathways to behavioral abnormalities seen in Crohn’s disease by inducing acute colitis in male adult mice and evaluating anxiety and depressive-like behaviors (Haj-Mirzaian et al., 2017). They found that colonic inflammation from the treatment led to the infiltration of inflammatory cells and activation of toll-like receptor signaling genes in the intestines (Haj-Mirzaian et al., 2017). The affected mice exhibited anxiety and depression, which seem to be associated with heightened inflammatory gene expression and disrupted mitochondrial function in the hippocampus (Haj-Mirzaian et al., 2017). This suggests a communication pathway where the brain can detect gut inflammation, potentially explaining the onset of anxiety and depression in Crohn's disease patients. The mechanisms by which neuroimmune interactions influence sickness behaviors associated with systemic inflammation remain unclear. Nevertheless, increased levels

of pro-inflammatory cytokines and nitrosative stress can disrupt the mitochondrial respiratory chain, resulting in a deficiency of cellular energy (Morris & Maes, 2014). Given the essential role of mitochondria in the excessive generation of reactive oxygen species during disease states, several studies indicate that impaired mitochondrial function and inflammatory responses may significantly contribute to the onset of depression (Agam & Toker, 2015).

Additionally, stress can aggravate IBD directly, by triggering the adrenal cortex to release corticotropin-releasing hormones, and indirectly, by activating the sympathetic nervous system while suppressing the vagus nerve (Ge et al., 2022a). This leads to an increase in proinflammatory cytokine production and promotes the polarization of M1 macrophages and the transformation of Treg cells into a pro-inflammatory phenotype. Furthermore, stress can cause dysbiosis, which reduces the production of SCFAs by gut bacteria and compromises the integrity of the intestinal barrier (Ge et al., 2022a).

In conclusion, the etiology and the long-term effects of IBD on mental health remain elusive. Gaining insight into the cellular and molecular mechanisms driving the brain's response to colitis could reveal new therapeutic targets.

The physiopathological roles of glial cells

Glial cells were firstly described in the 19th century by Rio-Hortega, Virchow and Ramon y Cajal with a unique function as cells that surround neurons. During the following years, many other functions have been added to the characterization of glial cells. In the CNS, glia can be divided into three main subtypes: microglia, astrocytes and oligodendrocytes. With the purpose of this study, we focus on microglia and astrocytes for their fundamental roles in physiopathological conditions.

Microglia display immune and non-immune functions

Microglia are the resident immune cells within the CNS. They originate from yolk-sac progenitors and infiltrate the brain during development, specifically around embryonic day 9 in mice (Ginhoux et al., 2010). These cells are highly dynamic, capable of rapidly altering their morphology and molecular profile to adapt to changes in cerebral tissue (Hanisch & Kettenmann, 2007). Across different species, microglia display regional and temporal diversity, particularly in terms of their density, morphology, and the expression of various markers throughout different regions of the CNS (Grabert et al., 2016). Additionally, microglia interact with various other cells in the CNS, including neurons, astrocytes, and oligodendrocytes (Matejuk & Ransohoff, 2020; Peferoen et al., 2014; Szepesi et al., 2018).

The investigations of the microglia properties in physiopathological conditions have included pharmacological or genetic ablation of microglia. Many studies utilizing ablation methods have shown that microglia can transiently repopulate, typically within approximately one week. However, the precise origin of the proliferating cells in the adult brain remains a topic of debate. Current knowledge of repopulating microglia shows sources in local Nestin-expressing progenitors, infiltrated macrophages, or from surviving microglia (Elmore et al., 2014; Hashimoto et al., 2013; Y. Huang et al., 2018; Sieweke & Allen, 2013). While these studies provide compelling evidence for microglial repopulation, the subject remains contentious, likely due to the techniques employed in microglia depletion. Specifically, pharmacological methods often target molecular

pathways that overlap with those of peripheral macrophages and monocytes, complicating the isolation of microglial cells.

Contrary to previous beliefs, microglia do not exist in a bipolarized state. Instead, they represent a continuum of stages characterized by variations in gene expression, morphology, motility, migration, metabolism, receptors, secretome, phagocytosis, proliferation, and cell death (Prinz et al., 2019) (Figure 5). Today, the use of advanced techniques in transcriptomics, epigenomics, proteomics, and imaging analysis allows for a detailed description of the functional states of microglia.

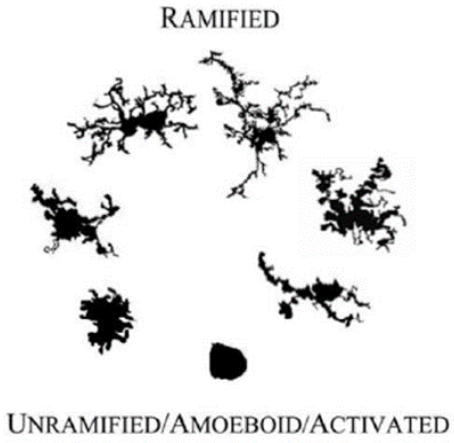


Figure 5 - Microglia exist in a continuum of morphofunctional states. (from Karperien et al., 2013).

The morphology of microglia is closely linked to their functional role. Pio del Rio Hortega was the first to highlight their morphological changes in response to CNS pathology. Within minutes after an injury, microglia swiftly transform into a characteristic ameboid shape, significantly altering their gene expression (Walker et al., 2014). It is now understood that the microglial response to brain damage involves multiple intermediate stages, encompassing motility, migration, phagocytosis, proliferation, and cellular death (Prinz et al., 2019). Furthermore, these responses are not uniform; reactive microglia can also exhibit a hyper-ramified morphology in reaction to acute stress or premature aging (Hellwig et al., 2016; Tay et al., 2017).

Under physiological conditions, microglia survey the parenchyma playing a crucial role in the maintenance of brain homeostasis. In fact, they can support neuronal plasticity,

synaptic pruning, phagocytosis and programmed cell death (Figure 6) (Sierra et al., 2019).

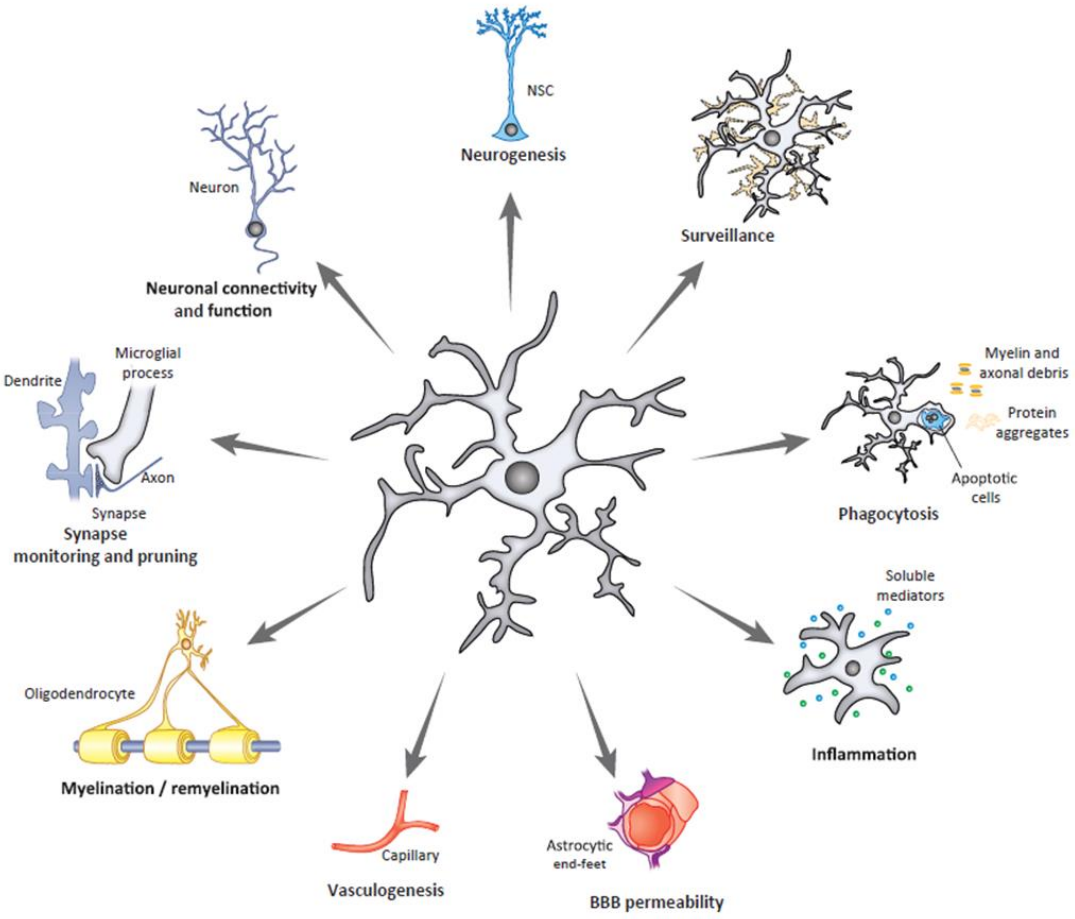


Figure 6 - Microglia have multiple roles in physiological conditions. (from Sierra et al., 2019).

By defined microglial domains, which are well-delimited regions of the microenvironment that do not overlap, microglia constantly oversee the brain parenchyma. They scan the microenvironment by adapting their morphology and motility. In particular, microglia adaptation to the brain parenchyma has been attributed to several factors, including purinoceptors, ion channels, and neurotransmitters (Cronk & Kipnis, 2013; Tremblay et al., 2010). Moreover, they detect a variety of stimuli or damage through the rapid movement of their filopodia (Nimmerjahn et al., 2005). In addition to these, they have the capability to phagocyte microorganisms, dead cells, and cellular debris (Sierra et al., 2019).

Microglia undergo substantial transformations in response to pathological conditions, which include changes of morphology associated with specific functions (Figure 7) (Edler et al., 2021).

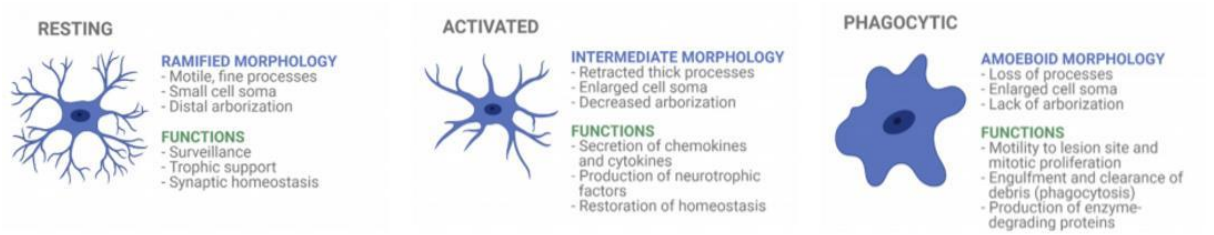


Figure 7 - Morphofunctional changes of microglia in CNS pathophysiological conditions. (Modified from Edler et al., 2021).

As resident immune cells within the brain, they have important innate immune functions (Sierra et al., 2019). In this context, they are capable of both secreting and responding to cytokines and other factors derived from cells. All these processes are fundamental for CNS homeostasis and when microglial functions become disrupted, they may contribute to the onset and progression of various neurological and neurodegenerative disorders (Salter & Stevens, 2017). They are equipped with a variety of receptors, referred to as the microglial sensome, which allows them to detect invading pathogens, chemokines, cytokines, metabolites, and changes in pH or extracellular matrix composition (Hickman et al., 2013). In mice, this sensome comprises purinergic receptors, such as P2y12, chemokine receptors, including Cx3cr1, and Toll-like receptors (Davalos et al., 2005; Hickman et al., 2013). Additionally, microglia exhibit chemotaxis toward injury sites, primarily mediated by purinergic receptor P2y12 in response to ATP or ADP (Davalos et al., 2005; Nimmerjahn et al., 2005). Upon arrival at the injury site, microglia swiftly proliferate and alter their morphological and functional properties. The reactivity and proliferation, known as microgliosis, are often associated with secretion of proinflammatory cytokines and cell death mechanisms. Interestingly, reactive microglia can present different morphological properties. Some studies found that microglia reactivity is associated to a more “amoeboid” morphology, but other demonstrated that they can also express a hyper-ramified morphology, especially under stress, in states of premature aging, or in germ-free animals lacking normal microbiota (Erny et al., 2015; Hellwig et al., 2016; Prinz et al., 2019; Riazi et

al., n.d.). Reactive microglia also release pro-inflammatory cytokines, including IL-1β, TNFα, IL-18, IL-6, and IL-23, affecting neuronal and peripheral immune cells during neuroinflammatory processes. They can also produce reactive oxygen species and nitrogen species that can be damaging to both oligodendrocytes, which primarily form the myelin, and neurons. Furthermore, microglia can contribute to the development of neurotoxic astrocytes by secreting TNFα and C1q (Liddelow et al., 2017). An excessively active phagocytic response, characterized by the engulfment of synapses and neurons via receptors for complement, fractalkine, and purines, may be implicated in neurological and psychiatric disorders.

Moreover, microglia communicate with astrocytes for the maintenance of neuronal functions and brain homeostasis. Releasing CSF1, IL-34, TGFβ and cholesterol, astrocytes provide trophic support to microglia (Borst et al., 2021). Together, they also coordinate the clearance of apoptotic cells and debris. In the process of synaptic pruning, astrocytes identify redundant synapses and release IL-33, which serves to recruit microglia (Borst et al., 2021). Subsequently, microglia are able to engulf these synapses, playing a crucial role in synapse remodelling during postnatal development stages. This process is essential for the evolution and maintenance of a functional network in the CNS (Borst et al., 2021).

Astrocytes are key regulators of brain homeostasis

Astrocytes are star-shaped glial cells found in the CNS that develop from a population of radial glial cells surrounding the ventricular zone during early brain development, similar to neurons and oligodendrocytes (Rowitch & Kriegstein, 2010). In the brain, astrocytes are an integral part of the BBB regulating its permeability (Abbott et al., 2006).

In addition to the regulation of BBB permeability, these cells are essential for regulating blood flow, providing metabolic support to neurons, recycling neurotransmitters, and facilitating synaptic transmission (Figure 8) (Khakh & Deneen, 2019). In response to neuroinflammation or neurodegenerative diseases, astrocytes undergo changes in their morphology, function, and molecular characteristics (Hasel & Liddelow, 2021).

This transformation significantly disrupts brain homeostasis and impacts the survival of other cells.

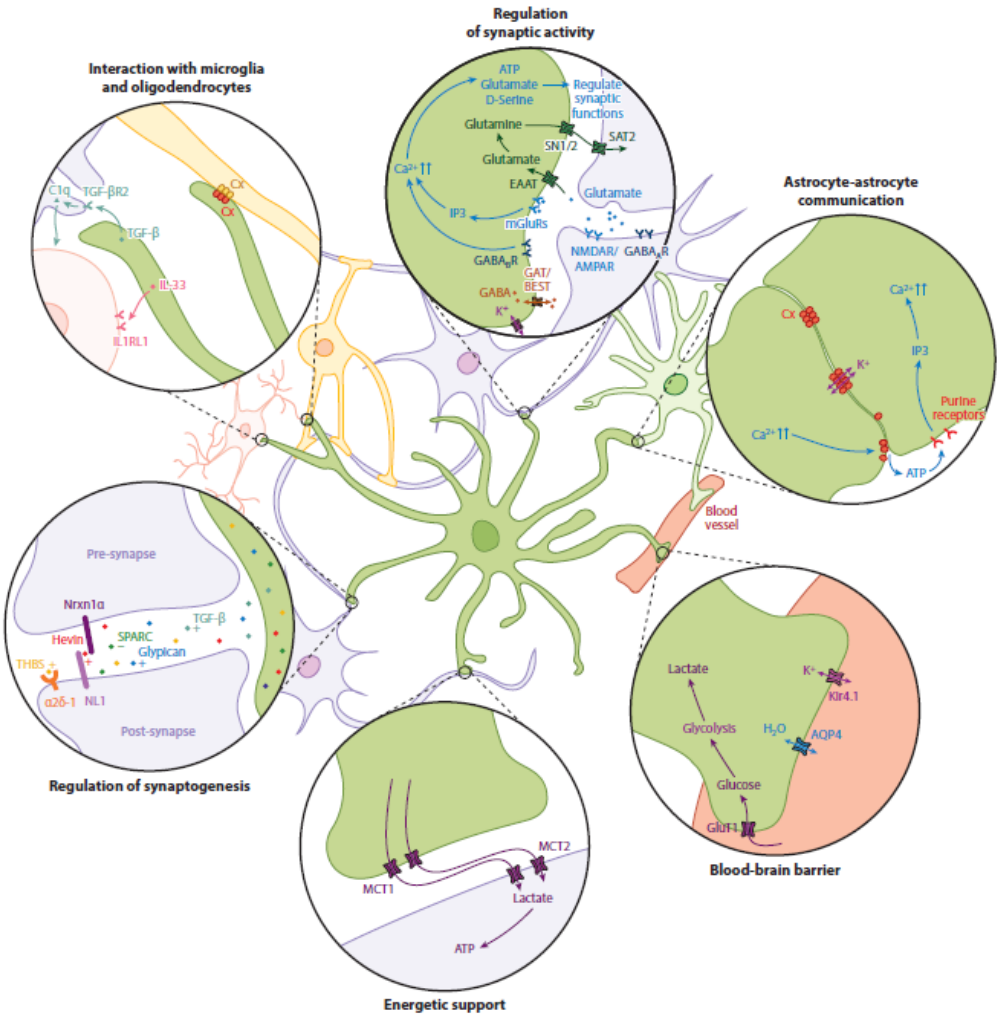


Figure 8 - Astrocytes functions in physiological conditions. (from Khakh & Deneen, 2019).

Ramón y Cajal and his contemporaries carefully documented the morphological variety of astrocytes in the human brain, suggesting the potential existence of different astrocyte subtypes, even if they referred to astrocytes as neuroglia. One approach to understanding the cellular diversity within a specific subpopulation is to combine molecular markers with morphological criteria as key defining characteristics. A common marker used to identify astrocytes is glial fibrillary acidic protein (Gfap). However, Gfap expression varies significantly across different brain regions and even within local areas, influenced by the functional state and affected by various intra- and

extracellular signals (Khakh & Deneen, 2019; Sofroniew, 2009). Additionally, Gfap labelling does not extend to all branches of astrocytes, nor does it label fine processes and cell bodies, potentially leading to an underestimation of astrocyte quantification (Bushong et al., 2001; Khakh & Deneen, 2019).

Astrocytes communicate with each other both through releasing factors, but also by calcium signals. They possess ion channels that generate inward currents but do not produce action potentials like neurons (Goenaga et al., 2023). Instead, their excitability is based on increases in intracellular calcium, which facilitates communication both among astrocytes and between astrocytes and neurons (Goenaga et al., 2023). Calcium fluctuations can occur spontaneously or be induced by neurotransmitters like glutamate or purines. In response to elevated calcium levels, astrocytes can release glutamate and GABA, influencing neuronal activity. Additionally, calcium signals can spread to neighbouring astrocytes via gap junctions, creating "calcium waves" (Halassa et al., 2007; Nedergaard et al., 2003; Perea et al., 2009; Shigetomi et al., 2008; Volterra & Meldolesi, 2005).

As previously mentioned, astrocytes can also interact with other neuronal and glial cells. One of the fundamental roles of astrocytes is forming the BBB and regulating their integrity, along with other cell types, including neurons, pericytes and microglia. The interactions at the BBB are regulated by various molecular factors, which dysregulation leads to immune cell infiltration exacerbating pathological conditions in the brain. Interestingly, astrocytes produce retinoic acid to enhance the expression of Tjp1, which encodes for Zo-1 a tight junction protein (Han et al., 2021). Moreover, astrocytes communicate with microglia to maintain brain homeostasis. One molecular signaling includes IL-33 and its receptor on microglia. In particular, astrocytes produce IL-33 to enhance microglial-mediated engulfment of excitatory synaptic proteins during brain development (Vainchtein et al., 2018). *In vitro* studies have shown that astrocytes release IL-34, TGF$\boldsymbol{\beta}$, and cholesterol for the survival of ramified microglia (Bohlen et al., 2017). Instead, in the presence of inflammation the bidirectional communication among astrocytes and microglia play pivotal roles. In particular, microglia can trigger astrocytes to express neurotoxic phenotypes. Research on rodent models of sepsis has revealed that microglia release pro-inflammatory factors like IL-1$\boldsymbol{\alpha}$, TNF$\boldsymbol{\alpha}$, and

C1q, which activate reactive astrocytes that then promote apoptosis in neurons and oligodendrocytes (Liddelow et al., 2017). In contrast, microglia and other immune cells produce TGF**β**, aiding in the modulation of astrocyte activity to reduce neuroinflammation following stroke or infection (Cekanaviciute et al., 2014). Additionally, astrocytes interact with peripheral immune cells in conditions like experimental autoimmune encephalitis, where gut microbiome metabolites trigger microglia to release inflammatory cytokines that further activate astrocytes and facilitate immune cell infiltration (C. J. Jensen et al., 2013; Rothhammer et al., 2018).

Under physiological conditions, astrocytes contribute also to neurotrophic support by releasing essential molecules, including cholesterol and lactate, as well as through the reuptake of neurotransmitters, such as glutamate (Z. Chen et al., 2023). However, in pathological states the reactive astrocytes show alteration of these roles affecting neuronal vitality. This can occur, for instance, through reduced lactate transfer from astrocytes to neurons or impaired neurotransmitter uptake, leading to excitotoxicity associated with elevated glutamate levels (Z. Chen et al., 2023).

Astrocytes play a critical role in the metabolism of the CNS. Taking part of the BBB, astrocytes regulate the influx of glucose into the brain and serve as the primary storage site for glycogen, particularly in regions with high synaptic density to provide essential support for neuronal activity during episodes of hypoglycemia (A. M. Brown et al., 2004; Suh et al., 2007). Other evidences suggested that astrocytic glycogen is regulated by neurotransmitters and by neuronal activity and also that hypoglycemia induce the breakdown of glycogen to lactate in astrocytes, which is then transferred to neurons to be used as aerobically as fuel (A. M. Brown et al., 2004; Pellerin et al., 2007; Rouach et al., 2008; Suh et al., 2007; Voutsinos-Porche et al., 2003).

Synaptic transmission in physiopathological conditions

Synaptic transmission is a crucial unidirectional process for neuronal communication that occurs at chemical synapses in vertebrates through neurotransmitter release. Chemical synapses are specialized structures, characterized by a narrow gap (20-40 nm) between presynaptic and postsynaptic terminals. Presynaptic terminals contain synaptic vesicles filled with neurotransmitters and specialized proteins for their release. The release of neurotransmitters is induced by action potentials that cause Ca^{2+} influx, leading to vesicle fusion with the presynaptic membrane. Released neurotransmitters bind to postsynaptic receptors, affecting the postsynaptic membrane potential. This can result in depolarization or hyperpolarization, allowing for potential generation of new action potentials, if depolarization meets the threshold at the axonal initial segment, where high density of voltage-gated Na^{+} channels is (Kandel et al., 2019). Neurotransmitter action is transient, quickly removed from the synaptic cleft via reuptake, degradation, or diffusion (Marx, 2014). Moreover, synaptic transmission can be influenced by various factors, including the presence of neuromodulators, the overall synaptic activity, and changes in receptor density. This modulation can affect the strength and efficacy of synapses, a concept known as synaptic plasticity, which is important for learning and memory.

Although current knowledge on the dynamics of synaptic transmission has described numerous neurotransmitters in the CNS, the majority of rapid synaptic responses in the adult brain are mediated by the excitatory glutamate and the inhibitory GABA neurotransmitters (Kandel et al., 2019). After their release, these neurotransmitters bind specific postsynaptic receptors, which can be distinguished in ionotropic (ion channels) and metabotropic (GPCR). Through the rapid ion flux, ionotropic receptors mediate the rapid response of the postsynaptic terminals. In particular, glutamate ionotropic receptors are AMPA, kainate and NMDA and they trigger excitatory postsynaptic currents (EPSC). While all the glutamatergic ionotropic receptors let Na^{+} influx, the NMDA constitutively induces the entrance of Ca^{2+}, too. In the adult synapses, AMPA receptors are largely composed of GluA1-3 subunits that form

GluA1/2, the most abundant, and GluA2/3 heterodimers (Greger et al., 2017; Wenthold et al.,1996). The contribution of each of these AMPA subunits to synaptic transmission is still not fully understood. In particular, GluA1 subunits appear to be mostly localized in the postsynaptic sites tightly within the synapse, while GluA2 subunits seem to be more localized in the extrasynaptic AMPA pool (Chater & Goda, 2022). Moreover, the pattern of their distribution in the synaptic and extrasynaptic spaces is modulated by the activity (Chater & Goda, 2022). Silencing the presynaptic terminal input, the levels of GluA1 are reduced, and they are absent in such inactive synapses, with no effect on GluA2 (Chater & Goda, 2022; Kopec et al., 2007). In contrast, at active synapses GluA1 is restricted, while in the extrasynaptic areas of the dendritic surface, GluA1 increased surface diffusion rates (Greger et al., 2017). Moreover, in some conditions, synapses can adapt and change the expression of AMPA subunits, producing the Ca^{2+} permeable AMPA receptors. Some forms of the synaptic plasticity are determined by Ca^{2+} influx mediated by NMDA receptors. In general, the increase of intracellular Ca^{2+} concentration induces the activation of second messengers in processes that usually are at the basis of memory establishment (Lau et al., 2009). In case of pathological conditions, the intracellular Ca^{2+} content is excessive inducing the cellular death and brain lesions (Berliocchi et al., 2005). Instead, GABA binds the ionotropic GABA-A receptor inducing the entrance of Cl^- in adult synapses determining the inhibitory postsynaptic currents (IPSC) and the hyperpolarization of postsynaptic terminals (Kandel et al., 2019).

An important aspect of synapses is that each afferent, excitatory or inhibitory, determines just little variation of membrane potential. Hence, neurons must integrate multiple inputs to reach the threshold potential and propagate the action potential. The mechanism underlying this integration is called summation and it depends on the passive properties, which are the time and space constants. In general, excitatory synapses are usually localized at dendrites, while the inhibitory synapses are predominantly on the soma, where they can block the excitatory afferents (Kandel et al., 2019). The sum of all these afferents at the level of the initial segment of the axon determines the establishment of an action potential.

In the context of neurotransmission, neurotransmitters such as glutamate and GABA, along with neuromodulators like acetylcholine, affect not only communication between neurons but also the Ca^{2+} waves in astrocytes (Araque et al., 2014; Semyanov et al., 2020). These Ca^{2+} waves can lead to the release of gliotransmitters, which can temporally and spatially modulate the neuronal excitability (Araque et al., 2014; Semyanov et al., 2020). Specifically, the release of glutamate from astrocytes in the hippocampus affects both evoked EPSCs and IPSCs, modifies neuronal excitability, and increases the frequency of miniature and spontaneous excitatory and inhibitory PSCs (Araque et al., 1998, 2014).

Traditionally viewed as supportive cells in the CNS, glial cells, particularly astrocytes and microglia, are increasingly recognized for their active roles in regulating neuronal activity and synaptic transmission. The concept of the "tripartite synapse" emerged in the 1990s, emphasizing the involvement of astrocytes alongside presynaptic and postsynaptic neurons in critical functions such as potassium buffering and glutamate reuptake (Araque et al., 1998). Advances in live imaging have shown that microglia interact directly with synapses, leading some researchers to propose a "quad-partite synapse" model, highlighting the complexity of these interactions (Schafer et al., 2013). Microglia modulate synaptic function by detecting synaptic activity through various receptors and engaging in processes like phagocytosis of inactive synapses, which is vital for synaptic remodeling and behavioral outcomes (Badimon et al., 2020; Crapser et al., 2021; Cserép et al., 2021; Nguyen et al., 2020). The influence of microglial activity is particularly significant during development and in response to sensory experiences (Tremblay et al., 2010). In mature CNS, microglia are involved in influencing synaptic transmission and plasticity via paracrine signaling. Key signaling pathways, such as Cx3cr1, play a role in the context-dependent functions of microglia, while factors released by glia, such as TNF$\boldsymbol{\alpha}$, are important for synaptic scaling, which is a type of synaptic plasticity that modulates the strength of all excitatory synapses in a neuron, either increasing or decreasing their effectiveness, in order to maintain stable firing rates (Paolicelli et al., 2011; Rogers et al., 2011; Stellwagen & Malenka, 2006; Turrigiano, 2008; Turrigiano & Nelson, 2004). Regarding astrocytes in the quad-partite synapses, they actively regulate neuronal excitability by releasing gliotransmitters in response to increased intracellular Ca^{2+} levels, affecting neurotransmitter release

(Halassa et al., 2007; Nedergaard et al., 2003; Perea et al., 2009; Shigetomi et al., 2008). Moreover, astrocytes are crucial for the physiological development of synapses and the maintenance of synaptic functions in adulthood. Interestingly, *in vitro* studies have shown that pro-inflammatory stimuli or co-culturing astrocytes with pro-inflammatory microglia can alter the secretion of synaptogenic molecules by astrocytes (Liddelow et al., 2017).

In the presence of inflammation, synaptic transmission can be altered. For example, when acute organotypic hippocampal slices were exposed to the proinflammatory agent LPS, microglial activation resulted in an increased frequency of AMPA receptor-mediated sEPSCs in CA1 neurons (Pascual et al., 2012). The authors also suggested that the impact of microglia is probably indirect. Specifically, LPS appears to activate microglia, prompting them to release ATP, which then binds to P2Y1R on astrocytes. This interaction ultimately amplifies excitatory transmission via a metabotropic glutamate receptor 5 (mGluR5)-dependent mechanism (Pascual et al., 2012). Microglia not only affect glutamatergic signaling but also regulate GABAergic transmission following injury. When an injury occurs, ATP is released, prompting microglia to release BDNF and resulting in a depolarizing shift in the Cl^- reversal potential, which alters the GABA activation. As a result, there is increased GABA receptor activity, leading to hyperactivity and heightened allodynia, which is a behavioral response to pain (Coull et al., 2005; Tsuda et al., 2003).

Notably, bowel inflammation affects synaptic transmission in the brain, particularly through processes related to neuroinflammation and changes in synaptic plasticity. Research has shown that peripheral inflammation, such as that seen in IBD, can lead to significant alterations in hippocampal synaptic function, potentially contributing to behavioral issues like anxiety and cognitive dysfunction (Haj-Mirzaian et al., 2017). Studies indicate that excitatory synaptic transmission in the hippocampus of animals with colitis is enhanced, marked by increased AMPA receptor activity and modifications in long-term potentiation (LTP) and long-term depression (LTD) (Nyuyki & Pittman, 2015; Riazi et al., 2015). Additionally, acute gut inflammation has been associated with a shift toward immature dendritic spines in the hippocampus, which correlates with decreased neural activity (Matisz et al., 2022). These synaptic changes related to

bowel inflammation are believed to contribute to the cognitive impairments and mood disorders frequently seen in patients with IBD (Nyuyki & Pittman, 2015). Studies on the mechanisms underlying the synaptic changes and sickness behaviors associated with IBD did not provide a clear explanation of the processes and further investigation need.

Animal models of colitis

Since the bidirectional communication among gut and brain involves different peripheral pathways, to date colitis cannot be entirely studied *in vitro* and it is necessary the contribution of *in vivo* studies using animal models. Animal models of colitis can be distinguished in chemically-induced and transgenic mice. The former helps the researcher to focus not on a few altered pathways, but on a more general view of the colitis-induced alterations. Instead, the transgenic mouse model of colitis has the limitation that it permits to study few genetic pathways related to colitis. Among the chemically-induced animal models, studies focused on Dextrane Sodium Sulfate (DSS), TNBS or DNBS-induced colitis (Wirtz et al., 2017). It is also possible to induce an acute colitis, mimicking the acute alterations such as the colitis induced by infections, or chronic colitis, which model the long-lasting effects of colitis in humans.

Even if DSS-induced mouse model of colitis is the most used because it is easily reproducible, it is less reliable, because mice it is difficult to evaluate how much substance mice is self-administered by drinking water, and the establishment of colitis needs from 4 to 8 days of continuing self-administration (Wirtz et al., 2017). Compared to DSS and TNBS, which is toxic, the DNBS-induced acute colitis is considered more reliable, reproducible, and not toxic for experimenters (Morampudi et al., 2014; Wirtz et al., 2017). The mechanism of action of DNBS primarily involves its role as an hapten molecule that induces an immune response in the intestinal epithelium mimicking the bowel inflammation observed in patients with Crohn's disease. DNBS has to be injected with ethanol because it destroys the mucus layer increasing the permeability of gut epithelium and the entrance of DNBS in the gut wall (Figure 9). Here, DNBS triggers a Th1/17-type immune response, characterized by the production of pro-inflammatory cytokines such as TNFα and IL-1β, which contribute to mucosal inflammation and tissue damage (Wirtz et al., 2017). DNBS induces an initial bowel inflammation that peaks at day 3 and ameliorates in 5-7 days with complete recovery after 6 weeks (Morampudi et al., 2014; Qiu et al., 1999; Wirtz et al., 2017).

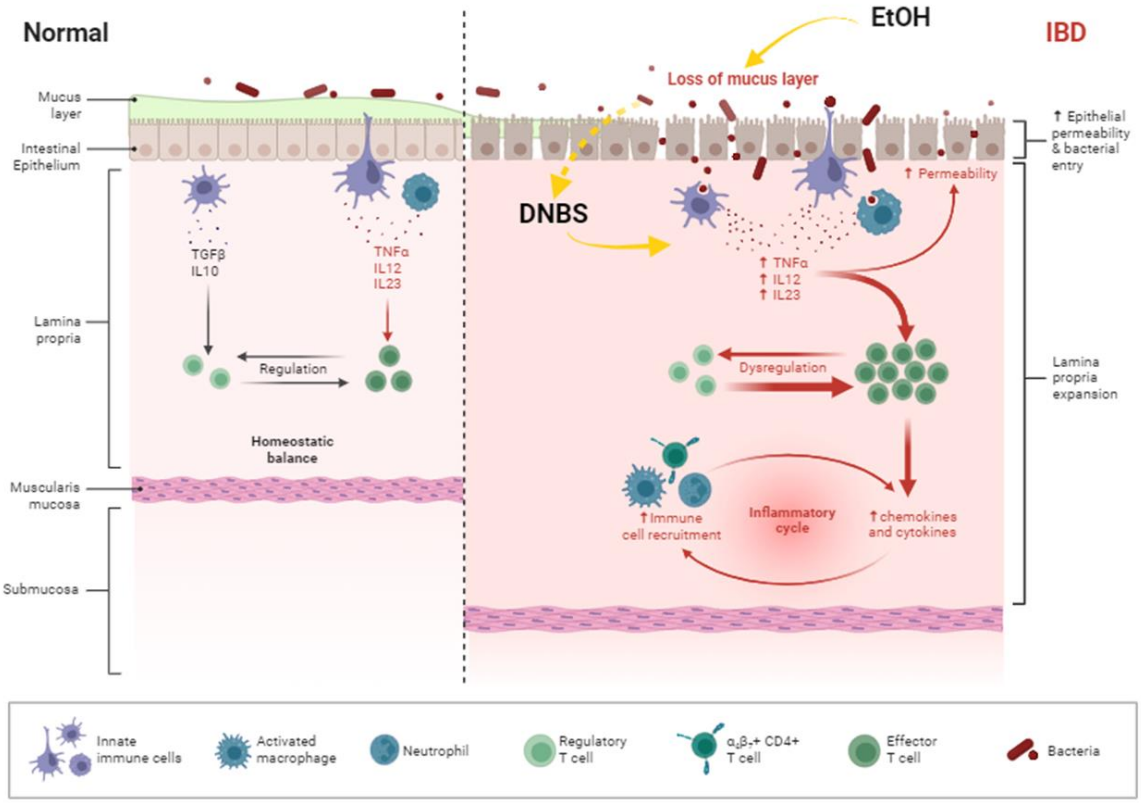


Figure 9 - DNBS is a chemically-induced model of colitis. DNBS is dissolved in EtOH/saline solution to destroy the mucus layer increasing epithelial permeability and the entrance of DNBS in the gut wall, where it triggers inflammation.

Chapter 2: Aims of the study

IBD are a group of autoimmune relapsing-remitting diseases characterized by an abnormal immune response in the intestinal tract. Beyond the gut, IBD can lead to extra-intestinal effects, including depression and anxiety, linked to brain dysfunction (Günther et al., 2021). It is predicted that by 2025, 1 in 4 individuals will manifest one of these autoimmune and inflammatory disorders (Malard et al., 2021). Understanding how intestinal inflammation influences synaptic transmission and glial responses is crucial for developing treatments for the neuropsychiatric and neurodegenerative disorders associated with the chronic extra-intestinal effects of IBD. Specifically, certain brain regions, such as the hippocampus, respond to bowel inflammation by modulating neuronal and glial activity (Riazi et al., 2008, 2015). Additionally, bowel inflammation compromises the integrity of the blood-brain barrier, leading to the infiltration of immune cells and metabolites (Carloni et al., 2021; Gampierakis et al., 2021; Natah et al., 2005).

The aim of this study is to elucidate the cellular and molecular mechanisms underlying the observed alterations in the CNS during colitis. We hypothesize that colitis disrupts the gut metabolome, which subsequently impacts hippocampal metabolites and gene expression. These changes result in dysfunctional phenotypes in glial cells and synapses, thereby contributing to the behavioral abnormalities observed in IBD patients and animal models. To achieve this goal, we employed a mouse model of acute colitis, induced by intracolonic injection of 2,4-Dinitrobenzenesulfonic acid hydrate (DNBS), which causes progressive bowel inflammation peaking around the third day. All experiments were conducted on day 3 post-injection to capture the peak effects of colitis on the hippocampus.

First, to investigate whether bowel inflammation induces neuroinflammation and glial reactivity in the hippocampus, we analysed the hippocampal transcriptome along with the morphology and density of glial cells. Then, to assess whether bowel inflammation alters hippocampal synaptic functionality, we performed electrophysiological recordings of spontaneous and evoked glutamatergic and GABAergic postsynaptic currents in acute brain slices. Furthermore, to explore the potential mechanisms

underlying the observed brain alterations, we analysed gut and hippocampal metabolomes, derived from the same animals, that could potentially mediate hippocampal alterations resulting from peripheral inflammation.

Chapter 3: Material and methods

Animals

All the experiments were conducted on 2- to 3-month-old wild type (WT) C57BL6/J male mice maintained under 12-h light/dark cycle (light on at 7 a.m.) with food and water *ad libitum*. Procedures using laboratory animals were in accordance with the Italian and European guidelines and were approved by the Italian Ministry of Health in accordance with the guidelines on the ethical use of animals from the European Communities Council Directive of September 20, 2010 (2010/63/UE).

Mouse model of acute colitis

To develop an experimental mouse model of acute colitis, we treated WT mice with 2,4-Dinitrobenzenesulfonic acid hydrate (DNBS) (Morampudi et al., 2014; Wirtz et al., 2017). DNBS solution was freshly prepared as follows: 6 mg of DNBS were dissolved in 100 µl of 50% ethanol/saline. As negative controls, some animals were randomly chosen and treated with 100 µl of 100% saline. Moreover, since DNBS is dissolved in 50% ethanol, which increases the permeability of the gut epithelium and allows luminal bacteria to enter the gut wall - potentially inducing gut inflammation - an experimental group of mice treated with 50% ethanol/saline was added. This group helps differentiate the brain effects of DNBS-induced gut inflammation from the consequences of ethanol exposure. Before treatment animals were fasted for at least 4 hours, then they were weighed and randomly assigned to the experimental groups: DNBS-treated mice, 50% EtOH/Sal-treated mice or saline-treated mice. Only mice weighing more than 22 g were treated. Each animal received 100 µl of solution directly into the distal colon at 3-4 cm from the anus. After the treatment, animals were housed separately and we evaluated the disease activity daily. All experiments were conducted on day 3 post-treatment (Figure 10). The acute colitis mouse model was assessed both macroscopically and microscopically, as described below.

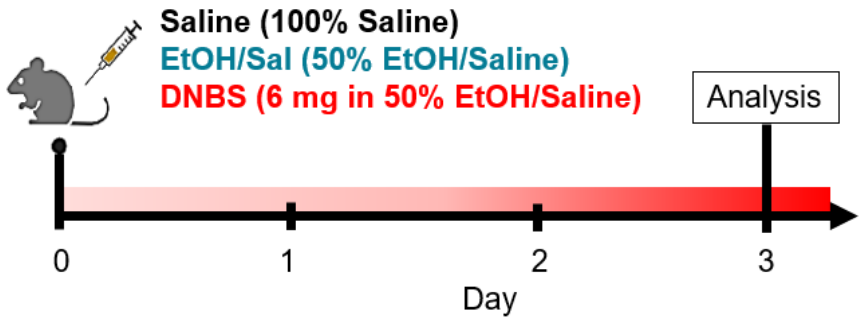


Figure 10 - Experimental timeline of DNBS-induced acute colitis mouse model. After fasting, mice were injected with 100 µl of DNBS or EtOH/Saline or Saline solutions and macroscopic validation of bowel inflammation was performed the following days. All the experiments were conducted at the peak of inflammation (day 3). The colour gradient in the time line indicates the time course of bowel inflammation, with the peak in red.

Macroscopic validation of the colitis mouse model

The acute colitis mouse model was macroscopically validated by evaluating the Disease Activity Index (DAI) each day following the treatment, and by measuring the colon length at the experimental day (day 3).

Disease Activity Index (DAI)

To evaluate the severity of the illness, animals were tested daily for the DAI, according to Wirtz et al., 2017. The DAI is calculated from the sum of scores assigned to three parameters: i) percentage of weight loss, ii) stool consistency, and iii) rectal bleeding. Scores range from 0 to 4, reflecting the progression of the disease, as detailed in the following table (Table 1).

Table 1

SCORE	WEIGHT LOSS (%)	STOOL CONSISTENCY	OCCULT/GROSS BLEEDING
0	None	Normal (solid)	Normal
1	1-5 %		
2	5-10 %	Loose (Soft/sticky)	Hemoccult (blood in stools)
3	10-15%		Blood in colon, start bleeding from the anus
4	> 15 %	Diarrhoea	Gross bleeding

Colon length

To evaluate whether colitis decreases colon length, after euthanasia the colon was gently extracted from the body on the day of sacrifice (day 3) by cutting at the level of rectum and tenue. Then, it was immersed in ice-cold 1X PBS to preserve tissue structure. Then, the mesentery was cut to allow for the distension of the colon and facilitate the measurement of its length.

Microscopic validation of the colitis mouse model

The severity of the gut damage was assessed microscopically by examining the distal portion of the colon. After measuring the colon length, it was washed in 1X PBS to preserve its integrity for histopathological analysis or permeability assay.

Histopathological analysis: Hematoxylin and Eosin staining

To assess the degree of colonic inflammation, the distal portion of colon was cut and post-fixed in 4% paraformaldehyde (PFA) (Thermo Fisher Scientific, Waltham, MA, USA) for 3 days, then cryo-sectioned into 15 µm transversal slices. The slices were stained with hematoxylin and eosin (H&E) (Sigma Aldrich, St. Louis, MO, USA) to evaluate the histopathological damage score according to Li et al., 2017. Criteria included: (i) distortion and loss of crypt architecture; (ii) infiltration of inflammatory cells; (iii) muscle thickening; (iv) depletion of goblet cells; and (v) absence of crypts (Table 2). Images were acquired using a Nikon Eclipse 80i microscope (Nikon Corporation, Tokyo, Japan) at 10X and 20X magnification, with a high-resolution digital camera (Nikon Digital Sight DS-U1). A total of 6 mice per group were analysed and the scores were summed to obtain the Histological Damage Score.

Table 2

SCORE	DISTORTION AND LOSS OF CRYPT ARCHITECTURE	INFILTRATION OF INFLAMMATORY CELLS	MUSCLE THICKENING	GLOBET CELLS DEPLETION	CRYPT ABSENCE
0	None	Normal	Normal	Absence	Absence
1	Mild	Mild	Mild	Presence	Presence
2	Moderate	Moderate	Moderate	-	-
3	Severe	Dense	Marked	-	-

Permeability of gut epithelium: immunofluorescence analysis of colonic sections

As explained before, to induce acute colitis in mice, DNBS was injected in combination with 50% of ethanol to destroy the mucosal layers and increase the permeability of the gut epithelium. To assess gut permeability, distal colonic sections were analysed for immunofluorescence of Zonula Occludens-1 (Zo-1), a tight junction protein of epithelium. The colon was post-fixed in 4% PFA and transversally cut into 15 µm sections. These sections were washed with 1X PBS and blocked with a solution composed of 1X PBS, 4% Normal Donkey Serum, 0.4% (Merck Millipore, St. Louis, MO, USA) TritonX-100 (Sigma Aldrich, St. Louis, MO, USA), and 1% Bovine Serum Albumin (BSA) (Sigma Aldrich, St. Louis, MO, USA) for 45 min. Subsequently, sections

were incubated at +4 °C overnight with the primary antibody mouse anti-Zo-1 (1:100; Invitrogen, Thermo Fisher, Waltham, MA, USA). Slices were then washed with 1X PBS and incubated in the dark at +4 °C with Texas Red-conjugated anti-mouse (1:400; Abcam, Cambridge, USA). Images of colonic sections were acquired using a microscope Nikon Eclipse 80i at 20X and 40X magnification with a high-resolution digital camera (Nikon Digital Sight DS-U1). A total of 6 mice per group were analysed. The relative fluorescence units (RFU) were quantified using ImageJ.

Immunofluorescence analysis on brain slices

To investigate whether colitis induces gliosis and changes in microglia morphology, brain slices from the three experimental groups were used for immunofluorescence analysis of microglia (Iba1$^+$ cells) and astrocytes (Gfap$^+$ cells) in the CA1 *stratum radiatum*.

Three days after treatment, mice were deeply anesthetized with 10 μl/g intraperitoneal injection of a freshly made solution composed of 4% Zoletil, 7% Rompun and 89% Saline. Under deep anesthesia, mice were transcardially perfused with 1X PBS and 4% PFA in 0.1 M pH 7.4 phosphate buffer (PB). Brains were removed, postfixed overnight in 4% PFA and incubated in 1X PBS with 30% sucrose for 24 h at +4 °C. Before being frozen, brains were placed for at least 2 h in chilled isopentane at -80 °C. The frozen brains were included in OCT and cut into 50 µm coronal slices using a cryostat (Leica).

Immunofluorescence was performed on free-floating slices. Sections containing the hippocampus were rinsed three times in 1X PBS for 10 minutes at room temperature (RT). To eliminate chemical modifications caused by post fixation in 4% PFA, coronal slices were kept 40 minutes at 90 °C with a warm Antigen Retrieval solution: 10 mM pH 6 NaCitrate Buffer (Sigma), 0.05 % Tween20 (Sigma) in pure water. After cooling down sections were rinsed three times with PBS 1 M for 5 minutes at RT on speed rocking and incubated for 1 h at RT on speed rocking with 3% Blocking solution, containing 3% Normal Goat Serum (Vector Labs), 0.3% Triton X-100 (Sigma) in PBS 1 M. Slices were then incubated overnight at +4 °C on speed rocking with primary

antibodies in 2% blocking solution: rat anti-Gfap (1:300, Abcam) and rabbit anti-Iba1 (1:500; Fujifilm Wako). The following day, free-floating slices were washed 3 times for 10 min in PBS at RT on speed rocking and incubated for 2 h at RT on speed rocking with secondary antibodies in 2% blocking solution: anti-rat Alexafluor 488 (1:500; Invitrogen) and anti-rabbit Alexafluor 647 (1:500; Abcam). In addition, slices were incubated for 20 min at RT with Hoechst (1:5000 in PBS 1 M; Invitrogen). At the end, slices were washed twice for 10 min in PBS 1 M on speed rocking at RT and then coverslipped with fluorescence mounting medium S3023 (Dako) and acquired the images by confocal microscopy.

Acquisition with confocal microscopy

To analyse Iba1$^+$ and Gfap$^+$ cells, images were acquired using a Nikon Eclipse Ti equipped with a X-Light V3 spinning disk (CrestOptics). The images were acquired with Metamorph software version 7.10.2. (Molecular Devices) using 20x and 60x objectives on hippocampal CA1 *stratum radiatum*. For density analysis, slices were sectioned in Z with a step size of 1 µm and acquired with 20x magnification, whereas for morphological analysis slices were sectioned in Z with a step size of 0.2 µm to obtain a total Z-stack of about 25–35 µm for astrocytes and microglia morphology analysis and acquired with 60x magnification within the CA1 *stratum radiatum*. Images were acquired with the same laser intensity and exposure time to maintain the conditions among the experiments. Nuclei were acquired with Laser 407 intensity 15 and exposure time 350 ms, Gfap$^+$ cells were acquired using laser 488 intensity 11 and exposure time 250 ms, Iba1$^+$ cells were acquired using laser 647 intensity 10 and exposure time 300 ms.

Analysis of Iba1$^+$ and Gfap$^+$ cell density

Cell density was analysed using ImageJ software on images acquired at 20x magnification. Channels of Iba1 or Gfap were merged with the nuclei channel and brightness and contrast were automatically adjusted. A maximal intensity Z-projection

of the stacks was made and inside the *stratum radiatum* a region of interest (ROI) was defined and the area (in μm^2) was calculated.

To quantify microglial density, the number of $Iba1^+$ $Hoechst^+$ cells was manually counted and reported as a number of somas per ROI volume (area x z-step x number of stacks). The same procedure was applied for the quantification of $Gfap^+$ $Hoechst^+$ cells within the volume of CA1 *stratum radiatum*.

Analysis of Iba1+ cells morphology

Analysis of microglia morphology in the CA1 *stratum radiatum* was performed using ImageJ software on images acquired at 60x magnification. Maximal intensity projections of Iba1 confocal images were obtained to analyse the morphological indicators of cell complexity: soma area, microglial domain and arborization domain. The microglial domain is the area described by unifying all the distant processes of the cell and it is related to the microglial scanning domain. The arborization domain is the area occupied by all the processes of the cell and it is an indicator of the ramification. Only cells whose body and processes were entirely contained in the image were included in the analysis. For each cell, the soma area, the microglial domain and the arborization domain were assessed.

NanoString nCounter gene expression Assay

Gene expression analysis was performed by NanoString, using the nCounter glial profiling panel that contains 740 mouse neuroinflammatory genes and 13 internal reference controls. Briefly, animals were deeply anesthetized and hippocampi were isolated using sterile 1X PBS and tools. Hippocampi were rapidly frozen at -80 °C. Using E.Z.N.A. total RNA kit I (omega, biotek) extraction protocol, a mean of 250 ng/µl RNA per experimental group was extracted from the hippocampi and hybridized to the capture and reporter probe sets at 65 °C for 20 h according to the manufacturer's instructions. Data were collected using the nCounter Digital Analyzer (NanoString). RNA counts were normalized using the geometric mean of the house-keeping genes

included in the panel, after validation against positive and negative controls, using the nSolver 4.0 software (NanoString). Fold changes were calculated comparing vehicles (100% saline) versus 50% ethanol/saline versus DNBS-derived samples.

Differential Gene Expression (DGE) was analysed to identify the number of genes differentially expressed among the groups. Using BioPlanet_2019 gene set library and EnrichR software, gene expression was analysed to determine the pathways associated with the genes. Statistical analysis was performed with One-way ANOVA followed by Holm-Sidak post hoc analysis.

Electrophysiology

Preparation of acute hippocampal slices

Under deep anesthesia with halothane, mice were decapitated and brains were rapidly removed from the skull and rinsed in ice-cold ACSF-NMDG solution (protocol Ting et al., 2018, continuously oxygenated with 95% O2 and 5% CO2 and containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 Hepes, 25 glucose, 5 Na ascorbate, 2 thiourea, 3 Na pyruvate, 10 MgCl2, 0.5 CaCl2, pH adjusted with HCl 12 N to 7.4. Wet hemispheres with ACSF-NMDG solution were separated and cerebellum, olfactory bulbs and a part of the dorsal cortex were removed. 300 um thick dorsal hippocampal transversal slices were cut in +4 °C ACSF-NMDG solution continuously oxygenated using a vibratome (Campden Instruments). Acute slices were transferred into an incubation chamber containing oxygenated ACSF-NMDG solution pre-heated at +32°C for 11 minutes and then into another incubation chamber containing oxygenated ACSF and allowed to recover for 1 h at RT (24 °C). The ACSF solution contains (in mM) 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 1.2 NaHPO4, 10 glucose, 26 NaHCO3. All recordings were performed on slices submerged in ACSF and perfused with the same solution in the recording chamber.

Whole-cell patch-clamp recordings

Recordings were performed at RT under constant perfusion of oxygenated external solution ACSF at 2 ml/min rate within a time window of 1–6 h after slice preparation. All the electrophysiological recordings were made with whole-cell patch-clamp configuration.

Excitatory and Inhibitory spontaneous and evoked excitatory postsynaptic currents (PSCs) from hippocampal CA1 pyramidal neurons held at -70 mV and 0 mV, respectively, using a patch clamp amplifier (Axopatch 200B, MolecularDevices, LLC). Data were filtered at 2 kHz, digitized (10 kHz), acquired using pClamp 10.0 software (Molecular Devices), and analysed offline using Clampfit 10 (Molecular Devices, LLC). Patch pipettes (3–5 MΩ) were filled with an intracellular solution. For the recordings of spontaneous excitatory postsynaptic currents (sEPSCs) intracellular solution contained (in mM): 135 Cs-methanesulfonate, 10 Hepes, 2 MgATP, 0.3 NaGTP, 0.4 CaCl2, 2 MgCl2, 2 QX-314, 5 BAPTA (pH adjusted to 7.3 with CsOH). For the recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) intracellular solution contained (in mM): 135 Cs-methanesulfonate, 10 Hepes, 2 MgATP, 0.3 NaGTP, 0.4 CaCl2, 2 MgCl2, 2 QX-314, 5 BAPTA, 8 CsCl (pH adjusted to 7.3 with CsOH). For experiments comparing the ratio of the amplitude or frequency between sEPCSs and sIPSCs, sPSCs were recorded from the same cell.

In experiments on evoked postsynaptic currents (ePSCs), electrical stimulation was applied by theta glass tubes (tip 15–20 µm) filled with an external solution. To evoke the excitatory local circuitry among CA3-CA1, Schaffer Collateral were stimulated with a stimulating electrode, connected to a stimulus isolation unit (Isostim A320, WPI), placed in the hippocampal CA1 *stratum radiatum*. For the recordings of eEPSCs, Bicuculline Methochloride 10 µM was added to the external solution. Patch pipettes were filled with an intracellular solution containing (in mM): 108 Cs-methanesulfonate, 5 TEA-Cl, 2.8 NaCl, 20 Hepes, 0.4 EGTA, 4 MgATP, 0.3 NaGTP, 10 Na2-phosphocreatine, 1 QX-314 (pH adjusted to 7.3 with CsOH and osmolarity adjusted to 290 mOsm with sucrose). Synaptic responses were elicited by stimulating for 100 µs at 0.1 Hz; the stimulus intensity was adjusted accordingly to the experiment. To

determine the paired-pulse ratio (PPR), AMPA-mediated EPSCs were evoked by paired-pulse stimulations with different inter-stimulus intervals (ISI) (in ms): 25, 50, 75, 100, 200, 300. For input/output curves, Schaffer collaterals were stimulated at increasing intensities (0.1–10 mA). Each pulse of a given intensity was repeated six times, to obtain an average response. The input/output curve of the AMPA component was determined at -70 mV. To determine the AMPA/NMDA ratio, stimulus strength was adjusted to obtain at -70 mV stable AMPA-mediated EPSC with an amplitude corresponding to the 50% of the maximum response. NMDA-mediated currents were recorded from the same neuron and using the same stimulus strength. For NMDA-mediated EPSC, we measured the EPSC Erev in each cell and corrected the holding potential for recording the NMDA-mediated currents consequently +40 mV + ERev, to avoid potential voltage-clamp errors. NMDA peak amplitude was measured with a delay of 25 ms from the AMPA peak. The AMPA/NMDA ratio was then calculated using the equation: ratio = AMPA EPSC amplitude/NMDA EPSC amplitude. I/V curves for EPSC were constructed by measuring current amplitude at two different time points; at the current peak (for AMPA component) and at 25 ms from the peak (for NMDA component).

To evoke the inhibitory local circuitry, the stimulating electrode was placed in the hippocampal CA1 *stratum oriens*, where interneurons are located, and the eIPSCs were recorded from CA1 pyramidal neurons. For the recordings of eIPSCs, ionotropic glutamatergic receptors were blocked by adding NBQX 10 µM and D-AP5 µM, antagonists of AMPA and NMDA receptors respectively, in the external solution. Patch pipettes were filled with an intracellular solution containing (in mM): 108 Cs-methanesulfonate, 5 TEA-Cl, 2.8 NaCl, 20 Hepes, 0.4 EGTA, 4 MgATP, 0.3 NaGTP, 10 Na2-phosphocreatine, 1 QX-314 (pH adjusted to 7.3 with CsOH and osmolarity adjusted to 290 mOsm with sucrose). To record the GABA-mediated IPSCs, pyramidal neurons were held at 0 mV holding potential. Synaptic responses were evoked by stimulating for 100 µs at 0.1 Hz; the stimulus intensity was adjusted accordingly to the experiment. To determine the PPR, GABA-mediated IPSCs were evoked by paired-pulse stimulations with different ISI (in ms): 25, 50, 75, 100, 200, 300, 700, 1000. For input/output curves, fibers in the *stratum oriens* were stimulated at increasing

intensities (0.1–10 mA). Each pulse of a given intensity was repeated six times, to obtain an average response.

Statistics

All data are presented as mean±SEM. GraphPad Prism 8.0.1. software was used for statistical analysis of hippocampal immunofluorescence and electrophysiological data. Statistical significance was determined by One-way and Two-way ANOVA (repeated measures when indicated); multiple comparison procedures were performed with Holm–Sidak post hoc. p values <0.05 were considered significant.

NMR-based metabolomics

Metabolomics analysis was performed on fecal water and hippocampus derived from the same animals. After the sacrifice, colon was extracted from animals and feces were rapidly collected using sterilized 1X PBS and tools to avoid environmental bacterial contamination. Same procedure was applied to isolate the hippocampus from the same animals. Samples were frozen at -80 °C.

Sample Preparation

Fecal samples were suspended in D_2O-PBS-NaN_3 solution (1.2 mL), vortexed until homogenization and then centrifuged at 11,000 x g for 15 min at 4°C. The supernatant was filtered through a cellulose strainer (100 µm pore size) and centrifuged again at same conditions. Finally, an aliquot of the supernatant (600 µL) was added with 60 µL of D_2O-PBS-NaN_3 solution containing 20 mM of the internal standard TSP (final concentration in the sample 1.82 mM).

All hippocampus samples were instead extracted following a modified Bligh-Dyer protocol from Rosito et al., 2024. In particular, each organ was initially ground in a mortar with liquid nitrogen and then added with a mixture of cold methanol, chloroform and water at a final ratio of 2:2:1, respectively. After an overnight storing at 4°C,

samples were centrifuged for 25 min at 4 °C at 10.000 x g; the upper hydrophilic and the lower organic phases were carefully separated and dried under nitrogen flow. Finally, the hydrophilic phase was resuspended in 0.7 mL of D_2O containing 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP, 2 mM) as an internal chemical shift and concentration standard, while the lower organic phase was resuspended in 0.7 mL of CDCl3 containing hexamethyldisiloxane (HMDSO, 2 mM) as an internal chemical shift and quantitative standard.

After pretreatment each sample was transferred into NMR precision tubes and analysed.

NMR experiments

^{1}H-NMR spectra were recorded at 25°C on a JEOL ECZR JNM spectrometer equipped with a magnet operating at 600.13MHz for the ^{1}H frequency. Spectra were acquired employing such parameters: 128 scansions, 64k data points and a spectral width of 15 ppm. Presaturation has been used for water signal suppression and the relaxation delay has been set to 7.723 s (15s of total acquisition time) guaranteeing the complete resonance relaxation between following scansions. Spectra have been processed by applying an exponential window function with a line broadening factor LB=0.3Hz. After applying the Fourier transformation, spectra have been manually phased and baseline corrected and referred to the chemical shift of the TSP methyl resonance at δ 0.00 ppm.

To allow metabolites identification, bidimensional experiments ^{1}H-^{1}H Total Correlation Spectroscopy (TOCSY) and ^{1}H-^{13}C Heteronuclear Single Quantum Correlation (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) were performed on selected samples. TOCSY experiments were conducted with a spectral width of 15 ppm in both dimensions, using a data matrix of 8192×256 points, a mixing time of 80ms, and a relaxation delay of 2s. HSQC experiments were performed with spectral widths of 15 ppm and 200 ppm for the proton and carbon dimensions, respectively, a data matrix of 8192×256 points and a recycle delay of 2s. HMBC experiments analogously have been acquired with a spectral width of 15 ppm and 200 ppm for the

proton and carbon dimensions, respectively, with a data matrix of 8 K×256 points, and a recycle delay of 3s. In addition, in order to confirm the resonances assignment different available databases were consulted (Wishart et al., 2007, 2022).

Metabolites quantitation has been carried out by comparing the integrals of specific resonances with the one of the internal standards (TSP or HDMSO for aqueous or organic fraction respectively) and normalized by the number of protons according to the general formula:

$$C_m = \frac{A_m}{A_{IS}} \times \frac{H_{IS}}{H_m} \times C_{IS}$$

where is the concentration of the metabolite, is the area of the metabolite signal, is the number of protons generating the metabolite signal, is Internal Standard (IS) concentration, is the area of the IS signal and is the number of protons generating the IS signal. For quantification only those signals that did not overlap with other resonances were chosen for integration and the final data were expressed as µmol/g of the original sample.

Statistical analysis of NMR-based analysis

With the aim to point out any spontaneous grouping of samples a first Principal Component Analysis (PCA) was carried out on the entire dataset after autoscaling. PCA is a multivariate statistical technique that enables the summarization of multiple variables in a dataset into a few factors, which capture most of the sample's information. The main idea behind PCA is to reduce the number of dimensions in the data while preserving as much variance as possible. We illustrate the PCA with the scores plot to see how the observations are distributed along the principal components. Clusters may indicate similar observations, while outliers may appear distant from other data points. Besides, to highlight the role of each metabolite in discriminating each of the three groups, different PLS-DA models have been built, using as validation procedure the Repeated Double Cross Validation method (Filzmoser et al., 2009).

Performances of models were then evaluated using validation parameters such as Accuracy, Precision, Sensitivity and Specificity. Significant variables for classification have been selected taking into account both the Regression Coefficients and the Variable Importance in Projection (VIP) criterion, considering only variables whose Regression Coefficients sign remained consistent during cross validation procedures and whose VIP value is greater than 1 (Chong & Jun, 2005).

Univariate analysis was also conducted on the data obtained. After testing each variable for normality and homoscedasticity of distributions between classes using Shapiro-Wilk and Brown-Forsythe tests (M. B. Brown & Forsythe, 1974); t-test or Wilcoxon test were applied accordingly (Stevens et al., 2007). All tests were two-sided, with statistical significance set at $p < 0.05$.

The whole dataset obtained from NMR analysis has been constructed using a low-level data fusion strategy as described in literature (Biancolillo et al., 2019) suitable for metabolomics analysis.

All Analyses were performed using in-house routines running under the MATLAB environment (The MathWorks, Natick, MA, USA).

Chapter 4: Results

DNBS treatment induces bowel inflammation

To study the effects of bowel inflammation on the hippocampus, we employed a mouse model of acute colitis chemically-induced by DNBS. DNBS is an hapten molecule that triggers an inflammatory response within the bowel wall, mimicking the pathophysiology of IBD. Previous studies have shown that DNBS treatment induces a progressive bowel inflammation that peaks around the third day following the intracolonic injection (Wirtz et al., 2017). Therefore, we characterized the effects of DNBS treatment on the third day, using a control group injected with 100 % Saline. Additionally, DNBS is dissolved in a solution containing 50% of ethanol, which disrupts the mucus layer and increases gut permeability. To differentiate the effects specifically due to DNBS-induced gut inflammation from the collateral effects of ethanol injection, we included an additional experimental group that received a 50% ethanol/saline injection.

Model validation

We validated the chemically-induced colitis model by analysing the effects of DNBS intracolonic injection both macroscopically and microscopically three days after the treatment.

Patients with IBD often experience unintended weight loss, rectal bleeding, diarrhea, and lower abdominal pain. Thus, we evaluated the presence of these symptoms by assessing body weight, stool consistency and rectal bleeding in the three experimental groups. The scores assigned to these parameters were summed to calculate the Disease Activity Index (DAI). We observed that both DNBS and EtOH/Saline treatments significantly increased the DAI starting from the first day following injection (Figure 11 A). Furthermore, since IBD is associated with a reduction in colon length (Ahn et al., 2020), we evaluated the colon length in the experimental groups. As shown in figure 11 B-C, only DNBS-treated mice exhibited a significantly reduced colon length

compared to the control group. Instead, EtOH/Saline injection does not change the colon length (Figure 11 B-C). To confirm the development of colitis in our experimental model, we performed histopathological analysis on the distal colon of the experimental groups. Microscopic features, in addition to the macroscopic hallmarks, support the diagnosis of colitis in patients (Villanacci et al., 2021). Our microscopic analysis revealed that DNBS- and EtOH/Sal-treatments significantly increase the histological damage score compared to the saline group, suggesting that both treatments severely damage the gut wall (Figure 11 D, E).

Another common feature of IBD is the presence of a leaky gut (Michielan et al 2015). Previous studies have shown that animal models of colitis exhibit increased gut permeability (Carloni et al., 2021). To verify the presence of a leaky gut in our experimental model, we analysed the expression of the tight junction protein Zo-1, expressed on the epithelial barrier in the distal colon by evaluating the Relative Fluorescent Unit (RFU). Our analysis revealed a significant reduction of Zo-1 expression in DNBS-treated mice compared to the saline group (Figure 11 F-G), while EtOH/Sal-treated mice showed a tendency in reduction that was not statistically significant (Figure 11 F-G).

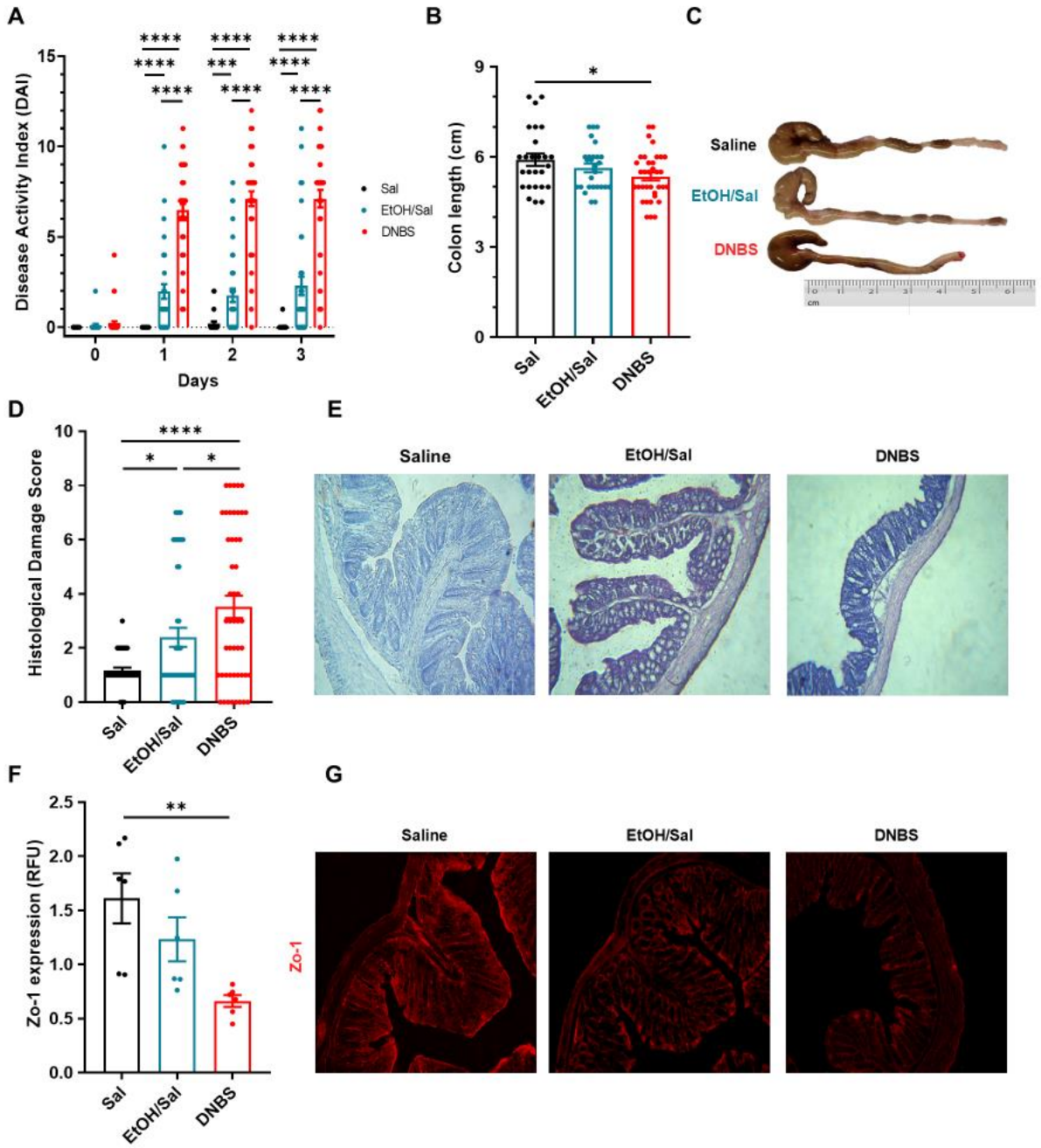


Figure 11 - While DNBS treatment induces colitis, EtOH/Saline results in mild bowel inflammation. *A-C) Macroscopic validation of the colitis mouse model reveals that DNBS treatment induces a progressively severe state of illness in mice.* A) The DAI significantly increases over time in DNBS (red, n=45 mice) and EtOH/Sal-treated mice (green, n=38) compared to Saline (black, n=33) group. Two-way ANOVA repeated measures: days ****, treatment ****, daysXtreatment ****. B) Colon length (in cm) is reduced in DNBS-treated mice (red, n=45 mice) compared to EtOH/Sal (green, n=38) and Saline (black, n=33). One-way ANOVA n.s. (p=0.0514). C) Representative images of colons collected from Saline, EtOH/Sal and DNBS-treated mice. *D-E) Microscopic validation of the colitis mouse model demonstrates that DNBS treatment severely damages gut wall.* D) Scatter plot of

histological damage score of distal colon shows significantly increased damage score in DNBS and EtOH/Sal -treated mice compared to Saline group. One-way ANOVA ****. N=Saline 40/5; EtOH/Saline 48/6; DNBS 48/6 villi/mice. E) Representative transversal sections of distal colon stained with H&E. Images were acquired at 20X magnification from Saline, EtOH/Sal and DNBS-treated mice. *F-G) Microscopic validation of the colitis mouse model demonstrates that DNBS treatment increases intestinal epithelial permeability*. F) Zo-1 expression (in RFU) is significantly decreased in the distal colon of DNBS-treated mice compared to Saline group. One-way ANOVA **. N=6 mice/group. One transversal section of distal colon per animal was analysed. G) Representative transversal sections of distal colon stained for Zo-1 (red). Images were acquired at 20X magnification from Saline, EtOH/Sal and DNBS-treated mice. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

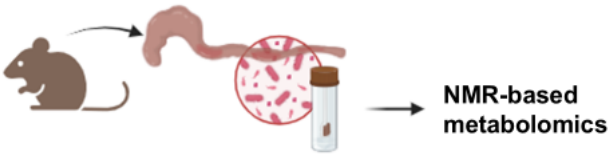
All together, these data demonstrate that DNBS treatment induces severe bowel inflammation, presenting symptoms and features similar to those observed in IBD patients. On the other hand, the injection of 50% EtOH/Saline leads to mild gut inflammation, characterized by less severe macroscopic and microscopic alterations.

Bowel inflammation alters gut metabolites

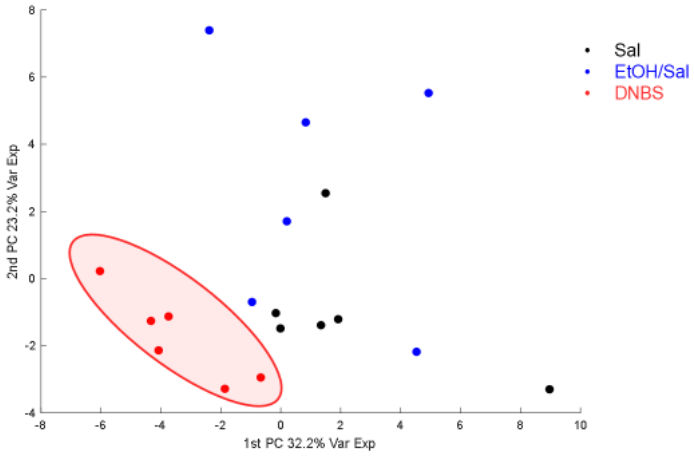
The bidirectional communication between the gut and the brain involves various pathways, with the gut microbiome playing a significant role. In fact, the progression of IBD is closely associated with alterations in the gut microbiome and its metabolites in both IBD patients and animal models (Franzosa et al., 2018; Ning et al., 2023; Sugihara & Kamada, 2024). Remarkably, metabolites derived from gut microbiota play a crucial role in the gut-brain axis, influencing brain function and the overall body health (Silva et al., 2020). Microbiota-produced metabolites, such as SCFAs, indoles and bile acids, can affect brain functions by modulating microglia, astrocytes and neurons, leading to mood and behavioral impairments (Deng et al., 2024; Kim, 2024). We evaluated changes of gut metabolites to define a possible mechanism underlying the brain effects observed in models and patients with colitis. To verify whether our models present dysbiosis, we performed Nuclear Magnetic Resonance (NMR)-based metabolomic analysis of fecal water, collected from the three experimental groups (Figure 12 A). We first analysed our dataset using Principal Component Analysis (PCA). The PCA of fecal metabolites revealed a distinct clustering of DNBS-treated samples (in red) compared to the other experimental groups (Figure 12 B). In total, we detected 42 gut metabolites.

Pairwise comparisons of the three groups using Partial Least-Squares Discriminant Analysis (PLS-DA) showed that DNBS treatment significantly reduced 17 metabolites compared to Saline group, and 14 metabolites compared to EtOH/Sal group (Figure 12 C). These metabolites included SCFAs, such as butyric and acetic acid, bile salts, and monosaccharides, all of which are essential for maintaining gut homeostasis and supporting brain health. Notably, previous studies have demonstrated that SCFAs and bile salts reduce neuroinflammation by modulating the properties of astrocytes and microglia (Erny et al., 2015; Spichak et al., 2021). In contrast, EtOH/Sal treatment resulted in the reduction of 6 metabolites, including bile salts, and an increase in 10 metabolites, such as monosaccharides, compared to Saline group (Figure 12 C). To gain further insight into how the experimental model affects metabolic pathways, we performed a Metabolite Set Enrichment Analysis (MSEA) of fecal metabolites identified by NMR in DNBS-treated mice. This analysis revealed that the metabolites associated with DNBS treatment were linked to SCFAs and free fatty acid metabolism (Figure 12 D).

A



B



C

	DNBS Vs Sal	DNBS Vs EtOH/Sal	EtOH/Sal Vs Sal
Bile salts 1	↓	↓	↓
Bile salts 2	↓	↕	↓
Bile salts 3	↓	↕	↕
Caproic acid	↕	↑	↓
Valeric acid	↓	↕	↓
Isovaleric acid	↕	↕	↕
Leu	↕	↕	↕
Val	↕	↕	↕
Ile	↕	↕	↕
Propionate	↕	↕	↕
2oxoisovalerate	↓	↕	↓
Ethanol	↕	↕	↕
3-H-3MBA	↕	↕	↕
Thr	↕	↕	↑
Acetoin	↓	↓	↕
2-AIB	↕	↕	↕
Ala	↕	↕	↑
Butyric acid	↓	↓	↕
Lys	↕	↕	↕
Acetic acid	↓	↓	↕
Glutamate	↕	↕	↕
Succinic acid	↕	↕	↕
U01	↕	↓	↕
TMA	↕	↕	↕
Choline	↕	↕	↑
Taurine	↕	↕	↕
Methanol	↕	↕	↕
Gly	↕	↕	↕
β-Arabinose	↓	↓	↑
β-Xylose	↓	↓	↑
Sugar 1	↕	↓	↑
Glucose	↓	↓	↕
α-Gal	↓	↓	↑
Uracil	↓	↕	↕
Fumarate	↕	↕	↑
4-HPA	↕	↕	↑
Tyr	↕	↕	↕
U02	↓	↓	↕
Phe	↕	↕	↕
Hyp	↓	↓	↕
Formate	↓	↓	↑
Nicotinate	↓	↓	↓

Accuracy	1 ± 0	0.88 ± 0.16	0.69 ± 0. 30
Precision	1 ± 0	0.84 ± 0.22	0.67 ± 0.41
Sensitivity	1 ± 0	1 ± 0	0.65 ± 0.41
Specificity	1 ± 0	0.75 ± 0.37	0.78 ± 0.35

D

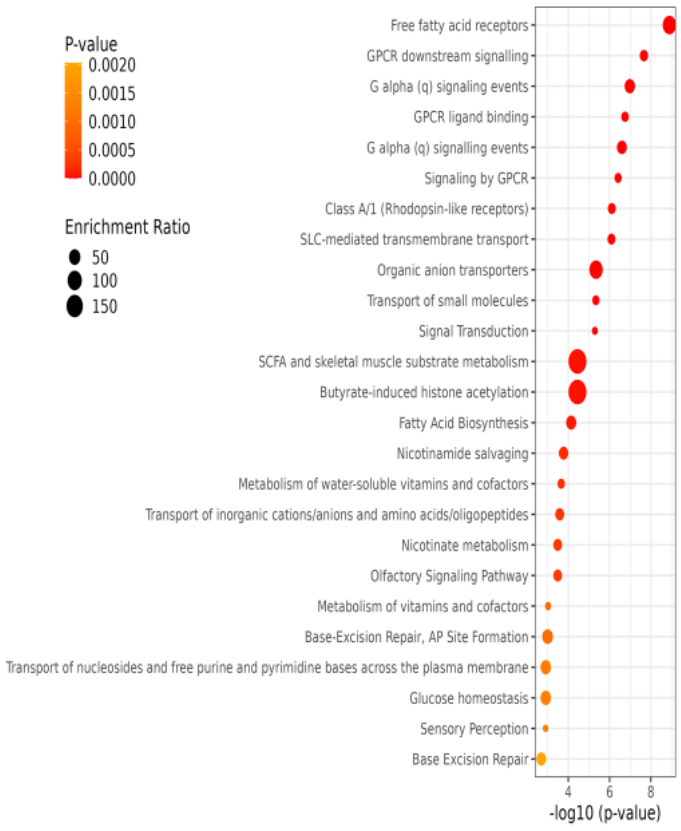


Figure 12 – Bowel inflammation alters gut metabolites. *A-D) DNBS and EtOH/Sal treatments differently alter gut metabolites, indicating shifts in metabolic processes related to gut health and dysbiosis associated with colitis.* A) Representative schematic of the fecal sample collection from the mouse colon. Created with Biorender. B) The scores plot of Principal Component Analysis (PCA) on fecal water metabolites shows that DNBS-treated mice form a distinct cluster compared to the other groups. The percentage of variance explained by each principal component is indicated on the respective axis labels. Blue dots: EtOH/Saline; red dots: DNBS; black dots: Saline. C) Table of gut metabolites (in rows) identified in fecal water using NMR analysis. The columns present the results of PLS-DA comparing DNBS vs. Saline, DNBS vs. EtOH/Sal, EtOH/Sal vs. Saline. At the bottom of each column are validation terms for the comparisons. In the table: ↑ indicates a significant statistical increase, ↓ indicates a significant statistical reduction, and ↕ indicates no statistical difference. D) Metabolite Set Enrichment Analysis (MSEA) of dysregulated fecal water metabolites in DNBS-treated mice reveals associations with various metabolic pathways. Data are presented as –log10(p value), where higher -log10 values indicate more statistically significant results. Each dot represents a metabolic pathway, with larger dots indicating greater representation of that pathway. According to the legend, the colour of the dots shifts toward red as the p-value decreases, indicating higher statistical significance. N=6 mice/group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Our data demonstrate that DNBS treatment disrupts gut metabolome with the reduction in gut metabolites essential for overall body health. On the other hand, EtOH/Sal treatment alters gut metabolites differently notably increasing monosaccharide production. Together, these findings suggest that the treatments modify microbiome composition, although further analysis is needed to confirm this hypothesis and characterize the specific alterations.

Colitis alters glial properties and gene expression in the hippocampus

Patients with IBD can develop extra-intestinal dysfunctions, including stress, depression and anxiety. Depressive-like behavior is linked to microglia and astrocytes which exert essential roles in the maintenance of brain homeostasis and neurotransmission (H. S. Kwon & Koh, 2020). Moreover, several studies on the brain effects of bowel inflammation have observed alterations of astrocytes and microglia (Carloni et al., 2021; Do & Woo, 2018; Riazi et al., 2008, 2015; Zhao et al., 2022). Nevertheless, the literature on the effects of bowel inflammation on microglia remains

discordant, and little is known about the role of astrocytes. Thus, we focused on studying glial properties to determine whether they could be altered by colitis induction, particularly investigating the effects of colitis on the hippocampus. This focus stems from the relationship between IBD-induced brain effects and hippocampal functions. Neurological dysfunctions, including memory and emotional disturbances, associated with IBD are linked to the hippocampus (Haj-Mirzaian et al., 2017). Moreover, inflammation and stress derived from IBD can trigger neuroinflammation in the hippocampus, influencing its functional properties. Previous studies have also connected changes in the gut microbiome with mood and cognition (Borre et al., 2014). Hence, exploring the connection between IBD and the hippocampus could lead to therapeutic approaches that address not only the gastrointestinal issues associated with IBD but also the cognitive and emotional challenges that may arise, potentially involving strategies aimed at managing inflammation and stress.

Based on these premises, we analysed the density of astrocytes and microglia in the hippocampal CA1 *stratum radiatum.* We performed immunofluorescence staining for Gfap, a marker of astrocytes, and Iba1, marker of microglia and monocyte-derived cells, in coronal brain slices from the three experimental groups. Then, we quantified the number of $Gfap^+$ and $Iba1^+$ cells in the CA1 *stratum radiatum*. Our results indicate that the density of $Gfap^+$ cells is significantly increased in DNBS-treated mice compared to the other groups, suggesting that colitis induces astrogliosis (Figure 13 A-B). Additionally, we found that the density of $Iba1^+$ cells in the CA1 *stratum radiatum* is significantly higher in DNBS-treated mice compared to the other groups, indicating that colitis leads to microgliosis (Figure 13 C-D).

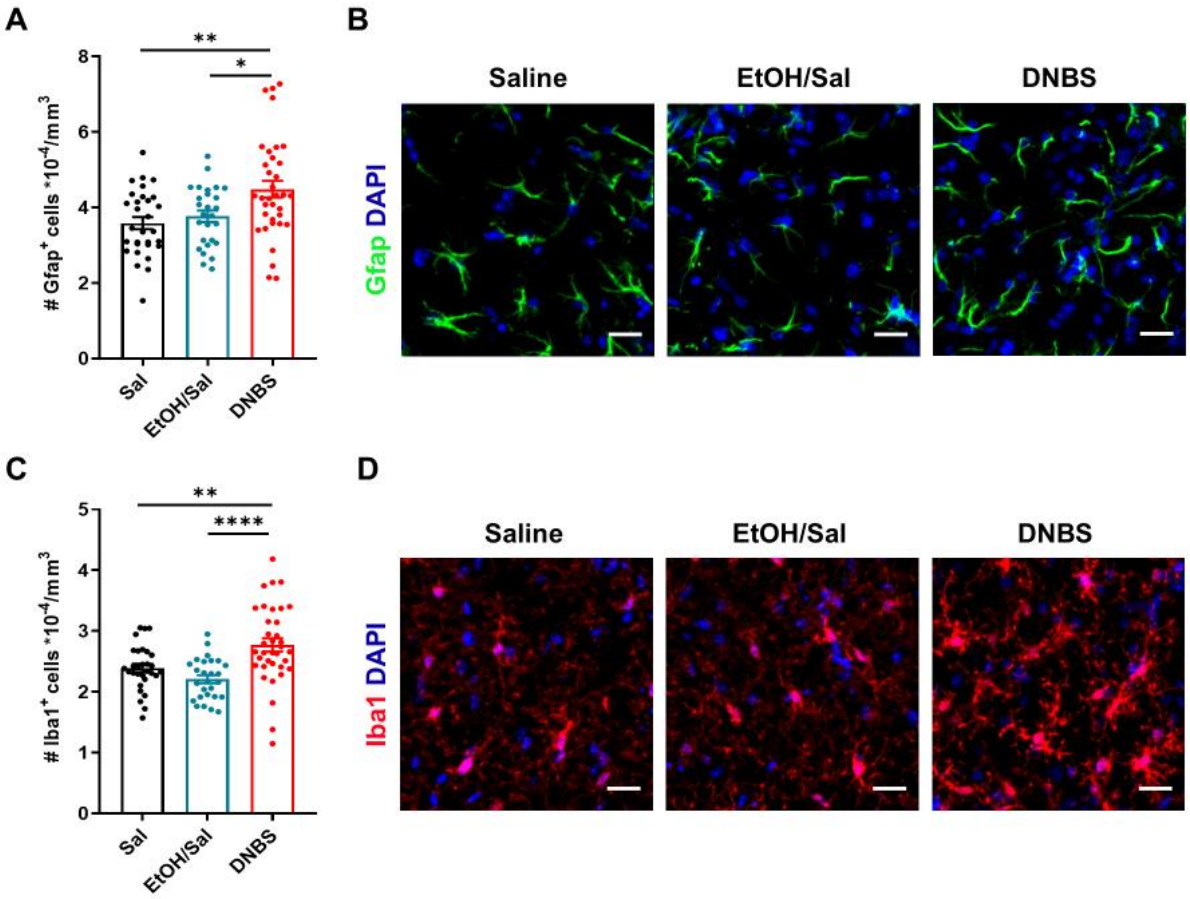


Figure 13 – Colitis induces both astrogliosis and microgliosis in hippocampal CA1 *stratum radiatum*. *A-B) DNBS treatment increases astrocyte density in hippocampal CA1 stratum radiatum.* A) Colitis induces a significant increase of astrocytes (# Gfap$^+$ cells *10^{-4}/mm^3) in CA1 *stratum radiatum* compared to the other groups. One-way ANOVA **. N = Saline 30/3, EtOH/Sal 27/3, DNBS 35/4 fields/mice. B) Representative immunofluorescence images of astrocytes (Gfap in green) density in the CA1 *stratum radiatum* of Saline, EtOH/Sal and DNBS-treated mice. Scale bar 20 µm. *C-D) DNBS treatment increases microglia density in the CA1 stratum radiatum.* C) Colitis significantly increases microglia density (# Iba1$^+$ cells *10^{-4}/mm^3) in the CA1 *stratum radiatum* compared to the other groups. One-way ANOVA ****. N = Saline 30/3, EtOH/Sal 27/3, DNBS 36/4 fields/mice. D) Representative immunofluorescence images of microglia (Iba1 in red) density in the CA1 *stratum radiatum* of Saline, EtOH/Sal and DNBS-treated mice. Scale bar 20 µm. * p<0.05, ** p<0.01, **** p<0.0001

Microglia are plastic cells that scan the environment and can dynamically adapt their morphofunctional properties under physiopathological conditions. Differently to what was believed about the M1- and M2-phenotypes, microglia exist in a continuum of different morphofunctional states. Previous studies have shown that, in animal models of colitis, microglia change their morphological properties (Carloni et al., 2021; Riazi et

al., 2008). Thus, we evaluated how colitis impacts microglia morphology in our experimental model. For this purpose, we analysed three parameters of Iba1$^+$ cells morphology: the area of the soma, the microglial domain area (reflecting the scanning domain), and the arborization domain area (related to microglia ramification). We observed that DNBS treatment leads to a reduction in both microglial and arborization domain areas, while the soma area remains unaffected (Figure 14 A-D).

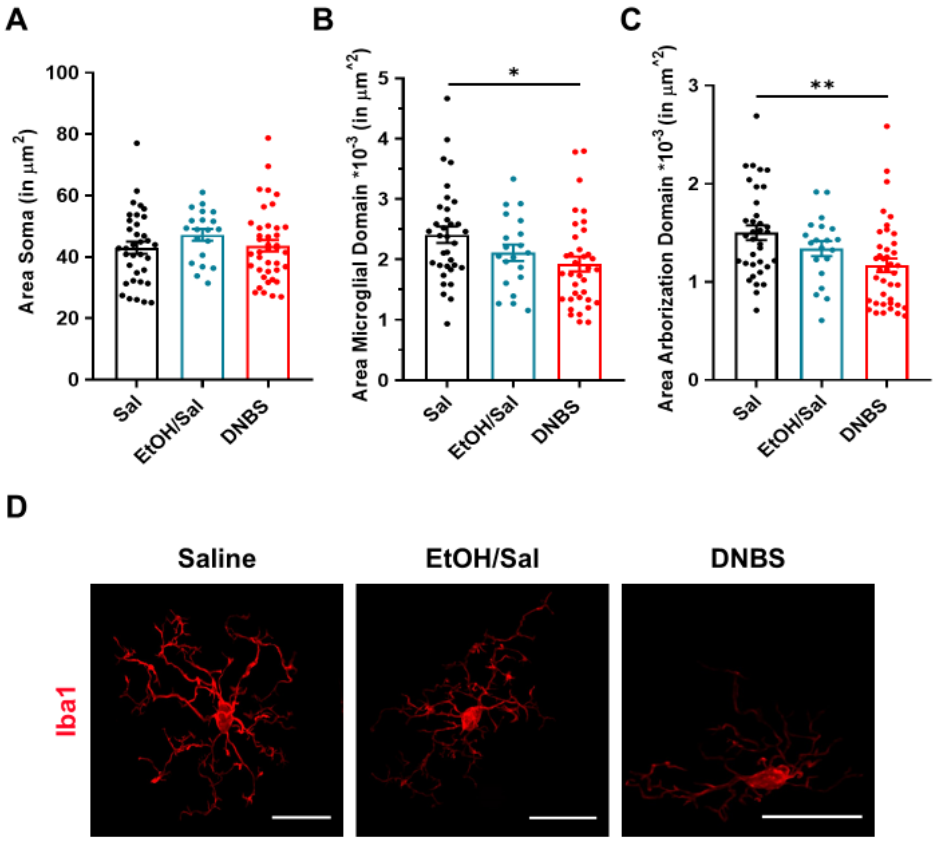


Figure 14 – Colitis alters microglia morphology in the hippocampal CA1 *stratum radiatum*. A) Colitis does not alter the soma area (expressed in µm^2) of Iba1$^+$ cells in the CA1 *stratum radiatum*. One-way ANOVA n.s. (p=0.3896). N = Saline 30/3, EtOH/Sal 27/3, DNBS 35/4 fields/mice. B) Colitis significantly decreases the area of microglial domain (expressed in µm^2) of Iba1$^+$ cells compared to Saline group, suggesting a reduction of the microglia scanning domain. One-way ANOVA *. C) Colitis significantly reduces the area of the arborization domain (expressed in µm^2) of Iba1$^+$ cells compared to Saline group, suggesting that colitis reduces microglia ramification. One-way ANOVA n.s. (p=0.3986). D) Representative images of microglia (Iba1$^+$ cells) from Saline (left-hand), EtOH/Sal (in the middle), DNBS (right-hand) -treated mice. Scale bar 20 µm. * p<0.05, ** p<0.01

It has been demonstrated that colitis increases the infiltration of peripheral immune cells and pro-inflammatory cytokines in the hippocampus, adversely affecting cognitive functions (Carloni et al., 2021; Gampierakis et al., 2021; Lee et al., 2023; Vitali et al., 2022). Moreover, TNBS-treated mice exhibit a compromised BBB that correlates with the extent of tissue/gut damage (Natah et al., 2005). To evaluate whether DNBS-induced colitis leads to hippocampal neuroinflammation, we analysed gene expression in the hippocampus using the nCOUNTER Glial profiling panel from NanoString, which allows for the simultaneous profiling of 750 genes. Our analysis identified different transcriptional profiles in the hippocampus among the three experimental groups. Specifically, when performing pairwise comparison analysis of the experimental groups, we observed that DNBS-treated mice show 77 differentially expressed genes (DEGs) compared to the Saline group and 35 compared to the EtOH/Sal group (Figure 15 A). Furthermore, EtOH/Sal treatment alters the expression of 28 genes compared to the Saline group (Figure 15 A). These data indicate that DNBS-induced colitis significantly modifies hippocampal gene expression more than EtOH/Sal and control groups. Interestingly, some of the altered genes are uniquely differentially expressed, while others are shared among the groups, suggesting that there may be both specific and common pathways affected by the treatments. Next, we focused on the comparison between DNBS and Saline, noting that among the 77 DEGs, 16 are upregulated and 61 are downregulated. To determine whether these altered genes are associated with specific dysregulated pathways in the hippocampus of DNBS-treated mice, we performed the enrichment analysis using the BioPlanet_2019 gene set as reference. We found that these genes are linked to the immune system, fatty acid metabolism, synaptic transmission and glial function (Figure 15 B). To verify the influence of DNBS treatment on the regulation of gene expression we normalized the expression levels of the 77 genes in DNBS to those in the control group, calculating the percentage of their up- or down-regulation. Remarkably, some of the downregulated genes in DNBS are associated with the immune system (such as *Lair1*, *Jak2*, *Tyrobp*, *Vim*), neurotransmitter release (*Rims1*), tight-junctions (*Tjp1*), glia and myelination (*Gfap*, *Sox10*), and cellular metabolism (*Hadh*, *Mmut*); other genes, associated with glutamatergic and GABAergic synaptic transmission, like *Gria1*, *Gad1*, *Cacna2d1* are upregulated (Figure 15 C). Additionally, we analysed the 28 DEGs in EtOH/Sal compared to Saline. We found that, among the downregulated genes, a few

are related to the immune system (i.e. *Jak2*, *Prkcq*) and cellular metabolism (*Mmut*), whilst the genes associated with synaptic transmission (such as *Grin3a*, *Gabra3* and *Gria2*) are upregulated (Figure 15 D).

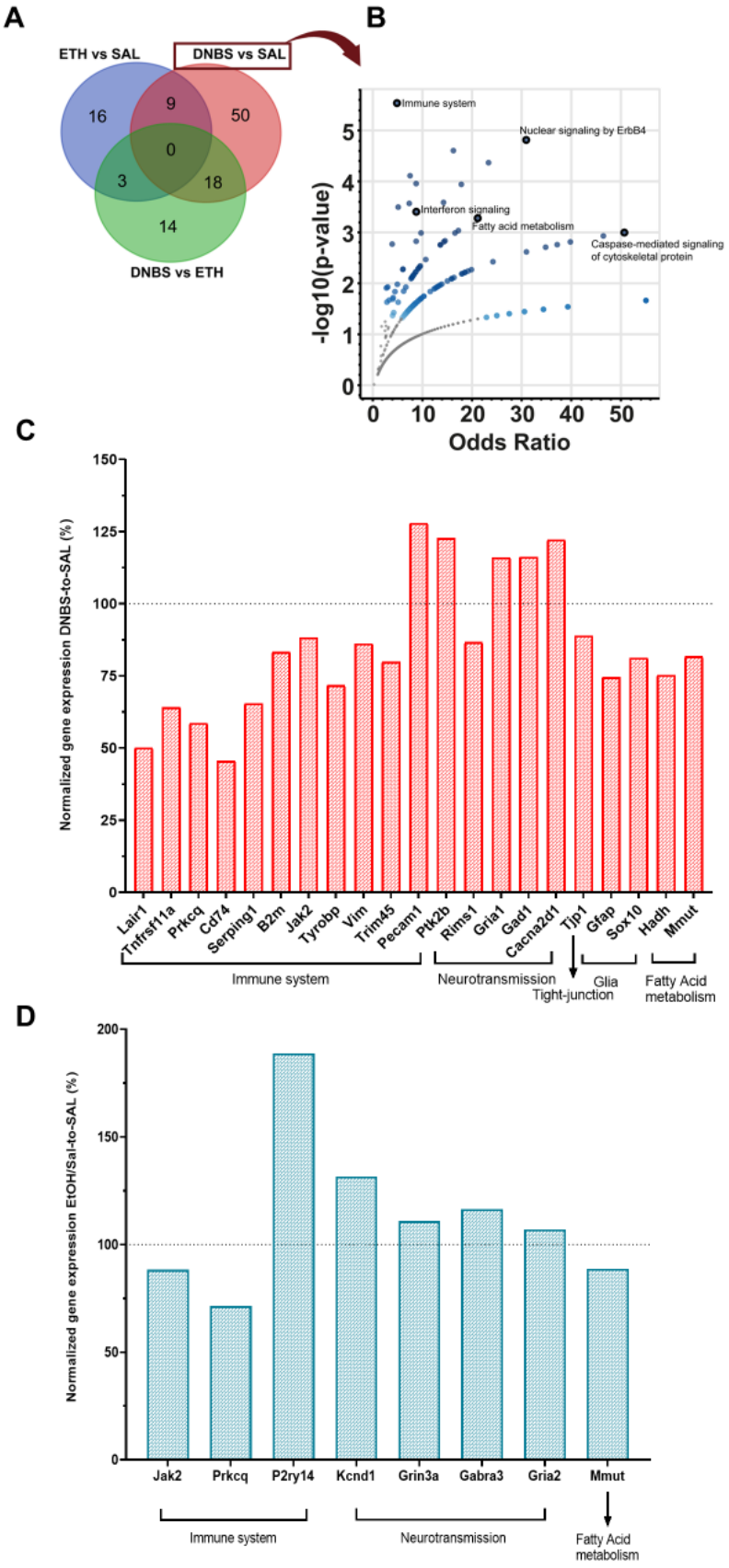

A
ETH vs SAL
DNBS vs SAL
16
9
50
0
3
18
14
DNBS vs ETH
B
Immune system
Nuclear signaling by ErbB4
Interferon signaling
Fatty acid metabolism
Caspase-mediated signaling of cytoskeletal protein
-log10(p-value)
Odds Ratio
C
Normalized gene expression DNBS-to-SAL (%)
Lair1
Tnfrsf11a
Prkcq
Cd74
Serping1
B2m
Jak2
Tyrobp
Vim
Trim45
Pecam1
Ptk2b
Rims1
Gria1
Gad1
Cacna2d1
Tjp1
Gfap
Sox10
Hadh
Mmut
Immune system
Neurotransmission
Tight-junction
Glia
Fatty Acid metabolism
D
Normalized gene expression EtOH/Sal-to-SAL (%)
Jak2
Prkcq
P2ry14
Kcnd1
Grin3a
Gabra3
Gria2
Mmut
Immune system
Neurotransmission
Fatty Acid metabolism

Figure 15 – Bowel inflammation dysregulates gene expression in the hippocampus. *A)* Venn diagram showing the number of DEGs detected in the hippocampus of the three experimental groups. *B-C) DEGs identified in DNBS-treated mice are related to the immune system, neurotransmission and fatty acid metabolism.* B) Volcano plot showing the gene ontology terms (DNBS vs SAL) identified through enrichment analysis. Each point represents a single term. Coloured dots represent terms significantly different in DNBS-treated mice compared to the Saline group. C) Bar graph showing the normalized gene expression of DNBS relative to Saline (%). We reported the downregulation of genes associated with the immune system, tight junction, glia and fatty acid metabolism, and the upregulation of genes related to glutamatergic and GABAergic synaptic transmission. N=4 mice/group. D) Bar graph showing normalized gene expression of EtOH/Sal relative to Saline (%). EtOH/Sal-treatment alters the expression of genes associated with the immune system and fatty acid metabolism, and upregulates genes related to synaptic transmission. N=4 mice/group.

Collectively, these results indicate that DNBS-induced colitis leads to gliosis and alteration in microglial morphology in the hippocampus. Moreover, bowel inflammation dysregulates hippocampal gene expression associated with key processes such as immune response, synaptic transmission, epithelial barrier permeability, myelination and fatty acid metabolism. This highlights the potential for gut inflammation to influence neurobiological processes, suggesting that the interplay between the gastrointestinal system and the brain may play a critical role in the pathophysiology of conditions such as neuroinflammation and cognitive dysfunction.

Colitis alters hippocampal excitatory and inhibitory synaptic transmission

IBD is primarily characterized by chronic inflammation of the gastrointestinal tract. Moreover, emerging evidence highlights a significant interplay between IBD and neurobiological processes, particularly regarding cognitive and emotional functions (Abautret-Daly et al., 2018). It was observed that gut inflammation is accompanied by increased cytokines in the periphery and in the brain, which can modulate brain functionality (Abautret-Daly et al., 2018; Riazi et al., 2008, 2015; Zonis et al., 2015). In addition, gut inflammation affects astrocytes, microglia and hippocampal transmission (Riazi et al., 2008, 2015; Zonis et al., 2015). These findings suggest that systemic inflammation associated with IBD can influence hippocampal function, potentially

disrupting neurotransmission. Alterations in synaptic transmission can lead to neurological symptoms, including anxiety, depression, and cognitive impairment, which are frequently reported by patients with IBD. Previous studies have shown that in a rat model of colitis, hippocampal excitability is increased, making these animals more susceptible to seizure (Riazi et al., 2008, 2015). Moreover, it is well-established that microglia and astrocytes can impact synaptic transmission, particularly in the context of pathological conditions (Basilico et al., 2022; Cornell et al., 2022; Henstridge et al., 2019; Lei et al., 2024; Paolicelli et al., 2011; Raghuraman et al., 2019; Rosa et al., 2020). We hypothesized that in the colitis model, the dysregulation of hippocampal glial properties and the observed alterations in gene expression may be associated with altered synaptic transmission. Hence, using whole-cell patch-clamp electrophysiological recordings we studied the excitatory and inhibitory spontaneous activity of hippocampal CA1 pyramidal neurons in acute brain slices. First, we recorded spontaneous excitatory synaptic currents (sEPSC; holding potential -70 mV). Our results indicate an increase in the peak amplitudes of sEPSCs in DNBS-treated mice compared to the other experimental groups (Figure 16 A-C). However, this increase is not reflected in the mean of sEPSC peak amplitudes, where only a tendency is observed in the DNBS-treated mice (Figure 16 A). In contrast, the cumulative probability distribution of sEPSC peak amplitudes is shifted to the right in DNBS-treated mice compared to the other experimental groups (Figure 16 B), indicating an increase in current amplitudes. To highlight changes in event frequency, we also analysed the Inter-Event Interval (IEI), which is the inverse of frequency (Figure 16 D-E). We found that the mean of sEPSC IEI in DNBS-treated mice is significantly reduced with respect to the EtOH/Sal group (Figure 16 D), suggesting an increase in sEPSCs frequency. The cumulative probability distribution further shows that sEPSC IEI is significantly reduced in the DNBS group compared to the others (Figure 16 E).

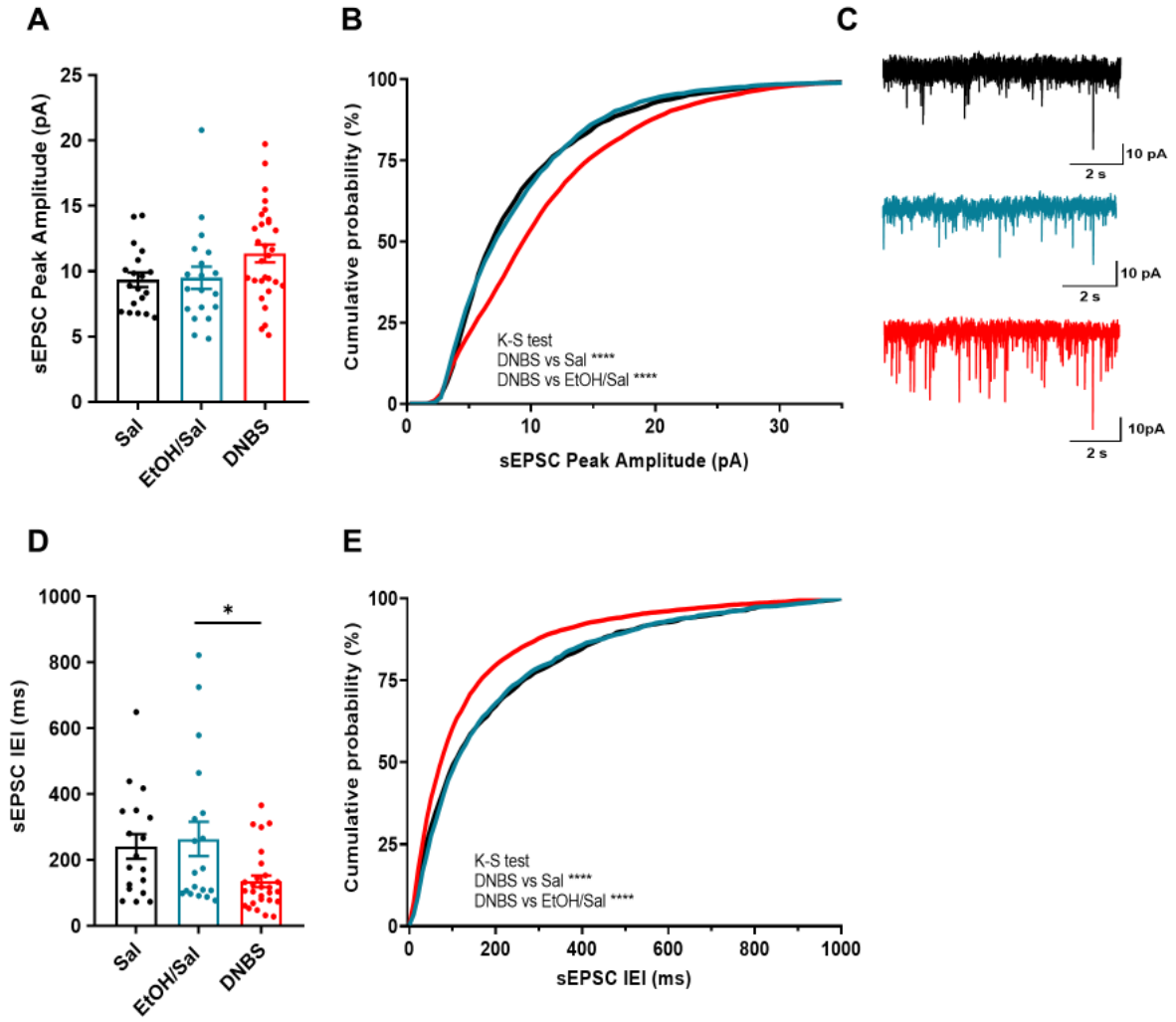


Figure 16 – Colitis increases glutamatergic synaptic transmission in CA1 pyramidal neurons. *A-B) DNBS treatment significantly increases the amplitude of glutamatergic synaptic currents in CA1 pyramidal neurons.* A) The scatter plot of sEPSC peak amplitudes shows a tendency for increased peak amplitudes in DNBS-treated mice. One-way ANOVA n.s. (p=0.0736). N= Saline 19/7; EtOH/Sal 19/9; DNBS 29/11 cells/mice. B) The cumulative probability distribution of sEPSC peak amplitudes shows that DNBS-treated mice have significantly increased sEPSC peak amplitudes compared to the other groups. K-S test. C) Representative traces of sEPSC: black is saline; green is EtOH/Sal; red is DNBS; scale bar 10 pA, 2s. *D-E) DNBS treatment significantly increases the frequency of glutamatergic synaptic currents in CA1 pyramidal neurons.* D) The scatter plot of sEPSC IEI reveals that IEI is significantly reduced in DNBS-treated mice compared to EtOH/Sal group, and there is a tendency for reduction when compared to saline-treated mice. One-way ANOVA *. N= Saline 18/7; EtOH/Sal 19/9; DNBS 27/11 cells/mice. E) The cumulative probability distribution of sEPSC IEI shows that IEI is significantly reduced in DNBS-treated mice compared to the control group. K-S test. * p<0.05, **** p<0.0001

All together, these data suggest that colitis increases the glutamatergic transmission in CA1 pyramidal neurons, increasing both the amplitude and the frequency of excitatory synaptic currents.

The activity of CA1 pyramidal neurons is modulated by a local circuit established by GABAergic afferents from inhibitory neurons, influencing both synchrony and overall network dynamics (Topolnik & Tamboli, 2022). We studied GABAergic transmission by recording the spontaneous inhibitory postsynaptic currents (sIPSC) from CA1 pyramidal neurons. To do so, we held the potential of CA1 pyramidal neurons at 0 mV. We found that both DNBS and Eth/Sal reduce the amplitude and IEI of sIPSCs in CA1 pyramidal neurons (Figure 17 A-E). In particular, for the amplitude, differences among groups are not revealed by the analysis of sIPSC mean (Figure 17 A), but by cumulative probability distribution, where we found a significant reduction of the amplitudes in DNBS- and EtOH/Sal-treated mice compared to control (Figure 17 B). Conversely, we observed that gut inflammation increases the frequency of sIPSC, as shown by significant reduction in the mean and cumulative probability distribution of sIPSC IEI in CA1 pyramidal neurons from DNBS- and EtOH/Sal-treated mice (Figure 17 D-E).

These results demonstrate that both treatments inducing inflammatory bowel responses alter GABAergic transmission in the hippocampus, causing a decrease of IPSCs amplitudes, along with an increase in the frequency of events.

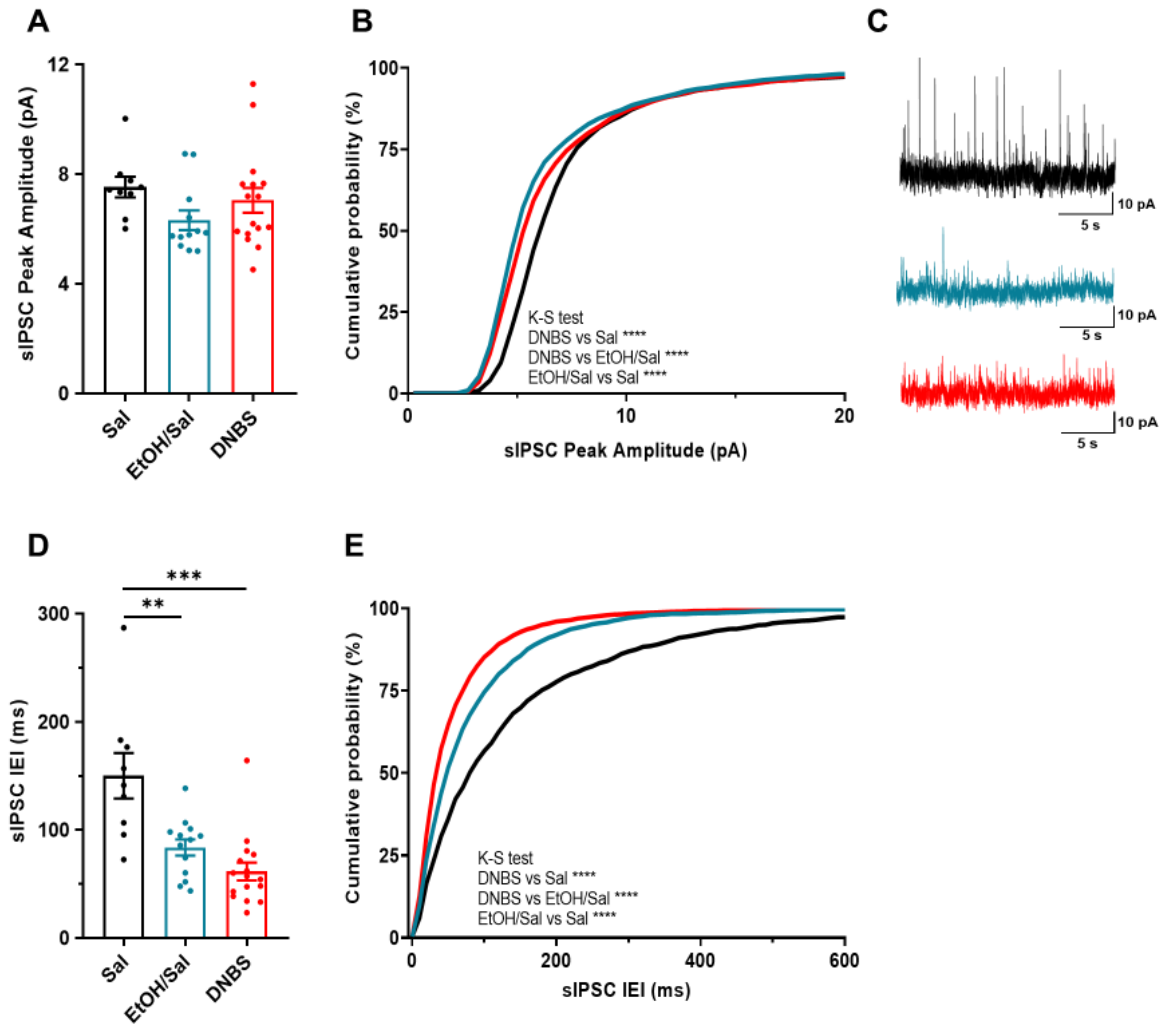


Figure 17 – Bowel inflammation alters GABAergic synaptic transmission in CA1 pyramidal neurons. *A-B) Colitis and mild gut inflammation significantly reduce the amplitude of GABAergic synaptic currents in CA1 pyramidal neurons. A*) The scatter plot of sIPSC peak amplitudes shows a tendency for reduction in DNBS and EtOH/Sal-treated mice compared to the control group. One-way ANOVA n.s. (p=0.1868). N= Saline 9/4; EtOH/Sal 13/6; DNBS 16/7 cells/mice. B) The cumulative probability distribution of sIPSC peak amplitudes shows that DNBS and EtOH/Sal-treated mice have significantly decreased sIPSC peak amplitudes compared to the control group. K-S test. C) Representative traces of sIPSC: black is saline; green is EtOH/Sal; red is DNBS; scale bar 10 pA, 5 s. *D-E) Colitis and mild gut inflammation significantly increases the frequency of GABAergic synaptic currents in CA1 pyramidal neurons. D*) The scatter plot of sIPSC IEI reveals that IEI are significantly in CA1 pyramidal neurons of DNBS and EtOH/Sal-treated mice compared to the control group, meaning that the frequency of sIPSC is higher in presence of bowel inflammation. One-way ANOVA ****. N= Saline 18/7; EtOH/Sal 19/9; DNBS 27/11 cells/mice. E) The cumulative probability distribution of sIPSC IEI shows that IEI is significantly reduced in DNBS and EtOH/Sal-treated mice compared to the control. K-S test. ** p<0.01, *** p<0.001, **** p<0.0001

Based on our results, we speculated that bowel inflammation leads to a shift in the excitation-to-inhibition ratio (E/I), specifically pushing the CA1 pyramidal neurons towards increased excitation. To confirm this, we compared the glutamatergic and GABAergic synaptic currents recorded from the same CA1 pyramidal neurons, at -70 and 0 mV holding potentials, respectively (Figure 18 A). As expected from our previous results, we found that the ratio between the peak amplitudes of sEPSC-to-sIPSC is significantly increased in DNBS-treated mice compared to the Saline group (Figure 18 B). On the other hand, no statistical differences were observed among the groups in the E/I ratio for IEIs (Figure 18 C). These data confirm that colitis shifts the synaptic activity recorded from CA1 pyramidal neurons toward excitation.

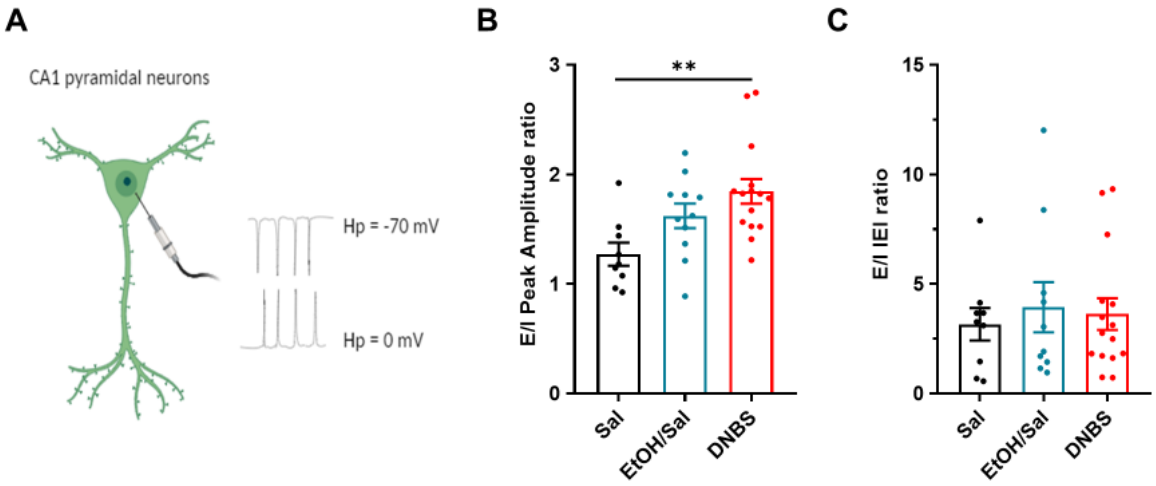


Figure 18 – Colitis shifts the synaptic transmission of CA1 pyramidal neurons toward excitation. A) Schematic of excitation-to-inhibition (E/I) ratio recordings from CA1 pyramidal neurons at holding potentials -70 and 0 mV, respectively. B) Scatter plot of the E/I ratio of peak amplitudes shows a significant increase in DNBS-treated mice compared to the saline group, indicating that colitis induces a shift toward excitation in CA1 pyramidal neurons. One-way ANOVA **. N = Saline 9/4; EtOH/Sal 11/6; DNBS 15/6 cells/mice. C) Scatter plot of the E/I ratio of IEI shows no significant differences among groups. One-way ANOVA n.s. (p=0.8477). ** p<0.01

Together, these data suggest that bowel inflammation alters both glutamatergic and GABAergic synaptic transmission in the CA1 pyramidal neurons of the hippocampus, shifting the neuronal activity towards excitation.

Colitis modifies short-term plasticity at excitatory and inhibitory hippocampal synapses

In light of the aforementioned results regarding the effects of bowel inflammation on spontaneous activity, we aimed to investigate the functional properties of local excitatory and inhibitory circuits in the hippocampal CA1 region. To achieve this, we performed whole-cell patch-clamp recordings to study the evoked glutamatergic and GABAergic synaptic transmission in CA1 pyramidal neurons by stimulating the Schaffer collaterals in the *stratum radiatum* and the interneurons in the *stratum oriens,* respectively.

First, we evaluated the glutamatergic evoked activity by electrically stimulating the Schaffer collaterals from the CA3 region. Specifically, we focused our analyses on three parameters, which are the Input/Output curve (I/O) of AMPAR-mediated responses, the Paired-Pulse Ratio (PPR) and the AMPA/NMDA ratio (Figure 19 A-F). To evaluate the strength of the functional connectivity between CA3 and CA1 neurons, we placed the stimulating electrode on the Schaffer collaterals and recorded from the CA1 pyramidal neurons held at -70 mV. The Schaffer collaterals were stimulated at increasing stimulus intensities to elicit the AMPAR-mediated excitatory postsynaptic currents (eEPSCs) and build a stimulus-response curve. We observed no significant differences in the AMPA-mediated I/O curve, suggesting that colitis does not alter functional connectivity at CA3-CA1 synapses (Figure 19 A-B). Then, to evaluate the short-term plasticity at CA3-CA1 synapses, we analysed the PPR of AMPA-mediated currents. Short-term plasticity is crucial for understanding synaptic transmission dynamics, as it reflects the ability of synapses to modulate their strength in response to the frequency of incoming signals. Again, we stimulated the Schaffer collaterals while recording from the CA1 pyramidal neurons. To obtain the PPR, we applied a paired stimulation of fibers using the same stimulus intensity with varying inter-stimulus intervals (ISI) and calculating the ratio of the second response to the first. Our analysis revealed that DNBS-treated mice have increased PPR compared to the other experimental groups, suggesting that colitis increases the paired-pulse facilitation at CA3-CA1 synapses (Figure 19 C-D).

Finally, we investigated the AMPA/NMDA ratio to assess the relative contribution of AMPA and NMDA receptors in excitatory synaptic currents, providing information about organization of the postsynaptic terminals and synaptic maturation. From the same CA1 pyramidal neuron, we recorded the evoked AMPA- and NMDA-mediated currents. Our analysis reveals that bowel inflammation does not alter the AMPA/NMDA ratio (Figure 19 E-F).

Together, these findings indicate that colitis impairs short-term plasticity at CA3-CA1 synapses, while not affecting the overall connectivity or observable postsynaptic changes.

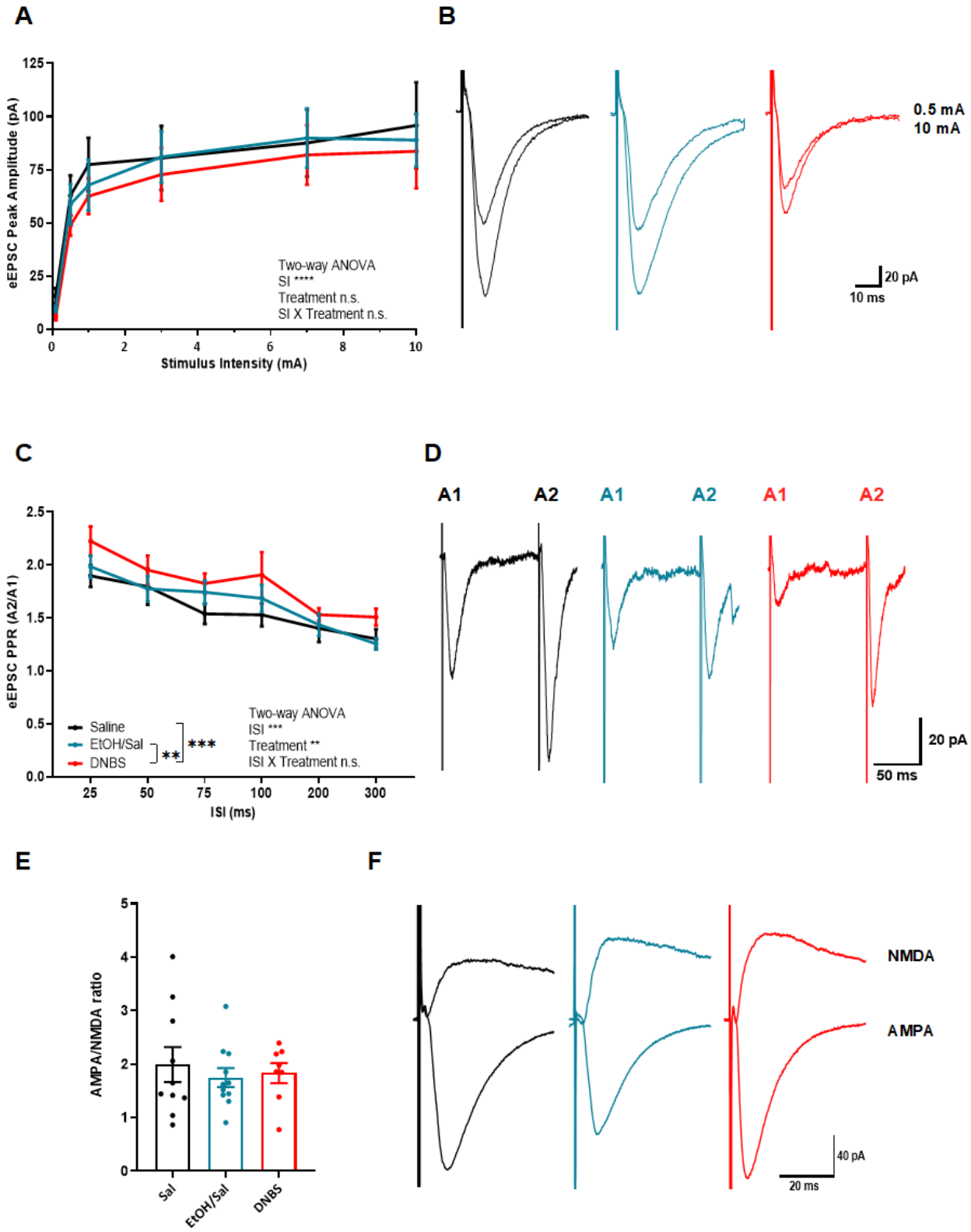

A
B
eEPSC Peak Amplitude (pA)
Stimulus Intensity (mA)
Two-way ANOVA
SI ****
Treatment n.s.
SI X Treatment n.s.
0.5 mA
10 mA
20 pA
10 ms
C
D
eEPSC PPR (A2/A1)
ISI (ms)
Saline
EtOH/Sal
DNBS
**
***
Two-way ANOVA
ISI ***
Treatment **
ISI X Treatment n.s.
A1
A2
A1
A2
A1
A2
20 pA
50 ms
E
F
AMPA/NMDA ratio
Sal
EtOH/Sal
DNBS
NMDA
AMPA
40 pA
20 ms

Figure 19 – Colitis alters short-term plasticity at glutamatergic CA3-CA1 synapses in the hippocampus. *A-B) Colitis does not alter hippocampal CA3-CA1 functional connectivity.* A) I/O curve of AMPA-mediated excitatory postsynaptic currents evoked by stimulation of Schaffer Collaterals (eEPSC) shows no significant differences in the functional connectivity at CA3-CA1 synapses among groups. Two-way ANOVA. N= Saline 9/4; EtOH/Sal 16/8; DNBS 12/7 cells/mice. B) Representative eEPSC traces (average of 6 traces) recorded from CA1 pyramidal cells at −70 mV following Schaffer collateral stimulation at 0.5 mA and 10 mA. Saline (black), EtOH/Sal (blue) and DNBS (red) -treated mice. Scale bar 20 pA; 10 ms. *C-D) Colitis increases paired-pulse facilitation at CA3-CA1 synapses.* C) Paired-pulse ratio (PPR, A2/A1) of eEPSC is significantly increased in DNBS-treated mice compared to the other groups. Two-way ANOVA. N= Saline 11/4; EtOH/Sal 18/8; DNBS 14/9 cells/mice. D) Representative traces of eEPSC (average of 6 traces) recorded at -70 mV with ISI 100 ms following paired-pulse stimulation protocol. Scale bar 20 pA; 50 ms. *E-F) Colitis does not affect synaptic maturation of CA3-CA1 synapses.* E) Scatter plot of AMPA/NMDA ratio reveals no significant differences among groups. One-way ANOVA n.s. N= Saline 10/4; EtOH/Sal 11/6; DNBS 8/6 cells/mice. F) Representative traces of AMPA and NMDA-mediated eEPSCs (average of 6 traces) recorded at -70 mV and +40 mV+Erev, respectively. Scale bar 40 pA; 20 ms. ** p<0.01, *** p<0.001, **** p<0.0001

To evaluate whether colitis modifies local inhibitory synaptic transmission in CA1 pyramidal neurons, we studied the evoked GABA-mediated IPSCs in pyramidal neurons by stimulating the *stratum oriens*, where the majority of interneuron cell bodies and dendrites are located. We recorded evoked IPSC (eIPSC) by whole-cell patch-clamp, holding the potential of CA1 pyramidal neurons at 0 mV. To study the inhibitory functional connectivity, we analysed the I/O curve of GABA-mediated currents. Our results show that the I/O curve of eIPSC is not significantly affected by colitis (Figure 20 A-B). In addition, we investigated the short-term plasticity at the inhibitory synapses of CA1 pyramidal neurons. Note that contrary to excitatory currents, IPSCs are characterized by a paired-pulse depression, indicating a short-term depression of synaptic release (K. Jensen et al., 1999). As shown in figure 20 C-D, the PPR of GABA-mediated currents is increased in DNBS-treated mice compared to control, suggesting that colitis reduces short-term depression at inhibitory synapses.

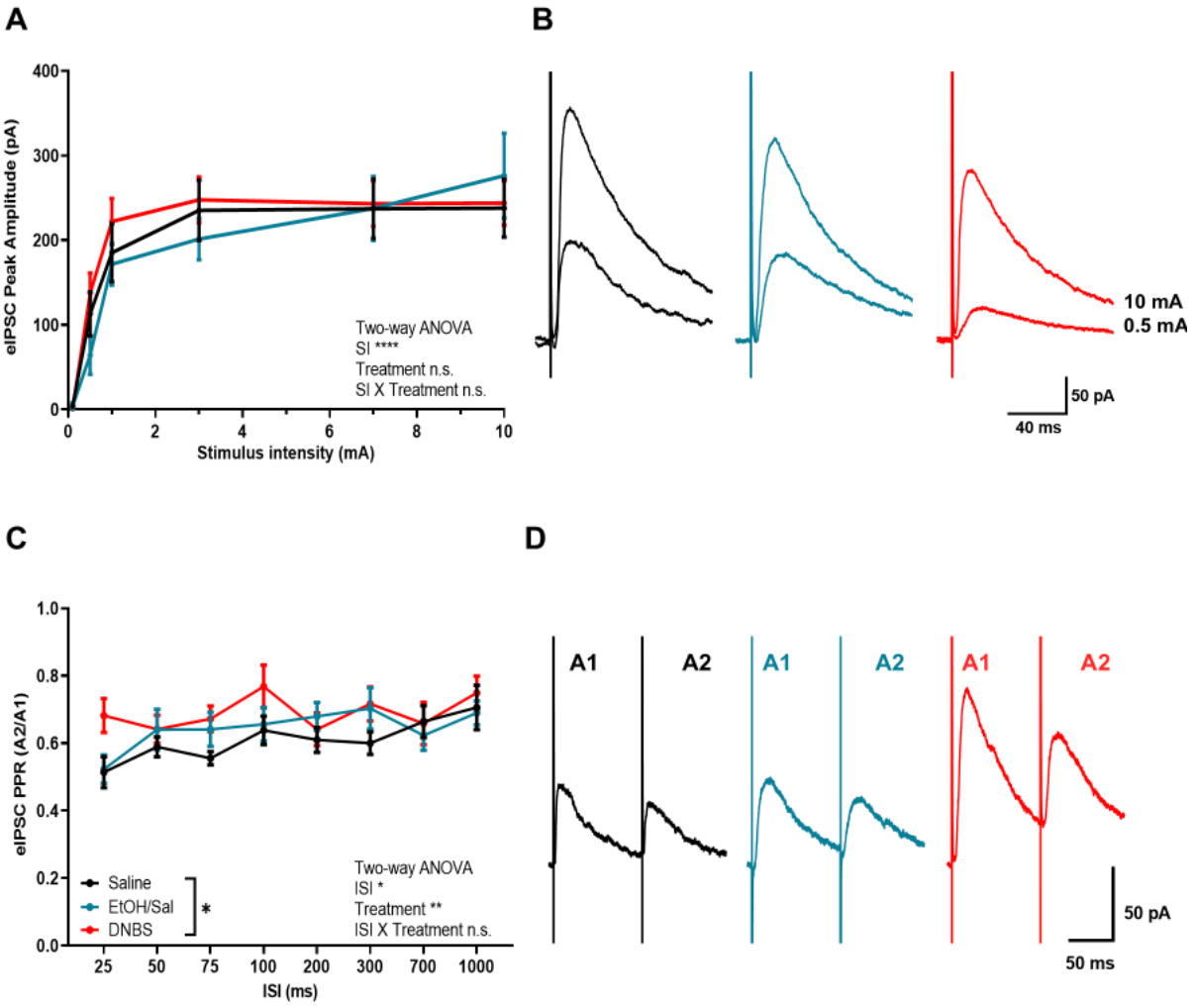


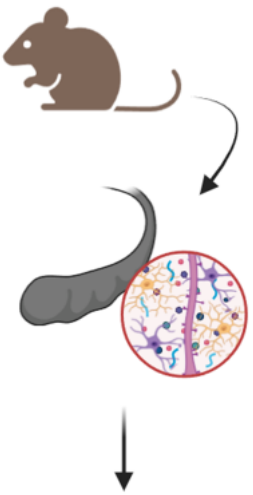
Figure 20 – Colitis alters short-term plasticity at GABAergic synapses of hippocampal CA1 pyramidal neurons. *A-B) Colitis does not impair the functional connectivity at inhibitory synapses.* A) I/O curve of GABA-mediated inhibitory postsynaptic currents evoked by stimulating *stratum oriens* (eIPSC) shows no significant difference among groups of functional connectivity at inhibitory synapses. Two-way ANOVA. Hp 0 mV. NBQX 10 µM, D-AP5 50 µM. N= Saline 13/4; EtOH/Sal 13/5; DNBS 15/4 cells/mice. B) Representative eIPSC traces (average of 6 traces) recorded from CA1 pyramidal cells at 0 mV following stimulation on the *stratum oriens* at 0.5 mA and 10 mA. Saline (black), EtOH/Sal (blue) and DNBS (red) -treated mice. Scale bar 50 pA; 40 ms. *C-D) Colitis alters short-term plasticity at inhibitory synapses.* C) Paired-pulse ratio (PPR, A2/A1) of eIPSC is significantly increased in DNBS-treated mice compared to the Saline group, meaning that colitis reduces paired-pulse depression at inhibitory synapses of CA1 pyramidal neurons. Two-way ANOVA. Hp 0 mV. NBQX 10 µM, D-AP5 50 µM. N= Saline 14/4; EtOH/Sal 13/5; DNBS 15/4 cells/mice. D) Representative traces of eIPSC (average of 6 traces) recorded at 0 mV with ISI 100 ms following paired-pulse stimulation protocol. Scale bar 50 pA; 50 ms. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$

All together these data indicate that colitis impairs both glutamatergic and GABAergic short-term plasticity in CA1 pyramidal neurons, suggesting a potential remodelling of neurotransmitter release probability

Bowel inflammation induces metabolic alterations in hippocampus

The dysregulated genes identified through transcriptomic analysis of the hippocampus suggest that bowel inflammation may significantly impact the immune system and cellular metabolism. Furthermore, alterations in cellular metabolism can occur in the presence of neuroinflammation, with metabolites serving as potential biomarkers for neuroinflammatory disorders (Yang et al., 2021). To elucidate the molecular mechanisms underlying the observed alterations in the hippocampus, we conducted NMR analysis of hippocampal metabolites derived from the three experimental groups (Figure 21 A). In total, we detected 37 hippocampal metabolites. When comparing the three groups using the PLS-DA, we found that DNBS treatment significantly reduces 13 metabolites while increasing 1 metabolite (total saturated fatty acids; SFA) compared to the Saline group (Figure 21 B). In contrast, compared to the EtOH/Sal group, DNBS reduces 9 metabolites and increases 1 (formate) (Figure 21 B). The reduced metabolites include amino acids (alanine, aspartate, glutamate), fatty acids (omega-9 and omega-6), neurotransmitters (GABA, glutamate), and products of cellular metabolism (i.e., lactate) (Figure 21 B). Conversely, EtOH/Sal treatment reduces 13 metabolites, such as lactate, acetate and fatty acids, while increasing 2 metabolites (propionate and total SFA) compared to the Saline group (Figure 21 B). Then, to determine which metabolic pathways altered in the colitis model, we conducted a MSEA on the DNBS-derived hippocampal metabolites identified by NMR. This analysis revealed that specific cellular metabolic pathways, particularly those related to alanine, aspartate, and glutamate metabolism, are significantly affected by DNBS treatment (Figure 21 C).

A



NMR-based metabolomics

B

	DNBS vs Sal	DNBS vs EtOH/Sal	EtOH/Sal vs Sal
Leu	↕	↕	↕
Ile	↕	↕	↕
Val	↕	↕	↕
Propionate	↕	↓	↑
U01	↕	↕	↕
U02	↕	↓	↕
Lactate	↓	↓	↓
Ala	↓	↓	↕
Acetate	↕	↕	↓
N-acetyl aspartate	↕	↕	↕
GABA	↓	↕	↕
Glutamate	↓	↓	↕
Gln	↕	↕	↕
Asp	↓	↓	↕
Crt	↕	↕	↕
Cholines	↓	↕	↕
Taurine	↓	↓	↕
Methanol	↕	↕	↕
Glycerol	↓	↕	↓
Myo-Inositol	↕	↕	↕
Beta- glc	↕	↕	↕
GXP	↓	↕	↓
AXP	↕	↕	↕
Fumarate	↕	↓	↕
Tyr	↕	↕	↕
Inosine	↓	↓	↓
Formate	↕	↑	↓
Niacinamide	↕	↕	↕
Tot Cholesterol	↕	↕	↓
Omega-9	↕	↕	↕
Tot SFA	↑	↕	↑
Omega-6	↓	↕	↓
Omega-3	↕	↕	↓
PE	↓	↕	↓
PC+LPC	↓	↕	↓
MAG	↕	↕	↓
Other Phospholipids	↕	↕	↓

Accuracy	0.68 ± 0.27	0.57 ± 0.25	0.55 ± 0.22
Precision	0.64 ± 0.38	0.45 ± 0.41	0.47 ± 0.32
Sensitivity	0.74 ± 0.39	0.52 ± 0.45	0.69 ± 0.42
Specificity	0.66 ± 0.41	0.62 ± 0.41	0.47 ± 0.41

C

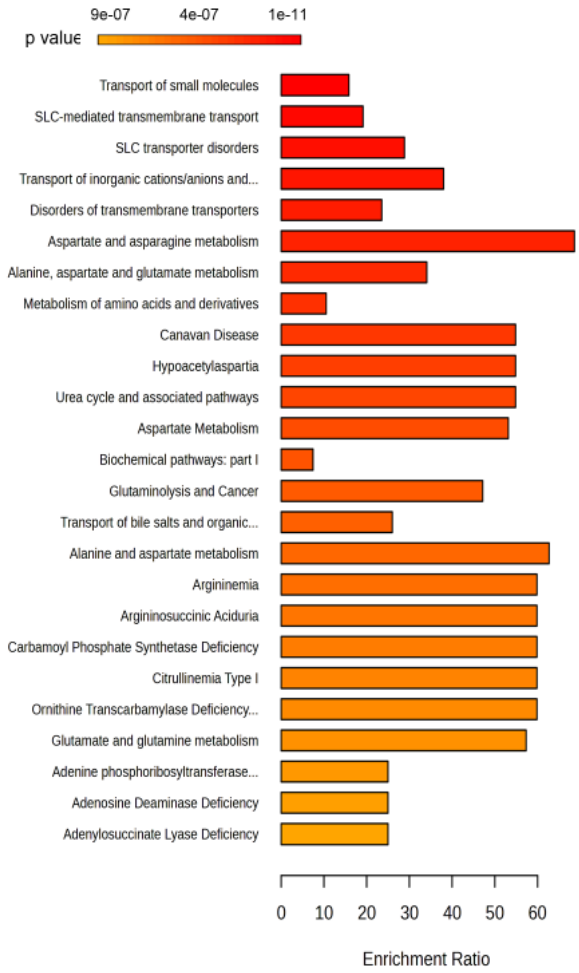


Figure 21 – Bowel inflammation alters hippocampal metabolites. A) Representative schematic of the hippocampal sample collection from mice for NMR-based metabolomics. Created with Biorender. B) Table of metabolites (in rows) found in hippocampus with NMR analysis. In the columns are reported the results of the PLS-DA comparing DNBS vs Saline, DNBS vs EtOH/Sal, EtOH/Sal vs Saline. At the bottom of the column are the validation terms of the comparisons: ↑ for significant statistical increase, ↓ for significant statistical decrease, ↕ no statistical difference. C) MSEA of dysregulated hippocampal metabolites of DNBS-treated mice finds associations with metabolic pathways. The legend of p-value shows that the lower is p value, meaning more statistically significant result, the colour of bars goes toward red. N=6 mice/group.

Together, these findings suggest that colitis induces a shift in hippocampal metabolism, resulting in the alteration of specific pathways related to non-essential amino acids, such as alanine (Ala), aspartate (Asp) and glutamate, as well as those associated with energy metabolism, such as lactate. These changes can be attributed to metabolic stress and disruption in energy metabolism. The observed alterations in hippocampal metabolites reflect the broader interplay between systemic inflammatory responses, metabolic changes, and neurochemical balances, all of which are disrupted in the context of colitis. Further analysis is necessary to elucidate the molecular and cellular mechanisms underlying the hippocampal effects of colitis in mouse models.

Chapter 5: Discussion and conclusions

In this study, we investigated the cellular and molecular mechanisms underlying the effects of colitis on the brain, highlighting new links between gut inflammation and its physiopathological consequences. Our findings reveal that DNBS-induced colitis not only triggers bowel inflammation and dysbiosis, but also affects the brain. In particular, in the hippocampus, we identified alterations in glial cells properties and gene expression. Additionally, we observed changes in hippocampal synaptic functionality and metabolomic profiles.

We found that DNBS treatment induces a progressive, daily, increase in characteristic features of colitis, peaking on the third day, as indicated by the Disease Activity Index. By the experimental day (day 3), the colons of DNBS-treated mice were significantly shortened and showed severe damage. Furthermore, in DNBS-treated mice, we observed a significant reduction in Zo-1 expression compared to controls, confirming the presence of a leaky gut in this model. These findings align with previous studies in humans and animal models, where it was reported that IBD induces sickness symptoms associated with bowel inflammation, including diarrhea, rectal bleeding and compromised gut barrier function (Ahn et al., 2020; Carloni et al., 2021; Villanacci et al., 2021). Our results indicate that DNBS is a reliable tool to mimic the effects of colitis in animal models. However, we observed considerable variability in the severity of DNBS-induced bowel inflammation among animals, underscoring the importance of validating DAI and colon length in each treated animal before conducting experiments.

Notably, we found that the EtOH/Saline group exhibited both macroscopic and microscopic effects on the gut, similar to those seen in DNBS-treated mice, though less severe. We attribute these effects to the damaging impact of ethanol on the mucus layer, consistent with prior findings (Morampudi et al., 2014). In fact, ethanol is intentionally included in the DNBS solution because of its detrimental effect, which aids in disrupting the mucus barrier and increasing the permeability of the intestinal epithelium (Morampudi et al., 2014). This disruption allows luminal microorganisms to penetrate the gut wall, potentially triggering immune cell reactivity. In our study, the mild inflammation and leaky gut observed in the ethanol-treated group likely reflect an

initial inflammatory response driven by microbial entry, but without the additional immune activation expected in DNBS-treated animals. Indeed, as a hapten molecule, DNBS not only facilitates microbial infiltration into the gut wall but also directly activates immune cells (Morampudi et al., 2014). This dual action likely results in more severe gut inflammation and tissue damage. Moreover, the role of proinflammatory cytokines, particularly TNFα, is central to this response. TNFα, released by activated immune cells, regulates the expression of tight junction proteins and can increase epithelial permeability (Michielan & D'Incà, 2015). Elevated TNFα and other proinflammatory cytokines have been detected in clinical studies and experimental models of IBD, further confirming the link between inflammation and increased gut permeability (Roberts-Thomson et al., 2011). Thus, the observed differences between the DNBS and EtOH/Saline groups likely stem from DNBS's stronger inflammatory response due to its haptenic nature. Including an EtOH/Saline control group was essential to rule out the possibility that any observed brain effects could be attributed to ethanol-induced collateral effects rather than DNBS-induced colitis. Despite prior studies using TNBS- or DNBS-induced colitis models often overlooking such controls (Haj-Mirzaian et al., 2017; Riazi et al., 2008, 2015), our inclusion of the EtOH/Saline group strengthens the specificity of our findings by ensuring that brain effects are associated directly with DNBS-induced gut inflammation. This approach confirms the value of DNBS as a reliable model for studying colitis, allowing us to differentiate the effects of gut inflammation from any ethanol-related effects, thereby adding to the robustness of our study.

The gut microbiota plays a crucial role in numerous metabolic and immune processes within the host, helping to maintain gut homeostasis. Disruption of the gut microbiome and its metabolites has been associated with the progression of IBD (Weingarden & Vaughn, 2017). In our analysis of fecal water metabolites identified in DNBS-treated mice, we observed a significant reduction in key SCFAs, including butyric acid, acetic acid, and valeric acid, as well as reductions in bile salts and certain carbohydrates, such as β-arabinose, β-xylose, glucose, and α-galactose. SCFAs are essential for gut health, as they regulate the expression of tight junction proteins and promote mucin secretion, thus reinforcing the mucus layer and sustaining the intestinal barrier (Dalile et al., 2019). This barrier is essential for protecting against inflammatory states, and its

impairment is commonly associated with IBD (Yu, 2023). SCFAs also have systemic effects by modulating the gut-brain axis. These metabolites can enter the bloodstream and cross the BBB, thereby influencing brain function and inflammation (Wenzel et al., 2020). Research indicates that SCFAs positively impact brain health, particularly linked to microglial and astrocytes (Dalile et al., 2019; Erny et al., 2015; Koh et al., 2016; Spichak et al., 2021; Wenzel et al., 2020). Furthermore, behavioral studies suggest that SCFAs may possess antidepressant properties and enhance learning and memory in animal models (Dalile et al., 2019). These roles are particularly relevant given that patients with IBD, and corresponding animal models, often exhibit deficits in emotional and cognitive behaviors, along with functional changes in associated brain regions, such as the hippocampus (Goodyear et al., 2023; Riazi et al., 2008, 2015). Although we did not conduct blood SCFAs analysis or metagenomics of microbiota, we speculate that the observed reduction of SCFAs in the fecal water of DNBS-treated mice could be linked to hippocampal dysregulation. In contrast, mice treated with EtOH/Saline did not display reductions in the most abundant SCFAs (butyric acid, acetic acid, and propionate), although minor SCFAs, such as valeric acid and caproic acid, were reduced. We hypothesize that different dysregulation of SCFAs in DNBS- and EtOH/Sal-treated mice may reflect a distinct composition of gut microbiome resulting from the presence of gut inflammation. This hypothesis should be confirmed through metagenomic analysis, to identify specific microbial changes associated with each treatment condition. These data also raise questions regarding the causal relationship between SCFAs levels, dysbiosis, and the adverse effects of colitis on brain function. Nevertheless, further investigation is warranted to elucidate the mechanisms related to the reduction of SCFAs in this model.

In the colitis model, we also observed a decline in bile acids in the fecal water. Bile acids, produced by hepatocytes and secreted into the intestine as primary conjugated bile acids, are largely recycled through enterohepatic circulation (Yang et al., 2021). The remaining primary bile acids are converted by gut microbiota into secondary bile acids that are either excreted with feces or reabsorbed in the colon (Yang et al., 2021). Secondary bile acids have demonstrated anti-inflammatory properties across various colitis animal models, indicating their importance in maintaining gut health (Cai et al., 2022; Jia et al., 2018; Yang et al., 2021). Dysbiosis disrupts bile acid transformations,

leading to reduced secondary bile acid production and decreased anti-inflammatory support in the gut (Yang et al., 2021). This reduction in bile salts in the colitis model likely reflects changes in microbiota composition. In fact, patients with IBD frequently exhibit less diverse microbiota profiles (Glassner et al., 2020). Given the microbiota's role in maintaining gut barrier integrity and immune homeostasis, mainly through the actions of SCFAs and bile salts (Yang et al., 2021), it may be possible that the reduction of SCFAs and bile salts could contribute to the impaired gut barrier integrity in our model.

Furthermore, the observed reduction of specific carbohydrates, including β-arabinose, β-xylose, and glucose in the fecal water, further suggests microbiota imbalance and gut barrier dysfunction in our colitis model. Specifically, β-arabinose supports the growth of beneficial bacteria that produce SCFAs (Gobbetti et al., 2000; Miyake et al., 2020; Xiang et al., 2024), while reductions in β-xylose may indicate impaired fiber fermentation and gut microbiota alteration associated with IBD (A. Andoh & Nishida, 2023; Gerasimidis et al., 2022; Sultan et al., 2021). In addition, reduced glucose levels in fecal water may indicate changes in absorption or microbiota-related glucose metabolism. Previous studies have shown that gut dysbiosis can exert a considerable effect on glucose metabolism, potentially leading to conditions like glucose intolerance and diabetes (Dong et al., 2020; Liao et al., 2019), a risk particularly heightened in IBD patients, where hyperglycemia has been linked to a compromised intestinal barrier function (Lees et al., 2011; Sang et al., 2022; Thaiss et al., 2018). While we lack blood data on glucose levels in this model, we cannot exclude that glucose absorption may be altered in the colitis model, resulting in lower glucose levels in fecal water. Ongoing analysis of blood metabolites will enhance our understanding of these phenomena.

In summary, these findings support our hypothesis that DNBS treatment compromises gut health. The observed decrease of specific gut metabolites in fecal water suggests changes in microbiota composition, along with compromised gut barrier function, and disrupted microbial metabolism. However, since our metabolomic analysis focused on fecal water, the decreased metabolite levels in the colitis model could result from either reduced microbial production or increased host absorption. Future metagenomic analysis will be essential to clarify these mechanisms, offering a more comprehensive

understanding of the microbiota composition and its impact on gut barrier integrity and metabolic balance in colitis models.

In parallel with gut inflammation in the colitis model, we observed notable alterations in the hippocampus, including gliosis and dysregulated gene expression, which collectively indicate shifts in neuroinflammatory response, metabolism, and synaptic activity. Our analysis primarily focused on the hippocampus, specifically the CA1 region, given its established link with neurological and cognitive disorders, such as depression, anxiety, and deficits in learning and memory, commonly seen in patients with IBD and in animal models (Craig et al., 2022; Ge et al., 2022b; Haj-Mirzaian et al., 2017). We cannot rule out that the brain effects caused by colitis induction might not be limited to the hippocampus, but represent a more general phenomenon, or involve other areas of the brain reported to be implicated in the gut-brain axis (Goodyear et al., 2023; Koren et al., 2021; Riazi et al., 2008).

In the hippocampus of DNBS-treated mice, we observed the presence of astrogliosis and microgliosis, together with changes in hippocampal gene expression, suggesting the alteration of immune response and the potential presence of neuroinflammation. Notably, the density of astrocytes in the CA1 *stratum radiatum* was increased, suggesting that colitis induces an enhancement of hippocampal CA1 astrocytes proliferation or reactivity following colitis induction. Our findings partially confirm previous studies in DSS-induced colitis models, where increased Gfap expression and the emergence of neurotoxic astrocytic phenotypes were observed in different brain regions, including hippocampus (Do & Woo, 2018; Gampierakis et al., 2021; Zhao et al., 2022). On the other hand, transcriptomic analysis on the entire hippocampus of DNBS-treated mice revealed a downregulation of *Gfap* mRNA, which could indicate a more complex astrocytic response. Variability in Gfap expression may reflect astrocytes' adaptive responses under pathological conditions, potentially involving a shift in their metabolic or functional activities (Escartin et al., 2021; Sofroniew, 2009). Moreover, spatial heterogeneity and temporal modulation of Gfap expression could also explain why we observed an increase of $Gfap^+$ cell density in CA1 *stratum radiatum* alongside decreased overall *Gfap* mRNA (Brenner & Messing, 2021; Endo et al. 2022). Indeed, Gfap expression alone does not comprehensively capture astrocyte

reactivity; further studies integrating immunohistochemical and transcriptomic analyses are necessary to clarify astrocytic roles in colitis-induced hippocampal alterations.

Furthermore, we observed an increase in Iba1$^+$ cell density in the CA1 *stratum radiatum*, suggesting that colitis may drive microglial proliferation or activation. Iba1 is broadly recognized as a microglial marker, though it is also expressed by peripheral macrophages and monocytes. Given that previous studies have reported the infiltration of peripheral immune cells, including macrophages, into the brain in colitis models (Carloni et al., 2021; Gampierakis et al., 2021), it is possible that our quantification of Iba1$^+$ cells in the *stratum radiatum* may be potentially overestimated, including infiltrated macrophages and monocytes. To differentiate resident microglia from infiltrating immune cells, further investigation is needed, such as flow cytometry analysis or BBB permeability assay. Despite these limitations, our data are in line with previous findings of microgliosis in colitis models, suggesting a reactive response of microglia to gut inflammation (Carloni et al., 2021; Gampierakis et al., 2021). In addition to changes in cell density, our data indicate that microglia in DNBS-treated mice adapted their morphology by reducing their process extension and arborization in the CA1 *stratum radiatum*, suggesting an environment-driven reactive state. Microglia are highly plastic cells which adapt their morphofunctional properties in response to changes in the surrounding microenvironment (Muzio et al., 2021; Wolf et al., 2017; Prinz & Priller, 2014; Colonna & Butovsky, 2017; Nimmerjahn et al., 2005). Previous work in a rat model of colitis also showed that microglia in the hippocampus retracted their process (Riazi et al., 2008). While we cannot definitively confirm the presence of neuroinflammation in the hippocampus following colitis induction, our results suggest that microglia are responding to changes in their local microenvironment, with process retraction being one hallmark of microglial shift to reactive state (Edler et al., 2021).

All together, these data suggest that the severe bowel inflammation induced by DNBS triggers significant changes in the hippocampal microenvironment, promoting gliosis and microglia reactivity, likely as part of broader neuroinflammation processes (Kölliker-Frers et al., 2021; Virtuoso et al., 2023; Woodburn et al., 2021). Notably, in the EtOH/Saline group, neither astrocyte or microglia density and morphology showed

significant alterations, suggesting that the mild gut inflammation alone may not be sufficient to provoke substantial changes in the hippocampal microenvironment linked to glial reactivity.

Glia reactivity and the release of cytokines can trigger neuroinflammatory processes that lead to neuronal damage and dysfunction through various mechanisms, including disruptions in cellular metabolism (Casaril et al., 2021; Witcher et al., 2021). Therefore, examining neuroinflammation is crucial for future research aimed at understanding the processes that drive brain changes following gut inflammation. However, it is insufficient to assess neuroinflammation in the hippocampus solely by evaluating glial properties, as it involves dysregulations across multiple levels, including molecular, cellular, and subcellular compartments. Indeed, using transcriptomics we screened the regulation of more than seven-hundreds genes related to glial and neuronal profiling. Our analysis of hippocampal transcriptomes revealed that colitis induces a dysregulation of genes associated with the immune system, epithelial barrier, glia, synaptic transmission and cellular metabolism. In particular, the downregulation of genes such as *Lair1*, *Prkcq*, *Jak2*, *Tjp1*, *Hadh*, and *Mmut* suggests cellular modulations in response to colitis. Lair1 (Leukocyte-associated immunoglobulin-like receptor 1) has been implicated in immune regulation and the modulation of inflammatory responses (Van Laethem et al., 2022). In animal models of lupus-like cognitive impairments with hippocampal neuron and dendrite loss, it has been reported that microglia respond to it by increasing the secretion of C1q and IL-10, with consequent downregulation of Lair1, an inhibitory receptor of C1q (Carroll et al., 2024). C1q is specifically involved in microglia-dependent synapse elimination. The downregulation of Lair1 in the colitis model may indicate a disrupted immune balance, potentially exacerbating neuroinflammation and contributing to microglial activation, which may impact neuronal survival and synaptic function. In parallel, Prkcq (Protein Kinase C Theta) is associated with T cell activation and cytokine production, influencing the immune response within the CNS (Zanin-Zhorov et al., 2011). Mice deficient of *Prkcq* exhibit reduced T cell and macrophage infiltration (Zanin-Zhorov et al., 2011). However, the observed downregulation of *Prkcq* in the colitis model complicates predictions regarding its exact role. Further investigations into peripheral immune cell infiltration and cytokine levels are warranted to understand the implications of *Prkcq* downregulation in this context.

In addition, Jak2 (Janus Kinase 2) is linked to JAK2/STAT3 signaling pathway, which is related to many cytokines production, playing a crucial role in the differentiation and function of immune cells (Jain et al., 2021). Studies indicate that inhibition of the JAK2/STAT3 signaling pathway can significantly reduce the expression of proinflammatory cytokines and mitigate the activation of microglia, as well as inflammatory pathways, thereby providing neuroprotection in various neuroinflammatory contexts (Qin et al., 2016; Zhu et al., 2021). The downregulation of these genes opens new questions about the dysregulation of the immune system in the hippocampus of the colitis model. To evaluate the presence of neuroinflammation or immune system alterations, additional experiments should be included to better comprehend these results.

In addition, in the colitis model the reduced expression of *Tjp1* (tight junction protein 1), which encodes the Zo-1 protein, indicates potential disruptions in blood-brain barrier integrity. Zo-1 is crucial for maintaining tight junctions within the BBB, and its decreased expression may allow neuroinflammatory agents to penetrate the CNS, potentially contributing to inflammation and cognitive dysfunction (Zheng et al., 2023). Moreover, the downregulation of *Hadh* (Hydroxyacyl-CoA Dehydrogenase) and *Mmut* (Mut Methylmalonyl-CoA Mutase), both key enzymes in fatty acids metabolism, could indicate a shift in cellular metabolic processes in response to inflammation. This pattern of downregulation in metabolic genes may point to a broader metabolic dysregulation within the hippocampus, possibly as an adaptive response to inflammation.

Consistently with the above discussed results on glial reactivity, in EtOH/Sal-treated mice we found less extensive gene dysregulation in the hippocampus. Among the downregulated genes, some, such as *Jak2*, *Pkcq*, and *Mmut*, reflect changes that may relate to inflammatory and metabolic shifts similar to those observed in DNBS-treated mice. This suggests that while EtOH/Saline treatment induces mild inflammation, it does not lead to the same level of immune response and metabolic disruption as observed in the colitis model.

Although we cannot directly confirm the presence of neuroinflammation in the hippocampus with immunofluorescence analysis, showing changes in microglial and

astrocytic densities, as well as altered microglial morphology (reduced scanning and ramification domains), together with dysregulated immune-related genes, point to a dysregulation of the microenvironment and altered immune response. Our findings also suggest the increase of BBB permeability, and alterations in cellular metabolism. Further studies examining cytokine levels, peripheral immune cell infiltration, and metabolic alterations will be critical to better comprehend the mechanisms underlying these hippocampal changes in response to bowel inflammation.

Remarkably, electrophysiological recordings showed that bowel inflammation alters glutamatergic and GABAergic synaptic transmission of CA1 pyramidal neurons in the hippocampus. In addition, the upregulation of genes involved in excitatory and inhibitory neurotransmission, such as *Gria1* (Glutamate receptor ionotropic, AMPA 1), *Gad1* (Glutamate decarboxylase 1), and *Cacna2d1* in the colitis mouse model points towards alterations in neuronal communication and plasticity.

Specifically, the increase in the amplitude and frequency of sEPSC observed in the colitis model is consistent with previous findings showing the alteration of AMPA-mediated excitatory transmission in hippocampal neurons in a rat model of colitis (Riazi et al., 2015). In particular, they observed increased mEPSC amplitude, insertion of Ca^{2+} permeable AMPA receptors (lacking GluA2-lacking), and modulation of NMDA receptor subunits. Moreover, they found that these alterations in glutamatergic transmission depended on the increase of TNFα derived from “activated” microglia. Given the established role of microglia in synaptic modulation (Basilico et al., 2022), we could speculate that the microglial morphofunctional changes observed in our study may contribute to the aberrant synaptic transmission described in the colitis model. In a model of microglial depletion, Basilico et al. found that repopulating microglia, showing altered morphology, are accompanied by synaptic alterations at CA1 pyramidal neurons (Basilico et al., 2022). However, other explanations for synaptic changes cannot be presently excluded, also in view of the potentially relevant other changes that we found in the hippocampus of colitis mice. Mechanistically, we hypothesize that the increased excitatory spontaneous activity of CA1 pyramidal neurons could be related to the observed upregulation of *Gria1*, probably representing the enhancement of postsynaptic glutamatergic receptor subunit GluA1, which could

lead to the increase of sEPSC amplitude. During synaptic plasticity, the insertion of GluA1 subunits at synapses enhances synaptic strength by stabilizing spine structure and increasing spine size (Kopec et al., 2007). Nevertheless, while gene expression data suggest elevated GluA1, this does not directly confirm changes in protein levels or receptor subunit insertion at synapses. Additional analyses of spine morphology and AMPA receptor subunit composition may help to link the increase of *Gria1* mRNA expression to its role during colitis. Moreover, spontaneous synaptic activity depends on different mechanisms occurring at pre- and postsynaptic sites, which can be distinguished by specific experiments, including the analysis of miniature excitatory postsynaptic currents (mEPSC). Indeed, spontaneous currents depend both on mEPSCs and on action potentials evoked by transmitter release. Thus, our present understanding of colitis-induced glutamatergic potentiation does not allow us to define the precise site of this regulation. We can only speculate that colitis induces a general increase of the excitatory activity in hippocampal CA1, which could be related to the overall dysregulation of hippocampal gene expression and microglia properties.

To further dissect these synaptic changes, we also recorded evoked excitatory postsynaptic currents, as the changes in some of their parameters are known to correlate with pre- or postsynaptic regulations. First, we evaluated the AMPA/NMDA ratio, a parameter that provides a measure of the relative expression of AMPAR and NMDAR at the synapse, and it is usually considered as an indicator of synaptic strength and synaptic maturation (Rao & Finkbeiner, 2007). Interestingly, despite the upregulation of *Gria1* mRNA in the hippocampus, we did not find changes in AMPA/NMDA ratio at CA3-CA1 synapses, making less likely the possibility of postsynaptic changes at CA3-CA1 synapses. This result contrasts with Riazi and colleagues' findings of a decreased AMPA/NMDA ratio in a rat model of colitis (Riazi et al., 2015). This discrepancy may stem from the animals used for modelling colitis, indicating different mechanisms among rat and mouse models. In alternative, it may reflect different experimental protocols. In fact, we chose the stimulus intensity to evoke 50% of AMPA current and we adjusted the holding potential of NMDA with the specific reversal potential of the cell. Conversely, Riazi and colleagues used the stimulus intensity to produce AMPA currents around 400 pA and they recorded NMDA-mediated currents at +40 mV, independently from the reversal potential of each cell (Riazi et al.,

2015). In our experience, this can give rise to errors in the determination of AMPA/NMDA ratio, due to incorrect voltage clamp of the cell, particularly in case of high amplitude currents. This makes it difficult to compare our data, at least in this respect.

Alongside gene expression changes, we also observed a reduction in hippocampal glutamate levels via NMR-based metabolomics, accompanied by downregulation of *Rims1* in the colitis mouse model. *Rims1* (Regulating Synaptic Membrane Exocytosis 1) encodes for a protein involved in the vesicles tethering to the active zone at presynaptic terminals and is required for the recruitment of Ca^{2+} channels to release sites (Brockmann et al., 2020). Its deletion alters the frequency and amplitude of excitatory synaptic transmission in hippocampal neurons (Brockmann et al., 2020). Together the reduced levels of glutamate in the hippocampus and the downregulation of *Rims1* could indicate that the release of glutamate for presynaptic terminals may be compromised. Nevertheless, we have shown that the spontaneous excitatory activity of CA1 pyramidal neurons is increased, rather than decreased as the *Rims1* downregulation could suggest. This discrepancy suggests that *Rims1* downregulation may not uniformly affect all hippocampal neurons or synapses. We hypothesize that the downregulation of *Rims1* in the colitis mouse model could be associated with the reduction of release probability at CA3-CA1 synapses, observed from the increase of PPR in DNBS-treated mice. PPR changes are in general correlated to short-term plasticity and changes in the release probability of neurotransmitters: when two stimuli arrive consecutively with a short time interval, the ratio among the second response and the first might highlight mechanisms of short-term plasticity (Citri & Malenka, 2008). Short-term plasticity depends on both pre- and postsynaptic mechanisms, and when the second response is higher than the first one, the activated synapses are facilitated (Blitz et al., 2004; Citri & Malenka, 2008; Jackman & Regehr, 2017). While Riazi and colleagues found no significant difference in the PPR at CA3-CA1 synapses in the colitis rat model (Riazi et al., 2015), our data show that colitis increases paired-pulse facilitation, indicating reduction of release probability of glutamate at this local circuit. It is possible that the incongruence derives from the different experimental approach, which in our case was set to evidentiate also small differences, while Riazi and coauthors only report data form a single delay, obtained by field recording.

Moreover, in the colitis mouse model *Cacna2d1* (Calcium voltage-gated channel subunit alpha2delta 1) gene, encoding for the auxiliary subunit of Cav1.3 (Campiglio & Flucher, 2015), which is implicated in synaptic plasticity and neurotransmitter release, is upregulated (Pilch et al., 2022; Szymanowicz et al., 2024). Its increase might influence calcium signaling pathways critical for synaptic strength and plastic changes in response to neuronal activity (Campiglio et al., 2015; Szymanowicz et al., 2024).

Even if at first glance the increase of spontaneous excitatory transmission and *Cacna2d1* upregulation, alongside with the reduction of glutamate level, *Rims1* mRNA and the release probability at CA3-CA1 synapses, appears counterintuitive, it can potentially be attributed to altered mechanisms at specific synapses, for instance related to the neurotransmitter release, or changes of synaptic numbers or morphology. Indeed, it has to be pointed out that spontaneous EPSC in CA1 depends both on Schaffer collaterals and commissural pathways (Megías et al., 2001) and specifically to a subset of fibers which are spontaneously active among these pools. On the other hand, the stimulation of Schaffer collaterals activates a specific group of CA3 fibers impinging on CA1 pyramidal neurons (O. Kwon et al., 2018). It is not infrequent that these two different subsets show different basal properties or are regulated in different or even opposite ways (Deivasigamani et al., 2023). Further analyses are needed to confirm and fully understand these data, since the relative expression of *Rims1* mRNA and the glutamate level refer to the entire hippocampus, rather than to the specific CA3-CA1 connection.

In the EtOH/Saline group we did not find alterations in the glutamatergic synaptic transmission in CA1 pyramidal neurons, nor at the specific CA3-CA1 synapses. However, we observed the upregulation of two genes deeply involved in glutamatergic transmission. Specifically, we found an increase in the expression of *Gria2*, encoding GluA2 AMPA receptor subunits. *Gria2* (Glutamate Ionotropic Receptor AMPA Type Subunit 2) encodes a subunit of the AMPA receptor, essential for fast synaptic transmission in the central nervous system. Upregulation of *Gria2* may lead to enhanced excitatory neurotransmission and synaptic plasticity, which could impact cognitive functions (Chater & Goda, 2022). The difference in the regulation of AMPA subunits mRNA following the two treatments can be associated with the specific

changes in synaptic activity. The upregulation of *Gria2* mRNA in EtOH/Saline injected mice could be indicative of a reduction in plasticity phenomena. Since very little data are available on this model, we cannot presently draw conclusions about the physiological changes associated with this upregulation. Both the upregulation of GluA1, in DNBS, and GluA2 mRNA subunits, in EtOH/Saline-treated mice, are potentially related to other pathways and further experiments are needed to comprehend these alterations.

The mechanisms underlying the changes in glutamatergic transmission observed in the colitis model, rather than in mice with mild bowel inflammation, will be investigated in future studies, focusing on the pathways involving microglia and astrocytes, given their roles in synaptic sculpting under pathological conditions (Cornell et al., 2022; Henstridge et al., 2019).

In addition, we found that gut inflammation alters inhibitory transmission of CA1 pyramidal neurons. In particular, the frequency of sIPSC increases with colitis, suggesting compensatory mechanisms of inhibitory synapses in response to the increase of excitatory transmission, and it can be addressed to the hippocampal upregulation of *Gad1* mRNA, which encodes the enzyme that synthesizes the inhibitory neurotransmitter GABA. The *Gad1* mRNA upregulation with colitis could be implicated in the increased production of GABA. Indeed, in a previous study on mouse hypothalamic networks, it was demonstrated that the level *Gad1* mRNA is a reliable indicator of GABA release (Dicken et al., 2015). Nevertheless, our NMR-based metabolomic analysis on the colitis mouse model found a significant decrease of GABA concentration within the hippocampus. This discrepancy might be explained through alteration of GABA metabolism in colitis: an increased degradation of GABA in the synaptic cleft or alteration of reuptake through GABA transporters could contribute to lower levels of GABA. If colitis affects these processes, it could result in a scenario where, even though *Gad1* mRNA is elevated, GABA concentration is reduced due to increased degradation or enhanced reuptake mechanisms (Bernstein & Quick, 1999). Previous study observed that in hippocampus of animal models of peripheral inflammation the number of inhibitory neurons and the vesicular GABA transporters result increased following the LPS injection (J. Chen et al., 2023). These data might

suggest that inhibitory neurons enhance the inhibitory inputs to CA1 pyramidal neurons in order to regulate the increase in their excitatory transmission. Recordings of spontaneous currents do not allow us to determine which synaptic mechanisms underlie the results. Similarly, we found that mild bowel inflammation, induced by ethanol injection, increases the sIPSC frequency of CA1 pyramidal neurons.

The reduced levels of GABA in the hippocampus of the colitis model could be also eventually related to the reduction of glutamate, since it is a metabolic precursor of GABA. We also observed that colitis reduces paired-pulse depression at inhibitory synapses formed among *stratum oriens* interneurons and the CA1 pyramidal neurons. Moreover, the *Rims1* downregulation may be also related to these observations at inhibitory synapses of the colitis model. We hypothesize that the neurotransmitter release of GABA in the synaptic cleft may be altered and further analysis might elucidate the mechanisms underlying these observations (Citri & Malenka, 2008; Debanne et al., 1996).

On the contrary, we observed that the amplitude of sIPSC is significantly reduced in the colitis mouse model. It is possible that it is related to changes at postsynaptic terminals in the sense of GABA-A receptor affinity or number. Nevertheless, spontaneous inhibitory activity can be influenced by different mechanisms and specific experiments, such as the recordings of miniature currents, can help to understand if the reduction of sIPSC amplitude refers to alterations in the postsynaptic receptors. Notably, in the EtOH/Saline group the amplitude of sIPSC is also deeply reduced, even more than in the DNBS-treated mice. Indeed, the sole injection of ethanol could affect the inhibitory synaptic transmission. We consider it more likely that these effects are not direct but by means of ethanol induced mild bowel inflammation. Indeed, with metabolomic analysis of the hippocampus we did not detect the presence of ethanol. In addition, ethanol is cleared from the body rather rapidly. One study quantified the concentration of ethanol in the brain of mice following peripheral injection, showing that it reaches zero concentration within one day (Golovenko et al., 2001). Hence, we tend to exclude the possibility that the alterations observed at inhibitory synapses are directly mediated by the presence of ethanol in the brain 3 days following its injection. On the contrary, in EtOH/Saline group *Gabra3* (Gamma-Aminobutyric Acid Type A

Receptor Subunit Alpha3) mRNA, encoding a subunit of the GABA-A receptor, is upregulated. Upregulation of *Gabra3* can lead to increased inhibitory signaling in the brain, potentially affecting anxiety, mood regulation, and overall neuronal excitability. Its upregulation is counterintuitive with the reduction of the amplitude of sIPSC. The upregulation of *Gabra3* does not correlate in an obvious way with physiological results. However, since it is not necessarily correlated to protein expression, further analyses including the hippocampal proteome are needed to understand its potential physiological impact in this model.

In summary, the dysregulation of hippocampal genes related to synaptic functionality does not provide explanations to all the electrophysiological results that we found in the CA1 pyramidal neurons. We hypothesize that they collectively could be related to altered excitatory and inhibitory balance, and they can ultimately affect various neuronal processes, including learning, memory, and possibly mood regulation. Nevertheless, we should investigate the protein contents in these animal models to fully understand the gene expression that we found.

Our NMR-based metabolomic analysis of hippocampus revealed that colitis reduces different metabolites related to lactate and energy metabolism, aminoacids and neurotransmitter dynamics, choline and phospholipid metabolism, fatty acids and membrane fluidity, inosine and neuroprotective functions. In particular, the reduction of lactate levels in the brain significantly impacts excitatory neuronal transmission by altering metabolic pathways and neurotransmitter release. We hypothesized that the colitis-induced increased excitatory and inhibitory synaptic transmission in CA1 could be due to the reduction of lactate level in the hippocampus. In fact, lactate has been shown to interfere with synaptic activity in various ways (Hollnagel et al., 2020; Kann, 2024; Lucas et al., 2018). Specifically, lactate reduce synaptic transmission in excitatory pyramidal cells and fast-spiking inhibitory interneurons, by lowering the release of neurotransmitters from presynaptic terminals, while the generation of action potentials in the axon remains stable (Hollnagel et al., 2020; Kann, 2024). Reduced lactate availability can impair presynaptic ATP production, resulting in fewer functional release sites for neurotransmitters and slower vesicle replenishment, ultimately hindering synaptic transmission (Lucas et al., 2018). Conversely, we also found that

the release probability of excitatory CA3-CA1 synapses is reduced and the neurotransmitter release at inhibitory *stratum oriens*-CA1 synapses is compromised in presence of colitis. These findings open new questions on the role of lactate reduction on synaptic transmission in our model.

Changes in the level of lactate are potentially very important in this model, also as a possible pathway of glia neuron communication, altering synaptic transmission. Indeed, astrocytes are one of the providers of lactate to neurons with the astrocyte-neuron lactate shuttle (Magistretti & Allaman, 2018). Astrocytes produce lactate via two main pathways: glycogenolysis and glycolysis (Magistretti & Allaman, 2018). Both processes are stimulated by activity-dependent signals from neurons, including noradrenaline, vasoactive intestinal peptide, adenosine, and potassium ions (K^+), which promote glycogenolysis (Magistretti & Allaman, 2018). In contrast, glucose uptake and lactate production through aerobic glycolysis are initiated by signals from glutamate, ammonium, nitric oxide, and K^+ (Magistretti & Allaman, 2018). Moreover, lactate also has anti-inflammatory effects on astrocytes, by reducing their production of TNF**α** (Xu et al., 2022). It is possible that the reduction of lactate is associated with more reactive phenotypes of astrocytes and in relation to neuroinflammation in the hippocampus. But specific experiments to evaluate the reactivity of astrocytes and the implication of the reduction of lactate are needed.

Our study on hippocampal metabolites showed that glutamate and GABA are reduced in the hippocampus of the colitis mouse model. Glutamate and GABA are not just neuronal neurotransmitters, but they can also bind microglia and astrocytes receptors acting on their modulatory functions in the neurotransmission (M. Andoh & Koyama, 2023a; de Ceglia et al., 2023; Favuzzi et al., 2021; Liu et al., 2022). In particular, astrocytes can sense and also release glutamatergic and GABAergic influencing the synaptic transmission (de Ceglia et al., 2023; Liu et al., 2022). Alterations in the extracellular glutamate and GABA contents were observed in neurological disorders but the current knowledge needs to be expanded (M. Andoh & Koyama, 2023; Liu et al., 2022). We speculate that in our model of colitis the reduction of glutamate and GABA in the hippocampal extracellular environment could be related to alteration in microglia and/or astrocytes uptake (Czapski & Strosznajder, 2021), which in turn can

modulate synaptic transmission of CA1 pyramidal neurons. Experiments including modulation of microglia or astrocytes responses to microenvironment will help us to better understand this intricate network.

Finally, we also quantify a reduction of inosine in the hippocampus of mice with bowel inflammation. Inosine is an adenosine metabolite which binds to adenosine receptors acting as neuroprotective and immunomodulatory, and it also has cognitive beneficial roles (Nascimento et al., 2021). We speculate that the reduction of inosine in hippocampus of DNBS- and EtOH/Saline-treated mice may be linked to the alterations observed in immune system and, probably, be linked to the cognitive impairment observed in previous studies in the colitis mouse model and patients with IBD.

In conclusion, these findings underscore a complex dysregulation in the immune response and in the synaptic transmission in the hippocampus during colitis. The downregulation of key immunological and metabolic genes points towards a dysregulated environment that may contribute to neuroinflammation and cognitive deficits. Conversely, the upregulation of genes associated with synaptic plasticity and neurotransmission suggests potential compensatory mechanisms at play. We highlight many missing points that should be investigated to better comprehend the intricate mechanisms occurring in the hippocampus in response to bowel inflammation. Currently, the present study is a correlative study of hippocampal changes in response to the acute phase of bowel inflammation. Together, these insights call for further investigation into the molecular pathways involved and their implications for understanding and treating neuroinflammatory conditions associated with gastrointestinal disturbances.

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