

KU LEUVEN

FACULTY OF PSYCHOLOGY AND
EDUCATIONAL SCIENCES

**Butyrate as a Potential Therapeutic Intervention
for Chemotherapy-Induced Cognitive and
Behavioural Impairments in a Hyper-CVAD Mouse
Model**

Master's thesis submitted for the
degree of Master of Science in
Master of Psychology: Theory and
Research by
Amber Gordts

Supervisor: Prof. Dr. Rudi D'Hooge
In collaboration with:
Ünal Batuhan Uysal

2025-2026



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Summary

Cancer is a common and debilitating disease, and its prevalence continues to increase. At the same time, advances in treatment have led to a growing population of cancer survivors. Chemotherapy remains a cornerstone of many cancer treatments, but its effects are not limited to cancer cells. Because chemotherapeutic agents can also affect healthy cells, many patients experience adverse effects during and after treatment. One clinically relevant complaint is chemotherapy-induced cognitive impairment (CICI), which refers to difficulties in domains such as attention, concentration, learning, memory, visuospatial skills, and executive functioning. However, chemotherapy-related complaints often extend beyond cognition and include broader physical, psychological, and sensory alterations. Together, these difficulties can negatively affect daily functioning and quality of life.

Therefore, the present study investigated to what extent the Hyper-CVAD polychemotherapy regimen induces cognitive, behavioural, and sensory alterations in a preclinical mouse model, and to what extent concurrent oral butyrate administration can alleviate these alterations. Unlike many preclinical studies that focus on single agents or simple combinations, this study used the clinically relevant multi-agent Hyper-CVAD regimen, increasing translational relevance. Butyrate, a short-chain fatty acid, was examined because previous research suggests beneficial effects on both gut and brain functioning, as well as on cognitive, behavioural, and motor impairments in other preclinical neurodegenerative disease models. To address the research questions, 72 adult female C57BL/6J mice were divided into four groups: saline with vehicle, saline with butyrate, chemotherapy with vehicle, and chemotherapy with butyrate. After three weeks of intervention alone and four weeks of concurrent intervention and treatment, mice underwent a test battery assessing locomotor activity (24-Hour Activity, Open Field), exploratory and anxiety-like behaviour (Open Field, Elevated Plus Maze), learning and memory (T-Maze, Passive Avoidance), and thermal nociceptive sensitivity (Tail Flick).

The findings partially supported the hypotheses. The Hyper-CVAD regimen induced weight loss and altered spontaneous locomotor activity in the 24-Hour Activity test, locomotor activity, exploration, and anxiety-like behaviour in the Open Field test, and thermal nociceptive sensitivity in the Tail Flick test. Butyrate mitigated these alterations most clearly in the Open Field and Tail Flick tests, while its attenuating effects on body weight and 24-Hour Activity were less consistent. No clear evidence was found that Hyper-CVAD or butyrate affected anxiety-like behaviour in the Elevated Plus Maze, spatial working memory in the T-Maze, or associative learning and memory in the Passive Avoidance test.

In conclusion, the Hyper-CVAD regimen induced alterations in several, but not all, assessed cognitive, behavioural, and sensory outcomes and butyrate showed partial mitigating potential. Butyrate therefore holds promise for further research on chemotherapy-induced cognitive, behavioural, and sensory impairments.

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This thesis is the result of two years of hard work. I would not have been able to complete it without the help and support of several important people, whom I would like to thank wholeheartedly.

First, I would like to thank my promotor, Prof. Dr. Rudi D’Hooge, for giving me the opportunity to learn more about this fascinating topic and to work on this thesis.

Second, I would like to sincerely thank Ünal Batuhan Uysal for being such a wonderful daily supervisor. I am grateful for the many hours we spent together in the lab, for our conversations about science, life, and everything in between, and for your guidance throughout this entire process. This thesis would not have been the same without you.

Third, I would like to express my gratitude to the other members of the Laboratory of Biological Psychology, especially those working on CICI, for being so kind and welcoming. Thank you for answering all my scientific questions and for the fun conversations in between.

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Finally, I would like to thank my parents for their unwavering support and belief in me throughout my academic journey. You lifted me up during the difficult moments and reminded me of what I am capable of. Your support has meant everything to me.

Approach and Own Contribution

My promotor, Prof. Dr. Rudi D’Hooge, proposed this thesis topic, which I selected through the faculty’s standard procedure. To be able to participate in the experimental work at the Laboratory of Biological Psychology, I obtained the FELASA B certificate during the first semester of the 2024-2025 academic year by completing the Laboratory Animal Science course. In that same period, I familiarised myself with the relevant literature, partly provided by my daily supervisor, Ünal Batuhan Uysal, and began drafting the literature review.

In the second semester of that academic year, my daily supervisor and I conducted the laboratory work together. The experimental protocols were designed by my daily supervisor as part of his doctoral research. My contribution to the experimental work included daily monitoring of the mice, such as weight measurements, tail marking, cage cleaning, and replenishing food and water. I also contributed to the delivery of the interventions through oral gavage, the preparation and administration of the chemotherapeutic agents, and the behavioural testing. During the summer, I completed the first draft of the introduction.

During the 2025-2026 academic year, I wrote the remainder of the thesis. My daily supervisor provided the datasets and introduced me to the basics of the analyses, after which I independently carried out the statistical analyses in R. I subsequently integrated the feedback I received on earlier drafts into the final version of the thesis.

I used generative artificial intelligence tools (ChatGPT and Copilot) in accordance with the guidelines of KU Leuven to improve clarity, phrasing, and language, troubleshoot R code, and identify potentially relevant literature. AI was only used as an assistive tool, the final thesis remains the product of my own work.

List of Abbreviations in Alphabetical Order

A β = amyloid beta

ALL = acute lymphoblastic leukaemia

ANOVA = analysis of variance

BBB = blood-brain barrier

BUT = butyrate

CBT = cognitive behavioural therapy

CHE = chemotherapy

CICI = chemotherapy-induced cognitive impairment

CIPN = chemotherapy-induced peripheral neuropathy

CMF = cyclophosphamide, methotrexate, and 5-fluorouracil

CNS = central nervous system

COX-2 = cyclooxygenase-2

CSF = cerebrospinal fluid

CT = computer-based training

CYC = cyclophosphamide

DEX = dexamethasone

DOX = doxorubicin

DTI = diffusion tensor imaging

EPM = Elevated Plus Maze

FFARs = free fatty acid receptors

GI = gastrointestinal

HDAC = histone deacetylase

IL-1 α = interleukin 1 α

IL-6 = interleukin 6

IL-12 = interleukin 12

i.p. = intraperitoneal

MESNA = 2-mercaptoethane sulfonate sodium

MTX = methotrexate

NaB = sodium butyrate

NAC = N-acetylcysteine

NHL = non-Hodgkin lymphoma

OF = Open Field

PA = Passive Avoidance

PGE2 = prostaglandin E2

PTX = paclitaxel

ROS = reactive oxygen species

SAL = saline

SCFAs = short-chain fatty acids

SEM = standard error of the mean

ST = strategy training

TNF- α = tumour necrosis factor alpha

VEGF = vascular endothelial growth factor

VEH = vehicle

Table of Contents

Summary	i
Acknowledgments	ii
Approach and Own Contribution	iii
List of Abbreviations in Alphabetical Order	iv
Butyrate as a Potential Therapeutic Intervention for Chemotherapy-Induced Cognitive and Behavioural Impairments in a Hyper-CVAD Mouse Model	1
Chemotherapy-Induced Cognitive and Behavioural Impairments	1
Challenges in CICI Research and the Role of Animal Models	2
Chemotherapy Agents and Their Effects on Cognition and Behaviour	4
Pathophysiological Pathways of CICI	6
Potential Therapeutic Interventions for CICI	10
Non-Pharmacological Interventions	10
Pharmacological Interventions	12
Targeting the Gut-Brain Axis to Ameliorate CICI	13
The Current Research Project	15
Methods	17
Animals	17
Timeline	17
Intervention and Treatment Protocol	18
Behavioural Test Battery	19
24-Hour Activity	19
Open Field	19
Elevated Plus Maze	20
T-Maze	20

Passive Avoidance	21
Tail Flick	21
Statistical Analyses	22
Results	23
Body Weight Changes	23
24-Hour Activity	24
Open Field	26
Elevated Plus Maze	30
T-Maze	31
Passive Avoidance	32
Tail Flick	34
Discussion	36
Weight Progression	36
Locomotor Activity	36
Anxiety-Like Behaviour	37
Learning and Memory	38
Nociceptive and Heat Sensitivity	40
Study Limitations, Strengths and Future Research	41
Conclusion	43
References	44

List of Figures

Figure 1: Pathophysiological Pathways of CICI	6
Figure 2: Modulatory Pathways of SCFAs	14
Figure 3: Timeline and Injection Schedule	19
Figure 4: Average Body Weight Curve.....	24
Figure 5: Results Total Beam Crossings 24-Hour Activity	25
Figure 6: Results Total Beam Crossings per Hour in the 24-Hour Activity Test	26
Figure 7: Results Distance Travelled Open Field	27
Figure 8: Results Open Field.....	29
Figure 9: Representative Open Field Trajectories.....	30
Figure 10: Results Elevated Plus Maze	31
Figure 11: Results T-Maze	32
Figure 12: Results Passive Avoidance Test	33
Figure 13: Results Change in Latency Passive Avoidance Test	33
Figure 14: Results Tail Flick Test	35

Butyrate as a Potential Therapeutic Intervention for Chemotherapy-Induced Cognitive and Behavioural Impairments in a Hyper-CVAD Mouse Model

Chemotherapy-Induced Cognitive and Behavioural Impairments

Cancer remains one of the leading causes of morbidity and mortality worldwide, with approximately 20 million new diagnoses and 9.7 million deaths reported in 2022. By 2050, this number is projected to rise by 77%, exceeding 35 million annual cases. Furthermore, cancer is extremely prevalent with lifetime risk estimates indicating that approximately one in five individuals will be diagnosed with cancer (World Health Organization, 2024). Parallel to this increase, advances in cancer treatment resulted in a continuously expanding population of cancer survivors (Gibson & Monje, 2019). Many different forms of cancer treatment exist, including hormone therapy, immunotherapy, radiation therapy, blood stem cell transplant, surgery, and chemotherapy. A treatment can be given in isolation, but oftentimes patients receive a combination of these treatments (National Cancer Institute, n.d.). Chemotherapy, a form of systemic therapy, remains a cornerstone in the treatment of many cancer types (American Cancer Society, 2016; Matsos & Johnston, 2019). It involves the administration of cytotoxic drugs designed to kill rapidly dividing cancer cells or inhibit their growth. Despite its life-saving effects, chemotherapy is also characterized by some serious side effects, as chemotherapy regimens lack selectivity and damage healthy cells as well (National Cancer Institute, 2025). Some of these adverse effects involve the nervous system. Many patients experience something called chemotherapy-induced peripheral neuropathy (CIPN), a condition characterised by symptoms such as altered sensations, pain, tingling, and numbness caused by chemotherapy-related nerve damage (Zajaczkowska et al., 2019). Beyond peripheral symptoms, increasing attention has been directed towards the cognitive consequences of chemotherapy exposure (Gibson & Monje, 2019; Matsos & Johnston, 2019). This phenomenon is better known as chemotherapy-induced cognitive impairment (CICI), also referred to as “chemobrain” or “chemofog” (Argyriou et al., 2011).

Argyriou et al. (2011) define CICI as: “the impairment of patients’ memory, learning, concentration, reasoning, executive function, attention, and visuospatial skills during and after discontinuation of chemotherapy” (p. 127). Estimates of people suffering from CICI vary between 17% and 75%, highlighting its substantial and widespread impact (Schagen et al., 1999; van Dam et al., 1998; Wieneke & Dienst, 1995). The experienced cognitive impairments vary from short-term, transient and subtle to long-term and sustained (Argyriou et al., 2011; Ferguson & Ahles, 2003). Boykoff and colleagues (2009) conducted focus groups

and interviews of breast cancer survivors and found that CICI often leads to feelings of frustration or fear, particularly when experiencing memory problems. It also increases dependency on others, requiring help with simple tasks, and diminishes work performance and opportunities due to decreased efficiency, focus and speed. These clinical observations indicate that cognitive sequelae of chemotherapy extend beyond the clinical setting and affect patients' personal and occupational lives (Boykoff et al., 2009). Consistently, Haywood et al. (2023) emphasise that CICI also disrupts relationships, psychological health, and overall social functioning, besides problems in vocation and daily functioning. CICI may alter intimate relations and parenting capabilities, undermine self-confidence, lower distress tolerance, reduce mental health, and contribute to communication difficulties and social withdrawal (Haywood et al., 2023). Taken together, this evidence makes it clear that CICI can diminish patients' quality of life by impairing cognitive, social and occupational functioning (Hodgson et al., 2013).

Importantly, chemotherapy-related complaints are rarely limited to cognition alone. Many patients also experience fatigue, characterised by high levels of cognitive, emotional, and/or physical exhaustion, as well as reduced physical activity, sleep disturbances, psychological complaints such as distress, anxiety and depressive symptoms, and sensory symptoms and pain, all of which may further reduce quality of life and interfere with daily functioning (Bower, 2014; Jacobsen et al., 1999; Jim et al., 2011; Konieczny et al., 2025; Kwan et al., 2012; Yi & Syrjala, 2017; Zajączkowska et al., 2019). These symptoms may interact with, exacerbate, or partly contribute to cognitive complaints, thereby increasing the overall burden experienced by chemotherapy-treated patients (Luo et al., 2023; Mayo et al., 2024). Together, these findings suggest that chemotherapy can induce a complex neuropsychological profile, encompassing cognitive, behavioural, psychological, and sensory side effects, underscoring the need to consider patients more holistically. Research investigating CICI should therefore also include behavioural, psychological, and sensory outcomes alongside cognitive outcomes to evaluate the broader impact of chemotherapy on overall functioning.

Challenges in CICI Research and the Role of Animal Models

Despite its clinical relevance, research on CICI involves considerable methodological and conceptual challenges. Several methodological difficulties have been highlighted in the literature, including relatively small sample sizes, variability in patient demographics, tumour type and location, the use of additional anticancer treatments (e.g., hormonal therapy), and

differences in treatment intensity (Seigers & Fardell, 2011). Such divergent samples make it hard to look at the effects of chemotherapy on cognition in isolation (Hodgson et al., 2013). Furthermore, many studies rely on cross-sectional rather than longitudinal designs. Without longitudinal baseline assessments, it becomes impossible to determine whether observed cognitive impairments are a consequence of chemotherapy or reflect pre-existing differences prior to treatment initiation (Hodgson et al., 2013). If such impairments were already present before treatment, they may reflect the psychological burden associated with a cancer diagnosis rather than the direct effects of chemotherapy (Schagen et al., 1999). Another methodological challenge in CICI research concerns the selection of the right control group. As Hodgson et al. (2013) note, healthy control participants do not experience the psychological burden associated with a cancer diagnosis. However, comparing chemotherapy-treated patients to cancer patients receiving different or no treatments also has limitations, as differences in disease severity and treatment burden may act as confounding variables. A longitudinal design, in which the same patients are assessed before and after treatment, could partly address this issue. Yet this approach also presents difficulties, particularly regarding the recruitment and retention of patients with a diagnosis like cancer. Furthermore, not all patients experience CICI, and the neuropsychological assessments used to evaluate cognitive performance are far from perfect (Hodgson et al., 2013).

These limitations highlight the importance of animal models in CICI research. Animal studies allow for objective investigation of CICI and its underlying mechanisms in the absence of confounding variables and cancer itself (Seigers & Fardell, 2011). Rodent models are particularly relevant in this context because they are widely used to investigate neurological, neurodegenerative, and psychiatric disorders that, like CICI, involve alterations in cognition, behaviour, and brain functioning, such as Parkinson's disease, Alzheimer's disease, Huntington's disease, ADHD, autism, depression, and schizophrenia (Ellenbroek & Youn, 2016; Nestler & Hyman, 2010). In these fields, rodent models have been widely used to investigate disease aetiology and pathophysiological pathways, identify genetic risk factors and potential biomarkers, and evaluate therapeutic interventions, as many of these questions cannot be experimentally examined in humans for ethical and practical reasons (Bansal, 2017; Ellenbroek & Youn, 2016; Nestler & Hyman, 2010; Richtand et al., 2022). Their value partly lies in the fact that rodents share relevant genetic, neuroanatomical, and behavioural characteristics with humans, while also allowing researchers to manipulate biological and environmental factors under controlled experimental conditions. In addition, rodents can be

genetically manipulated relatively easily, and a wide range of behavioural tests is available to assess different cognitive, affective, sensory, and motor functions (Baker et al., 2020; Haynes et al., 2022; Matsos & Johnston, 2019). Similarly, rodent models may help clarify the mechanisms underlying CICI and so support the development of interventions that can mitigate or reverse the adverse cognitive, behavioural, and sensory effects of chemotherapy, a step that is becoming increasingly important as cancer survival rates continue to rise.

Chemotherapy Agents and Their Effects on Cognition and Behaviour

Many different chemotherapy regimens exist, from single-agent cytostatic treatments to complex combination therapies. Polychemotherapy treatments have the advantage of having higher efficacy than single agents (DeVita & Chu, 2008). An example of a chemotherapy treatment that includes multiple drugs is Hyper-CVAD. Hyper-CVAD consists of six different agents: cyclophosphamide, vincristine, doxorubicin, dexamethasone, methotrexate and cytarabine and is used to treat aggressive haematological malignancies such as acute lymphoblastic leukaemia (ALL) and some non-Hodgkin lymphomas (NHL; Kantarjian et al., 2000; Younas et al., 2012; Siegel et al., 2018; Sapkota & Shaikh, 2023). Moreover, Hyper-CVAD has been linked to cognitive impairments in humans (Kotb et al., 2019).

Chemotherapeutic agents can be classified according to their chemical structure, mode of action and relationship to other chemo components. Cyclophosphamide is an alkylating agent that damages DNA throughout all phases of the cell cycle, ultimately preventing cellular replication of cancer cells and inhibiting tumour growth. Vincristine is a mitotic inhibitor belonging to the class of plant alkaloids, more specifically classified as a vinca alkaloids (American Cancer Society, 2025). It disrupts microtubule function, thereby preventing mitosis and inhibiting the proliferation of cancer cells (Chen et al., 2024; Orosz et al., 1999). Doxorubicin is an anthracycline, a group within the anti-tumour antibiotics, and works by preventing cell reproduction by binding with DNA and blocking enzymes essential for DNA copying (American Cancer Society, 2025). Dexamethasone is a corticosteroid. These agents can cause apoptosis in haematological neoplasms, are used in the management of allergic reactions, and help mitigate chemotherapy-related side effects (American Cancer Society, 2025; Faggiano et al., 2022). Lastly, methotrexate and cytarabine belong to the antimetabolites, which prevent cells from reproducing as they disrupt the formation process of DNA and RNA, by taking the place of normal building blocks and blocking replication (American Cancer Society, 2025).

Each of these agents has been shown to induce cognitive and behavioural impairments in animal models when administered individually. For instance, cyclophosphamide (CYC) has been shown to significantly impair spatial learning and memory in rats and to induce deficits in spatial working memory accompanied by increased anxiety-like symptoms in mice (Alhowail et al., 2019; Wu et al., 2017). Golchin et al. (2015) found that vincristine treatment induced impairments in spatial learning and memory and diminished explorative behaviours in rats. Doxorubicin (DOX) has been reported to impair episodic memory function and learned fear-association memory in rats (Ramalingayya et al., 2017, Christie et al., 2012). Another rat model showed that DOX can reduce short-term memory and spatial learning/memory (Tong et al., 2020). Tongjaroenbuangam et al. (2013) illustrated that chronic dexamethasone (DEX) administration in mice led to impaired spatial memory. Methotrexate (MTX) was able to reduce spatial working memory in rats (Lyons et al., 2011). Li and colleagues (2010) found impaired short-term spatial working memory, but normal object recognition memory in rats treated with acute or chronic MTX treatment. Rats treated with cytarabine had impaired long-term spatial memory, while performing normally on spatial learning and recent memory (Li et al., 2008).

In addition to studies examining the cognitive and behavioural effects of single agents, several studies have investigated the impact of combination chemotherapy regimens. One such combination is CYC and DOX. Philpot et al. (2019) found that this combination impaired spatial memory in mice, with deficits observed both during and after treatment. The same drug combination has also been shown to impair spatial working memory and spatial recognition memory, while concurrently increasing anxiety-like behaviour in rats (Kitamura et al., 2017). Another regimen that has been investigated is CYC, MTX, and 5-fluorouracil, also known as the CMF combination. Briones and Woods (2011) reported that CMF induced deficits in learning and memory function in rats. Comparable findings were observed in mice, where Anderson et al. (2020) found impaired long-term memory following CMF administration. A final relevant combination is MTX and cytarabine. Alexander et al. (2018) administered these agents intrathecally in mice and observed significant reductions in spatial memory retention. Likewise, Bisen-Hersh et al. (2013) found that this treatment impaired both acquisition and retention, as well as short-term recognition memory in mice.

Despite the many studies looking into the effects of single chemotherapy agents on cognition and behaviour and some into simple combinations, almost no studies investigate the effects of a full, clinically relevant multi-agent chemotherapy regimen, such as Hyper-CVAD,

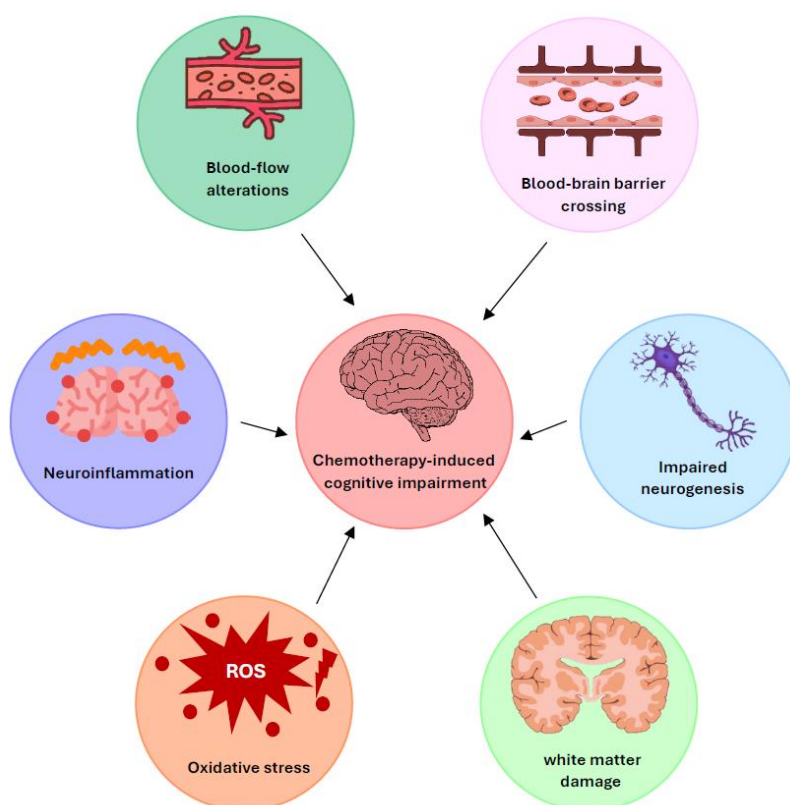
in animal models. This leaves a critical gap in understanding how the combined treatment may produce interactive or synergistic effects on cognition and behaviour that cannot be captured with single-agent or simplified combination studies alone.

Pathophysiological Pathways of CICI

The underlying biological mechanisms of CICI are not yet fully understood. Current evidence indicates that it arises from the interplay of multiple mechanisms that impact the central nervous system (CNS), both directly and indirectly. Chemotherapy may lead to both functional and structural changes in the brain, which could underlie the observed cognitive and behavioural difficulties. Animal and neuroimaging research has identified several potential pathophysiological pathways (Figure 1; Ahles & Saykin, 2007; Argyriou et al., 2011; Mounier et al., 2020).

Figure 1

Pathophysiological Pathways of CICI



Note. The figure illustrates the proposed pathophysiological pathways underlying CICI. Cancer-related factors may also contribute to cognitive impairments but are not depicted here, as it is not specific to chemotherapy.

Initially, it was assumed that most chemotherapeutic agents could not cross the blood-brain barrier (BBB) in sufficient concentrations to affect the brain. However, this view was revised after several conventional agents were detected in cerebrospinal fluid (CSF) and brain tissue at higher-than-expected concentrations. Within the CNS, these agents can exert direct neurotoxic effects by damaging key components of the cerebral parenchyma like neuronal axons, oligodendrocytes and microglia, leading to myelin loss and imbalances in neurotransmitter levels (Mounier et al., 2020). Dietrich et al. (2006) found that three common chemotherapeutic agents (carmustine, cisplatin, and cytosine arabinoside) had toxic effects on oligodendrocytes and CNS progenitor cells. When administered systemically in mice, these drugs caused heightened cell death and reduced cell division in the corpus callosum, the dentate gyrus of the hippocampus and the subventricular zone. Notably, these effects persisted for weeks after chemotherapy injections had ended. Taken together, this evidence suggests that the ability of chemotherapeutic agents to cross the BBB and directly damage neural cells represent a key mechanism underlying the neurotoxic effects associated with CICI (Mounier et al., 2020; Seigers & Fardell, 2011).

Several mainstream chemotherapy agents have been associated with increased cell death and reduced neurogenesis and cell proliferation in the hippocampus (Dietrich et al., 2006; Han et al., 2008; Mignone & Weber, 2006; Mondie et al., 2010; Mustafa et al., 2008; Seigers et al., 2009; Yang et al., 2010). Given the critical role of the hippocampus in learning and memory, chemotherapy-induced disruptions in hippocampal neurogenesis represent another potential pathophysiological mechanism for CICI (Mounier et al., 2020; Seigers & Fardell, 2011). Consistently, several studies have reported reduced performance on hippocampus dependent tasks and impaired neurogenesis following administration of chemotherapeutic agents (Mustafa et al., 2008; Nokia et al., 2012; Winocur et al., 2016; Winocur et al., 2018). However, some studies have suggested that alterations in hippocampal neurogenesis exert only limited or negligible impact on memory- and learning-related cognitive performance (Rola et al., 2004; Shors et al., 2002; Wojtowicz et al., 2008). Therefore, the discussion remains open, and other underlying biological mechanisms are now considered more likely explanations for CICI, consistent with evidence showing that cognitive impairments often involve functions not primarily mediated by the hippocampus (Ahles & Saykin, 2007; Mounier et al., 2020; Seigers & Fardell, 2011).

Another possible pathophysiological pathway of CICI is damage to white matter (Mounier et al., 2020; Seigers & Fardell, 2011). Multiple studies using diffusion tensor

imaging (DTI) showed that chemotherapy exposure could lead to a decrease in white matter integrity, and that this decrease in integrity was related to poorer cognitive functioning (Abraham et al., 2008; Deprez et al., 2011). De Ruiter and colleagues (2012) found that breast-cancer survivors who had received adjuvant high-dose chemotherapy had more white matter integrity loss nine years after treatment compared to non-chemotherapy receiving controls, likely due to axonal degeneration and demyelination. These patients also scored worse on neuropsychological tests, indicating the presence of CICI. Furthermore, animal studies confirm the toxic effects of chemotherapeutic agents on white matter. Mice receiving systemic 5-fluorouracil treatment showed intense myelin pathology and changes in the expression of Olig2, a transcriptional regulator which plays a central role in driving oligodendrocyte development and function (Han et al., 2008). Similarly, administration of methotrexate to rats resulted in white matter necrosis and degeneration, as well as a thinning within the lateral corpus callosum (Gregorios et al., 1989; Seigers et al., 2009).

Oxidative stress represents another potential pathophysiological pathway underlying CICI (Mounier et al., 2020; Seigers & Fardell, 2011). Oxidative stress occurs when antioxidant defence mechanisms fail to counterbalance the excessive generation of reactive oxygen species (ROS), resulting in cellular damage (Mounier et al., 2020; Salim, 2017). The brain is especially vulnerable to oxidative damage because of its high energy consumption, lipid-rich composition, and relatively limited antioxidant defence mechanisms (Hulbert et al., 2007; Salim, 2017). In the context of CICI, oxidative stress may contribute to cognitive dysfunction through several pathways, including DNA and RNA damage, lipid peroxidation, mitochondrial dysfunction, and altered antioxidant enzyme activity (Cauli, 2021; Salim, 2017). Several mainstream chemotherapeutic agents have been shown to increase oxidative stress and decrease antioxidant defence mechanisms, which in turn lead to lipid peroxidation, DNA and RNA damage, and cognitive impairments (Cauli, 2021; El-Agamy et al., 2018; Geller et al., 2001; Helal et al., 2009; Husain et al., 2001; Oboh & Ogunraku, 2010; Poprac et al., 2017; Uzar et al., 2006). Consistent with this, in rats treated with a combination of DOX and CYC, administration of N-acetylcysteine (NAC), an antioxidant, protected against these chemotherapy-induced cognitive deficits, suggesting that oxidative stress may mediate these effects (Konat et al., 2008).

Neuroinflammation is another possible pathophysiological pathway of CICI (Mounier et al., 2020; Seigers & Fardell, 2011). Following chemotherapy administration, the immune system releases proinflammatory mediators (Mounier et al., 2020). Yearly et al. (2023)

illustrated that the administration of cisplatin or MTX in rats led to a significant increase in proinflammatory cytokines, including interleukin 6 (IL-6), interleukin 12 (IL-12), and interleukin 1 α (IL-1 α). Similarly, El-Agamy et al. (2018) found that DOX administration in rats significantly increased levels of several pro-inflammatory mediators, namely prostaglandin E2 (PGE2), cyclooxygenase-2 (COX-2), and tumour necrosis factor alpha (TNF- α). The release of these mediators can induce systemic inflammation, which in turn may promote cytokine release and inflammation in the CNS, thereby contributing to cognitive impairments. This highlights an indirect pathway through which chemotherapy may give rise to CICI (Mounier et al., 2020; Seruga et al., 2008; Wilson et al., 2002). In addition, pro-inflammatory cytokines can cross and disrupt the BBB (Watkins et al., 1995; Wardill et al., 2016). Once in the brain, these mediators can trigger neuroinflammation and neurodegeneration by activating microglia and astrocytes, which subsequently release additional pro-inflammatory mediators (Kempuraj et al., 2016). Notably, microglial activation also contributes to impaired neurogenesis (Ekdahl et al., 2003). Together, these findings suggest that chemotherapy can induce cognitive impairments through both indirect and direct neuroinflammatory processes.

Chemotherapeutic agents like MTX reduce cerebral blood flow and glucose metabolism, likely due to their anti-angiogenic effects and vascular toxicity (de Vos et al., 2004; Mizusawa et al., 1988; Seigers et al., 2010b). This reduced cerebral blood flow in turn can lead to cognitive difficulties (Seigers et al., 2010b). Consistent with this, Silverman et al. (2007) found that breast cancer survivors who had received adjuvant chemotherapy five to ten years earlier showed altered cerebral blood flow compared to those who had not. Additionally, angiogenesis and neurogenesis seem to be closely associated (Palmer et al., 2000). This gave rise to the hypothesis that blood vessel density reduction caused by cytostatic agents may lead to less cell proliferation in the hippocampus, likely due to diminished energy availability and fewer proliferative signals (Mounier et al., 2020; Seigers & Fardell, 2011). Several studies confirm this hypothesis (Dietrich et al., 2006; Han et al., 2008; Mignone & Weber, 2006; Mustafa et al., 2008; Seigers et al., 2009). Blood-flow alteration thus serves as another potential pathophysiological pathway of CICI (Mounier et al., 2020; Seigers & Fardell, 2011).

Lastly, it is important to mention that cancer itself can induce cognitive impairment, even before the administration of treatment (Mounier et al., 2020; Seigers & Fardell, 2011). Hermelink and colleagues (2007) noted that some breast cancer patients had cognitive

impairment prior to the start of chemotherapy treatment. In a longitudinal study of breast cancer patients, Wefel et al. (2004) also found that a subgroup of women showed cognitive disturbances before the administration of systemic therapy. Furthermore, the presence of a tumour in rats suppressed cell proliferation in the hippocampus, highlighting a potential mechanism through which cancer may induce cognitive impairment (Seigers et al., 2010a; Seigers & Fardell, 2011). Another hypothesis is that cognitive dysfunction/neurodegenerative disorders and cancer may share some risk factors, like impaired DNA repair pathways, which could also contribute to pre-treatment cognitive decline (Ahles & Saykin, 2007; Padgett, 2020). In conclusion, chemotherapy can induce cognitive impairment through multiple pathophysiological pathways. CICI likely arises from an interplay of these mechanisms, alongside the effects of the cancer itself.

Potential Therapeutic Interventions for CICI

Given the multiple pathophysiological pathways implicated in CICI, a wide range of interventions have been explored. These approaches primarily aim to restore neural integrity, reduce neuroinflammation and oxidative stress, and enhance neuroplasticity. Broadly, current intervention strategies can be divided into non-pharmacological interventions and pharmacological interventions. Non-pharmacological interventions focus on behavioural, cognitive and lifestyle based strategies to promote functional recovery, whereas pharmacological interventions target specific molecular and cellular mechanisms underlying CICI.

Non-Pharmacological Interventions

Physical exercise has emerged as a promising intervention strategy for CICI, with evidence from both clinical and preclinical studies demonstrating its beneficial effects. Koevoets and Colleagues (2022) investigated the effects of a six-month exercise program combining aerobic and strength training with Nordic/power walking in breast cancer survivors 2-4 years post-chemotherapy. The intervention led to significant improvements in self-reported cognitive functioning, despite no effect on tested cognitive functioning. Brunet et al. (2025) found similar results. They compared a waitlist control group with a group receiving aerobic exercise during their chemotherapy regimen (12-24 weeks). Although the intervention did not result in measurable improvements in objective cognitive performance, participants reported notable enhancements in self-reported cognitive functioning. Animal studies further support these findings. A systematic review indicated that running wheel access improved

cognitive performance, neurotrophic factors and neurogenesis in the hippocampus of rodents after chemotherapy (Zimmer et al., 2016). Physical activity may alleviate cognitive impairments through multiple neurobiological pathways. It has been shown to boost neurogenesis, reduce neuroinflammation, stimulate synaptic plasticity, and prevent neural dysfunction (Liu et al., 2019). Exercise is also associated with changes in intracellular signalling, increased white matter integrity, reduced oxidative stress, altered cerebral blood flow through vascular endothelial growth factor (VEGF), and heightened levels of neurotrophic factors (Fabel et al., 2003; Liu et al., 2019; Marks et al., 2007; Radák et al., 2001; Seigers & Fardell, 2011). In conclusion, physical activity may simultaneously target several pathophysiological pathways underlying CICI.

Cognitive rehabilitation programs represent another promising non-pharmacological approach for mitigating CICI. The systematic review of Fernandes et al. (2019) indicated that all 19 studies included in their report showed amelioration in at least one cognitive outcome, either self-report or performance based. Memory showed the most frequent improvements, with executive functioning and processing speed also commonly enhanced. Importantly, the authors highlighted two different types of cognitive rehabilitation. The first one is computer-based training (CT), also referred to as cognitive (re)training or brain training, and includes computer exercises for cognitive skill rehabilitation. The second one is called strategy training (ST), cognitive behavioural training, or cognitive behavioural therapy (CBT). Next to cognitive skill rehabilitation it also aims to instil compensatory strategies and may involve elements such as psychoeducation, stress reduction, and peer-based support. Although it has the same name and may include elements also seen in a type of psychotherapy called cognitive behavioural therapy (CBT), it is nonetheless separate, as it is a form of cognitive rehabilitation (Fernandes et al., 2019). In addition to cognitive rehabilitation approaches, some studies have also reported positive effects on cognitive deficits through mindfulness-based interventions in breast cancer survivors (Cifu et al., 2018).

Another promising non-pharmacological intervention for CICI is environmental enrichment. In a study by Winocur and colleagues (2016), rats were housed for three months in either a standard or enriched environment and subsequently tested on multiple cognitive tasks. The enriched environment, which is designed to promote learning, social interaction, and sensory stimulation, resulted in significantly better performance on learning and memory tests compared to chemotherapy-treated rats housed under standard conditions. The rats in enriched cages scored almost as good as the saline group. The authors also found that

environmental enrichment boosted neuronal differentiation and growth during maturation, and thus neurogenesis in the hippocampus.

Pharmacological Interventions

CICI has many potential underlying pathways. Therefore, different pharmacological agents targeting different mechanisms may have the potential to mitigate CICI. Many pharmacological agents have been tested in preclinical rodent models, with those targeting oxidative stress and/or neuroinflammation appearing the most successful.

As oxidative stress arises from insufficient antioxidants defence mechanisms, administering agents with antioxidant properties represents a promising pharmacological strategy (Cauli, 2021; Mounier et al., 2020). This therapeutic potential was highlighted in a review by Cauli (2021), which showed that both CICI and oxidative stress were reduced after administration of NAC, gamma-glutamyl cysteine ethyl ester, polydatin, caffeic acid phenethyl ester, and 2-mercaptoethane sulfonate sodium (MESNA). Similarly, Raghu et al. (2021) reported that several natural compounds, including astaxanthin, catechin, C-phycocyanin, and dehydrozingerone, alleviated CICI through downregulation of oxidative stress.

Given the involvement of neuroinflammation in CICI, anti-inflammatory compounds represent another promising pharmacological avenue for intervention (Haller et al., 2023). In their systematic review, Haller et al. (2023) identified NAC, lithium, astaxanthin, memantine, melatonin, donepezil, and curcumin as particularly promising candidate treatments for CICI. Likewise, Raghu et al. (2021) highlighted several natural compounds, such as omega-3 fatty acids, resveratrol, berberine, and C-phycocyanin, as effective interventions because of their anti-inflammatory properties. Notably, some of these agents, including astaxanthin, NAC, and C-phycocyanin, appear to reduce both oxidative stress and neuroinflammation, highlighting the potential value of targeting multiple pathophysiological pathways simultaneously.

Despite these promising preclinical findings, no pharmacological intervention has yet been approved specifically for the treatment of CICI. Given the multifactorial nature of CICI, it is unlikely that a single, narrowly targeted agent will be sufficient to fully prevent or reverse its effects. Pharmacological interventions that influence multiple underlying mechanisms may therefore be particularly promising. One such approach involves targeting the gut-brain axis through short-chain fatty acids (SCFAs). This strategy holds considerable potential, as it can be implemented through relatively simple dietary interventions or supplementation. To fully

appreciate its therapeutic promise, it is essential to first understand the underlying mechanisms of SCFAs and consider evidence from related preclinical studies demonstrating their beneficial effects.

Targeting the Gut-Brain Axis to Ameliorate CICI.

The gastrointestinal (GI) tract hosts a diverse community of bacteria, viruses, archaea, and other microorganisms. This community is collectively referred to as the gastrointestinal microbiota and plays a crucial role in regulating immune function and metabolic processes (National Institutes of Health, 2018; Subramaniam et al., 2020). The gastrointestinal microbiota interacts bidirectionally with the central nervous system via a network of neuroimmune, neuroendocrine, and neural signalling pathways. Via these pathways, the brain can modulate gastrointestinal functions, while signals from the gut, in turn, can influence central processes including emotion, behaviour, and cognition. This dynamic interplay is known as the microbiota-gut-brain axis (Dalile, 2019; Subramaniam et al., 2020).

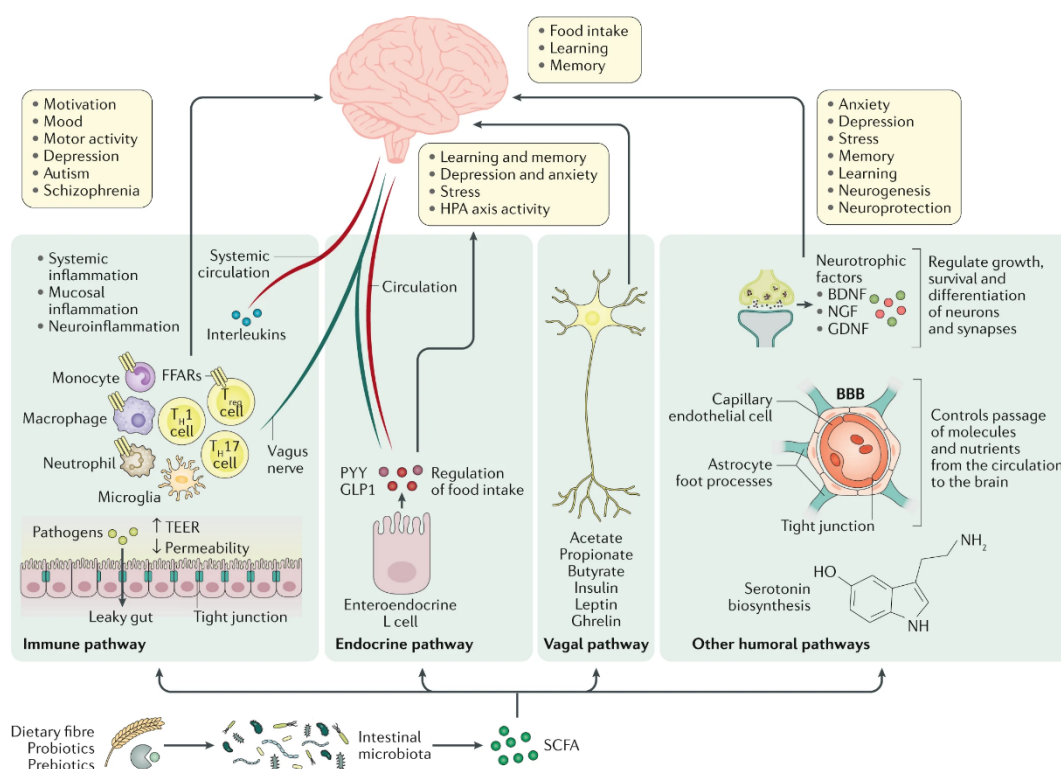
One key mediator of this communication is a group of microbial metabolites known as short-chain fatty acids (SCFAs), which are produced through the fermentation of dietary fibre by bacteria in the GI tract. The three most common SCFAs in humans are acetate, propionate, and butyrate (Dalile, 2019; Macfarlane & Macfarlane, 2003). While SCFAs have local effects within the gut, they are also thought to modulate the interaction between the gut microbiome and the brain through various pathways, either direct or indirect (Dalile, 2019). Firstly, SCFAs can influence brain functioning through the immune pathway. They can alter the barrier and mucosal immunity of the GI by influencing immune and epithelial cells through the inhibition of histone deacetylase (HDAC), which facilitates gene transcription, or by binding to free fatty acid receptors (FFARs). They also affect systemic inflammation by adjusting interleukin levels and contribute to neuroinflammation through microglial changes. Secondly, SCFAs may alter mood and cognition through the endocrine pathway as they elevate the concentration of gut hormones. Thirdly, they may act via the vagal nerve: when SCFAs bind to FFARs on vagal afferents, signals are transmitted to the brain. Lastly, a couple of other humoral pathways have been proposed. SCFAs can pass through the BBB, support its structural integrity, influence neurotransmission, and enhance serotonin and neurotrophic factor levels (Dalile, 2019). All these pathways are illustrated in Figure 2.

In addition to direct and indirect neurotoxic effects, chemotherapy agents are also known to disrupt gastrointestinal functioning and integrity (Jordan et al., 2018; Subramaniam

et al., 2020). Chemotherapy reduces the microbiome diversity and causes gastrointestinal toxicity, including constipation, diarrhoea, and gastrointestinal pain and bleeding (Subramaniam et al., 2020). Given the bidirectional interaction between the gut and the brain, it is likely that detrimental changes in the GI contribute to cognitive impairments. This hypothesis is supported by the frequent co-occurrence of CICI and gastrointestinal toxicity in patients (Jordan et al., 2018; Subramaniam et al., 2020). Treatments aimed at boosting the levels of SCFAs, either through changing diet or administration of probiotics, prebiotics, or SCFAs themselves, hold promise for mitigating both gastrointestinal and cognitive impairments, since they have been shown to be a key mediator of the gut-brain interaction.

Figure 2

Modulatory Pathways of SCFAs



Note. The figure summarizes immune, endocrine, vagal, and humoral pathways through which SCFAs may influence the microbiota-gut-brain interactions. From “The role of short-chain fatty acids in microbiota-gut-brain communication”, by B. Dalile, L. Van Oudenhove, B. Vervliet, and K. Verbeke, 2019, *Nature Reviews Gastroenterology & Hepatology*, 16, p. 468 (<https://doi.org/10.1038/s41575-019-0157-3>). Copyright 2019 by Nature Reviews Gastroenterology & Hepatology.

Several studies indicate that increasing the levels of SCFAs can help alleviate the burden of neurodegenerative disorders. For example, Zhou et al. (2023) found that acetate and butyrate supplemented diets postponed the onset of cognitive impairments in a mouse model of Alzheimer's disease. In the same study, fibre deprivation compromised hippocampal neurogenesis and hastened cognitive decline. Similarly, Fernando et al. (2020) showed that Alzheimer mice receiving a diet enriched with sodium butyrate (NaB) displayed superior memory abilities and reduced amyloid beta ($A\beta$) levels. Positive effects have also been seen in mice models for Parkinson's disease, with Zhang and colleagues (2023) showing that NaB improved motor and gut impairments. Likewise, Ferrante et al. (2003) tested a mice model for Huntington's disease. Their results indicated that injections of NaB resulted in longer survival, better motor functioning, and neuroprotection. Notably, Cristiano et al. (2022) extended this line of research to a chemotherapy-related model by examining the effects of oral NaB administration on paclitaxel (PTX)-induced side effects in mice. They found that butyrate alleviated depressive- and anxiety-like behavioural patterns, while also protecting microbiome diversity, gut integrity, and food consumption.

As highlighted by these studies, butyrate holds promise as a potential therapeutic agent for neurodegenerative disorders and chemotherapy-induced side effects. It fuels colonocyte metabolism, reinforces both gastrointestinal and blood-brain barriers, aids in intestinal movement, exerts anti-inflammatory properties, and promotes autophagy (Chakraborty et al., 2024). Together these properties of butyrate suggest that it could also be beneficial in mitigating CICI. However, to date, no studies have directly tested whether butyrate administration can alleviate cognitive deficits following the Hyper-CVAD chemotherapy regimen.

The Current Research Project

In this research thesis, we administered the Hyper-CVAD chemotherapy regimen to mice to investigate chemotherapy-induced cognitive, behavioural, and sensory alterations. Because this regimen combines multiple chemotherapeutic agents, it more closely reflects clinical chemotherapy exposure than models based on a single agent and may therefore provide a more representative picture of chemotherapy-related complaints. In addition, a butyrate intervention was administered to examine its potential to mitigate chemotherapy-related cognitive, behavioural, and sensory alterations. The mice underwent a behavioural test battery assessing overall locomotor activity, exploratory and anxiety-like behaviour, learning and memory, and thermal nociceptive sensitivity. Together, these tests aimed to capture a

broad neuropsychological profile of chemotherapy-induced alterations extending beyond cognition alone. The main research questions of this thesis were: To what extent does the Hyper-CVAD regimen induce cognitive, behavioural, and sensory alterations in adult female mice, and to what extent can concurrent butyrate administration mitigate these chemotherapy-induced alterations? We hypothesized that chemotherapy exposure would worsen performance across the behavioural test battery. Furthermore, we hypothesised that butyrate administration would attenuate these chemotherapy-induced alterations. More specifically, chemotherapy-treated mice receiving butyrate were expected to exhibit fewer impairments across the test battery than chemotherapy-treated mice receiving vehicle.

Methods

Animals

This study used 72 female C57BL/6J mice of 8 to 10 weeks old. These mice were housed per four in open-top cages, each enriched with shredded or toilet paper for nesting, and a wooden block for chewing. The mice had unlimited access to water and food. The environmental conditions were kept constant, with a 12-hour light/dark cycle (lights on between 7 a.m. and 7 p.m.), a humidity of 50-70%, and a temperature of $22 \pm 2^\circ\text{C}$. For logistical reasons related to the intervention and treatment period and the subsequent behavioural testing, the mice were split into 2 batches, the first one containing 32 animals and the second one 40. Every batch was split into four groups. Group abbreviations refer first to treatment condition (SAL or CHE) and second to intervention condition (VEH or BUT). The first group was the control group (SAL-VEH), in which the animals received water as intervention and got saline injections as treatment. The second group got butyrate as intervention and saline as treatment and represented the butyrate control group (SAL-BUT). The third group received water as intervention and the Hyper-CVAD polychemotherapy as treatment and functioned as the control chemotherapy group (CHE-VEH). Lastly, the final group (CHE-BUT) received butyrate as intervention and Hyper-CVAD as treatment. 48 animals made it to the behavioural testing, with 12 mice from each group. 22 animals were selected for immunohistochemistry testing directly after intervention and treatment ended and did not undergo behavioural testing. Two animals died during the intervention and treatment period, one from the SAL-BUT group, and the other from CHE-VEH group.

Timeline

After arrival in the laboratory, the mice got a seven-day acclimatisation period. This was followed by three weeks of intervention administration and then four weeks of concurrent intervention and treatment administration. Directly after came the behavioural testing. When behavioural testing was completed, the mice were humanely euthanised and their brains preserved for further immunohistochemistry testing, performed by my daily supervisor in a separate experiment. The KU Leuven Animal Ethics Committee approved all experiments, and the guidelines of the European Directive 2010/63/EU and Belgian Royal Decree of 29 May 2013 were closely adhered.

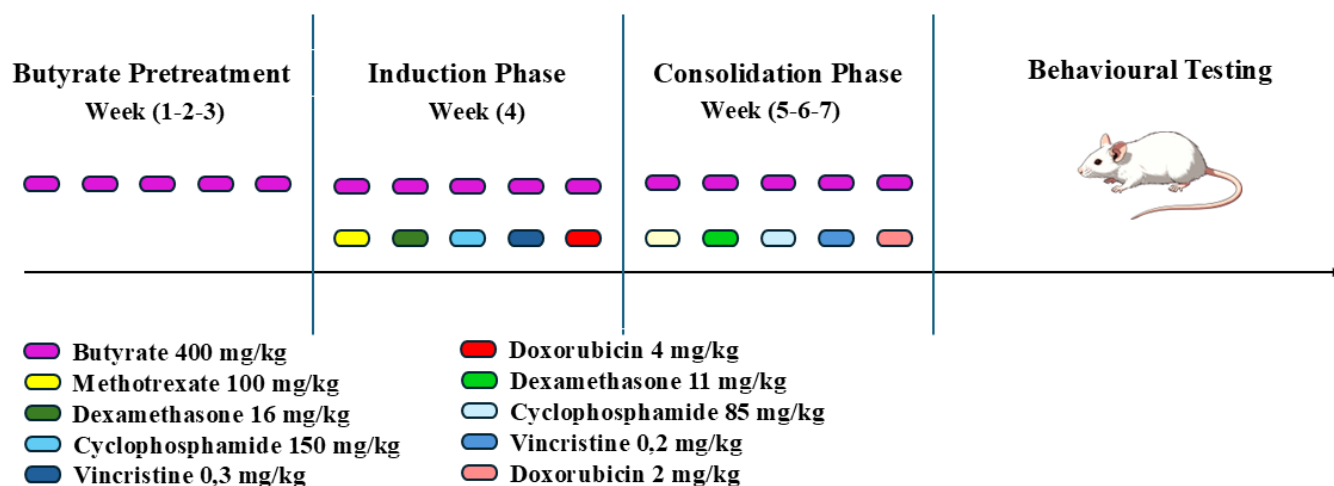
Intervention and Treatment Protocol

The intervention started three weeks before chemotherapy or saline treatment and continued throughout the four-week treatment phase. Mice in the butyrate groups received sodium butyrate (powder form) dissolved in water by oral gavage five days a week, from Monday to Friday. The solution was administered at 1% of body weight, corresponding to a dose of 400 mg/kg. The control groups received water through the same procedure.

Chemotherapy or saline treatment started after the three-week pre-treatment intervention phase. During this four-week combined phase, the animals received their intervention in the morning and their treatment in the afternoon, approximately four hours later. Mice in the chemotherapy groups received a Hyper-CVAD regimen, which in this study consisted of methotrexate on Mondays, dexamethasone on Tuesdays, cyclophosphamide on Wednesdays, vincristine on Thursdays, and doxorubicin on Fridays. The four-week treatment period included two phases. The first week was the induction phase in which higher doses of the chemotherapeutic agents were administered, followed by a consolidation phase of three weeks with lower doses of agents (See Figure 3 for a timeline and injection schedule). This schedule was used to closely mimic human protocols. The chemotherapeutic agents were diluted in saline, while mice in the non-chemotherapy groups received saline only. Both chemotherapy and saline were injected intraperitoneally (i.p.) at 1% of the mice's body weight. We weighed the animals daily to ensure we gave them the correct doses of intervention and treatment, and to closely check their weight progression. If an animal lost a considerable amount of weight, we added white chocolate or peanut butter to their cage. We closely followed the humane endpoint criteria established in our laboratory.

Figure 3

Timeline and Injection Schedule

**Behavioural Test Battery**

Once the intervention and treatment period had ended, the mice underwent behavioural testing to investigate whether there were any cognitive, behavioural, or sensory differences between the groups. The test battery included the 24-Hour Activity test, Open Field test, Elevated Plus Maze, T-Maze, Passive Avoidance test, and Tail Flick test, which were administered in this order.

24-Hour Activity

The 24-Hour Activity test assessed overall activity (D'Hooge et al., 2005; Naert et al., 2011). Mice were individually housed in specially equipped cages containing three infrared sensors. The system automatically captured horizontal and vertical beam crossings, with the number of crossings reflecting spontaneous locomotor activity. Environmental conditions were kept consistent with the home cages, including a 12-hour light/dark cycle, constant temperature, humidity, and noise levels, as well as unlimited access to food and water.

Open Field

In the Open Field (OF) test, each mouse was placed in the centre of a brightly illuminated large Plexiglass arena (50 cm x 50 cm), while its behaviour was recorded for 10 minutes. The OF assessed locomotor activity, exploration, and anxiety-like behaviour, leveraging the natural tendency of mice to remain close to the walls (thigmotaxis) when

introduced to a novel open space. Over time, a mouse will start to explore the middle as well, but more anxious mice will have less exploration (Callaerts-Vegh et al., 2015; Cartner & Shieh, 2015). A camera positioned above the arena recorded the movements of the mouse, which were subsequently analysed with the Any-maze™ Tracking System (Stoelting Co., IL, USA). Outcome measures included total path length, time spent in the centre vs. time spent in the periphery, number of centre entries vs. number of periphery entries, and latency to first centre entry. Before the test started, the mice got a 30-minute dark adaptation period. After each trial, the arena was cleaned with ethanol to remove olfactory cues.

Elevated Plus Maze

The Elevated Plus Maze (EPM) consisted of four arms extending from a centre in a cross-shaped configuration, set at a height of 30 cm above the surface. Two arms were open and two were enclosed by walls. Mice were placed in the centre of the maze facing a closed arm and were allowed to freely explore the apparatus for 10 minutes. A camera above the set-up recorded the behaviour of the mice. Any-maze™ software (Stoelting Co., IL, USA) was used to extract the time spent in the open and closed arms. From these values, relative open-arm time was calculated as the time spent in the open arms divided by the total time spent in both the open and closed arms, multiplied by 100. EPM assessed anxiety-like behaviour, as it relied on the natural tendency of mice to explore new environments, while avoiding open and elevated areas (Komada et al., 2008). After each trial, the apparatus got disinfected with ethanol to remove olfactory cues.

T-Maze

The T-Maze evaluated spatial working memory (d'Isa et al., 2021). The apparatus consisted of three enclosed arms arranged in a T-shape, 30 cm above the surface. A mouse was placed at the end of the starting arm (vertical arm) and was allowed to explore the maze for 10 minutes. When a mouse entered one of the goal arms (horizontal arms), the opposite arm was temporarily closed using a sliding door until the mouse re-entered the starting arm. This procedure corresponds to the spontaneous alternation version of the T-maze test. A spontaneous alternation was defined as entering the goal arm opposite to the one visited during the previous choice. Re-entering the same goal arm was considered a non-alternation. Selecting the alternate arm reflects intact spatial working memory, as the mouse retains information about the previously visited arm. This behaviour is considered adaptive, as exploratory tendencies increase the likelihood of locating food, shelter, and potential mates

(Deacon & Rawlins, 2006). Behaviour was recorded using a camera positioned above the apparatus, and the number of spontaneous alternations and non-alternations was extracted using EthoVision XT15 tracking software (EthoVision XT15; Noldus, Wageningen, The Netherlands). Performance was calculated as the proportion of spontaneous alternations relative to the maximum possible alternations. A minimum of two potential alternations was set as the inclusion criterion for the T-Maze analysis. After each trial, the maze was cleaned with ethanol.

Passive Avoidance

The Passive Avoidance (PA) test used a two-chamber apparatus separated by a sliding door. One chamber was brightly illuminated, whereas the other was completely dark and equipped with a grid floor through which electrical shocks could be delivered. The PA test consisted of two phases: a training phase and a test phase. During the training phase, a mouse was placed in the illuminated chamber. After 10 seconds, the sliding door opened, allowing the mouse to enter the dark chamber. Since mice naturally prefer dark environments (Ögren & Stiedl, 2015), they typically entered the dark compartment shortly thereafter. Upon entry, the sliding door closed and a mild electrical foot shock was delivered for two seconds at 0.5 mA. The aversive nature of the stimulus was confirmed by vocalisation responses. 24 hours later, the test phase was conducted, during which mice were again placed in the illuminated chamber and the latency to enter the dark compartment was recorded. This is a test evaluating associative learning and memory, as increased latency to enter the dark chamber during the test phase compared to the training phase indicates successful learning and retention of the dark room-shock association. To quantify this change, a change score was calculated by subtracting the training-phase latency from the test-phase latency.

Tail Flick

The Tail Flick test assessed thermal nociceptive sensitivity (Deuis et al., 2017). During testing, one experimenter immersed the tail of a mouse in a beaker containing heated water, while a second experimenter measured the latency to tail withdrawal (flick tail out of the water) using a stopwatch. This latency is viewed as a measure of thermal nociceptive sensitivity. We maintained the water temperature at three set points, namely 46, 48, and 50°C. The Tail Flick test was performed over three consecutive days, and the order of temperatures within each day was counterbalanced. For each mouse, latency measurements at each temperature were averaged across the three consecutive testing days.

Statistical Analyses

All statistical analyses were conducted in R (version 4.5.3). Two-way analyses of variance (ANOVAs) were used to analyse outcomes with one value per animal, with intervention and treatment as between-subject factors. These outcomes included the total number of beam crossings in the 24-Hour Activity test. They also included all Open Field parameters: total path length, time spent in the centre, number of centre entries, latency to first centre entry, time spent in the periphery, and number of periphery entries. Other outcomes analysed with two-way ANOVAs were relative time spent in the open arms in the Elevated Plus Maze, the proportion of spontaneous alternations in the T-Maze, and the change score in the Passive Avoidance test.

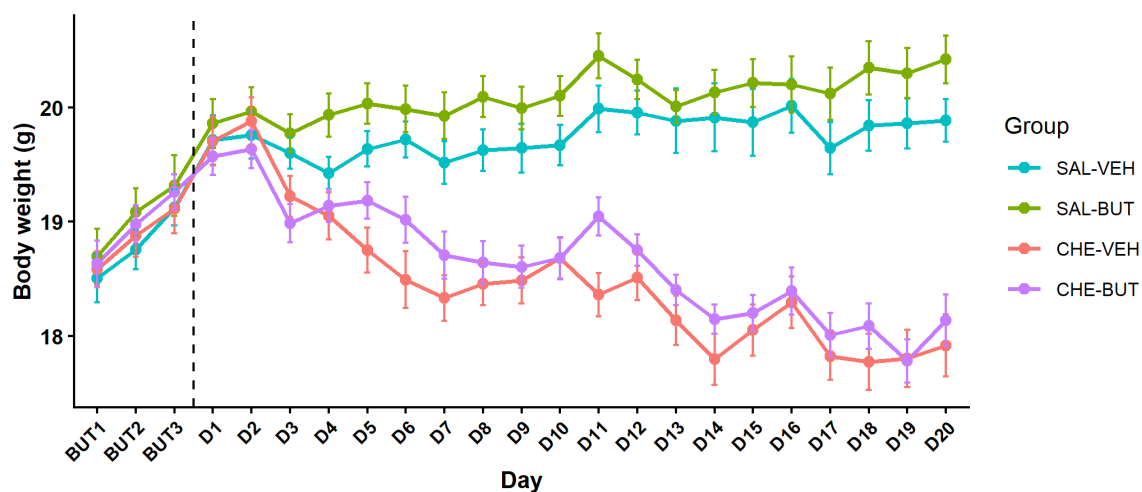
Mixed-design ANOVAs were used for outcomes with repeated measurements, again with intervention and treatment as between-subject factors. These analyses included body weight, with day as the within-subject factor. They also included the number of hourly beam crossings in the 24-Hour Activity test, with hour since placement in the cage as the within-subject factor, latency to enter the dark compartment in the Passive Avoidance test, with phase as the within-subject factor, and tail flick latency in the Tail Flick test, with temperature as the within-subject factor.

For each behavioural outcome, three a priori planned contrasts were performed using estimated marginal means: (1) CHE-VEH vs. SAL-VEH to assess the effect of chemotherapy relative to controls, (2) CHE-BUT vs. CHE-VEH to assess the effect of butyrate intervention within chemotherapy-treated animals, and (3) CHE-BUT vs. SAL-VEH to assess whether performance in chemotherapy-treated mice receiving butyrate differed from saline-treated controls. P-values were adjusted for multiple comparisons using the Bonferroni method. A significance threshold of $p < .05$ was used across all analyses. In all graphs, data are presented as group means $\pm$ standard error of the mean (SEM).

Results

Body Weight Changes

To examine the progression of body weight during the administration period, a mixed-design ANOVA was conducted (Figure 4). For the first three weeks, during which only butyrate was administered, body weight was averaged per week. Once treatment injections started, body weight was analysed per day. The ANOVA revealed a significant main effect of treatment ($F(1, 66) = 50.57, p < .001, \eta^2_p = .43$), a significant main effect of timepoint ($F(5.86, 386.85) = 22.34, p < .001, \eta^2_p = .25$), and a significant treatment x timepoint interaction ($F(5.86, 386.85) = 44.87, p < .001, \eta^2_p = .40$). These effects indicate that chemotherapy-treated mice (CHE-VEH and CHE-BUT) had lower body weights than saline-treated mice (SAL-VEH and SAL-BUT) overall, that body weight changed over time, and that the pattern of weight change differed between treatment groups. More specifically, saline-treated mice (SAL-VEH and SAL-BUT) generally maintained or gained weight over time, whereas chemotherapy-treated mice (CHE-VEH and CHE-BUT) showed a decline after treatment onset. Bonferroni-corrected planned contrasts indicated that the CHE-VEH group weighed significantly less than the SAL-VEH group from day five onward. The CHE-BUT group differed significantly from the SAL-VEH group from day seven onward, with an isolated earlier significant difference at day three. In contrast, the CHE-BUT vs. CHE-VEH contrast reached significance only at day 11. Together, these findings highlight the negative effect of chemotherapy on body weight. In contrast, there was no consistent evidence that butyrate protected against chemotherapy-related weight loss, as the CHE-BUT and CHE-VEH groups differed significantly only at day 11.

Figure 4*Average Body Weight Curve*

Note. Chemotherapy reduced body weight over time. Butyrate did not consistently alter chemotherapy-related weight loss, as the CHE-BUT and CHE-VEH groups differed significantly only on day 11. Data are presented as mean $\pm$ SEM.

24-Hour Activity

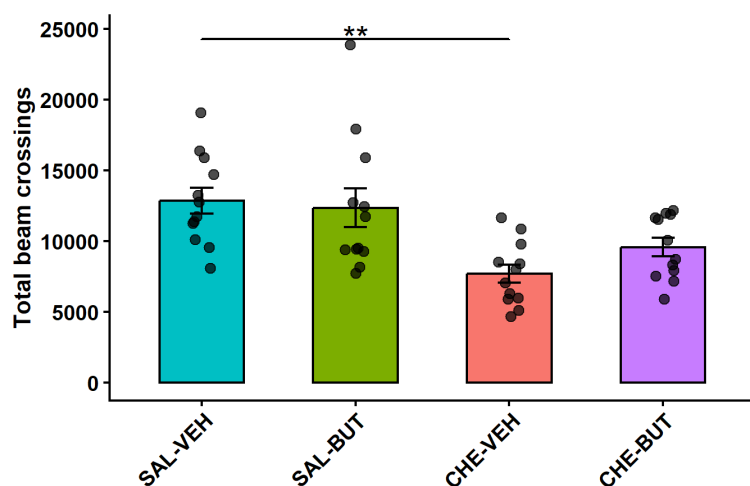
The 24-Hour Activity test assessed overall locomotor activity. First, a two-way ANOVA on total beam crossings revealed a significant main effect of treatment, $F(1, 44) = 17.60$, $p < .001$, $\eta^2_p = .29$ (Figure 5), indicating that chemotherapy reduced overall activity. Neither the main effect of intervention nor the treatment $\times$ intervention interaction was significant. Among the planned contrasts, only the CHE-VEH vs. SAL-VEH comparison survived Bonferroni correction ($p = .001$). The CHE-BUT group did not differ significantly from either the CHE-VEH group or the SAL-VEH group. Together, these findings indicate that chemotherapy reduced overall locomotor activity. The absence of a significant difference between CHE-BUT and SAL-VEH may suggest partial recovery in the butyrate-treated chemotherapy group. However, this interpretation warrants caution, as the CHE-BUT vs. CHE-VEH contrast was not significant.

Second, a mixed-design ANOVA on hourly beam crossings (Figure 6) revealed a significant main effect of treatment ($F(1, 44) = 16.94$, $p < .001$, $\eta^2_p = .28$), a significant main effect of hour ($F(7.69, 338.28) = 77.75$, $p < .001$, $\eta^2_p = .64$), a significant treatment $\times$ hour interaction effect ($F(7.69, 338.28) = 3.18$, $p = .002$, $\eta^2_p = .07$), and a significant intervention $\times$ hour interaction effect ($F(7.69, 338.28) = 2.12$, $p = .036$, $\eta^2_p = .05$). These findings indicate

that locomotor activity changed across the 24-hour recording period and that this temporal pattern differed as a function of treatment and intervention. This temporal variation is not unexpected, as mice are nocturnal animals and testing started between approximately 9 p.m. and 10 p.m. in the evening under a 12-hour light/dark cycle. However, because animals were placed in the cages at slightly different times across testing days, the hourly bins reflect time since placement rather than exact clock time. Planned contrasts showed that the CHE-VEH group was less active than the SAL-VEH group at hours 3, 4, 6, 9, 10, 12, 18, and 23. In addition, the CHE-BUT group was more active than the CHE-VEH group at hours 7, 9, and 12, while the CHE-BUT group showed lower activity than the SAL-VEH group at hours 2, 3, and 4. Together, these findings suggest that the CHE-VEH group showed reduced locomotor activity at several time points, whereas butyrate may have attenuated this reduction at specific hours. Although the CHE-BUT group often did not differ from SAL-VEH, suggesting possible partial recovery, this interpretation warrants caution because CHE-BUT did not consistently differ from CHE-VEH.

Figure 5

Results Total Beam Crossings 24-Hour Activity

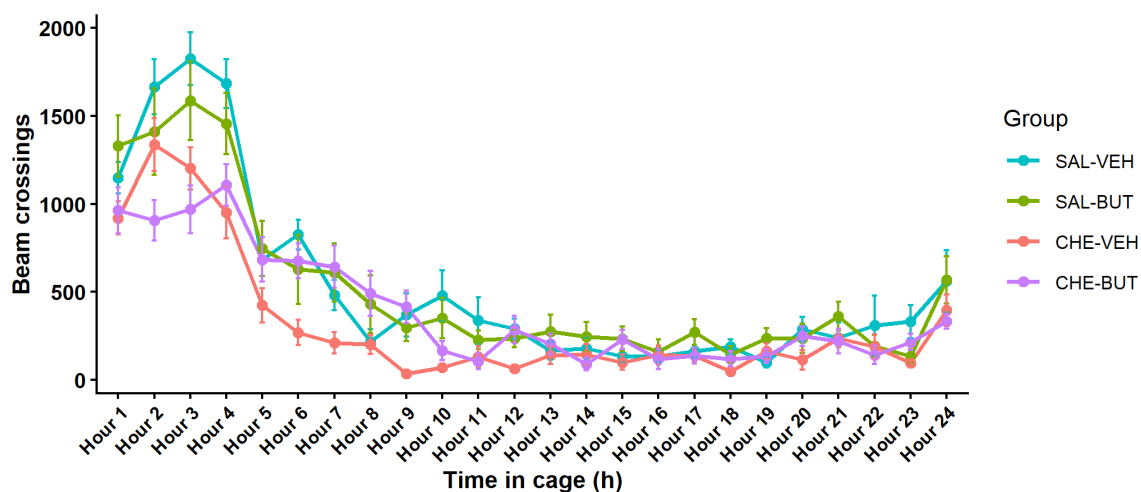


Note. Chemotherapy reduced overall locomotor activity in the 24-Hour Activity test, as the CHE-VEH group showed fewer total beam crossings than the SAL-VEH group. Although the CHE-BUT group did not differ significantly from the SAL-VEH group, it also did not differ significantly from the CHE-VEH group. Therefore, evidence for a butyrate-related attenuation of reduced locomotor activity remains inconclusive. Data are presented as mean $\pm$ SEM.

****** $p < .01$.

Figure 6

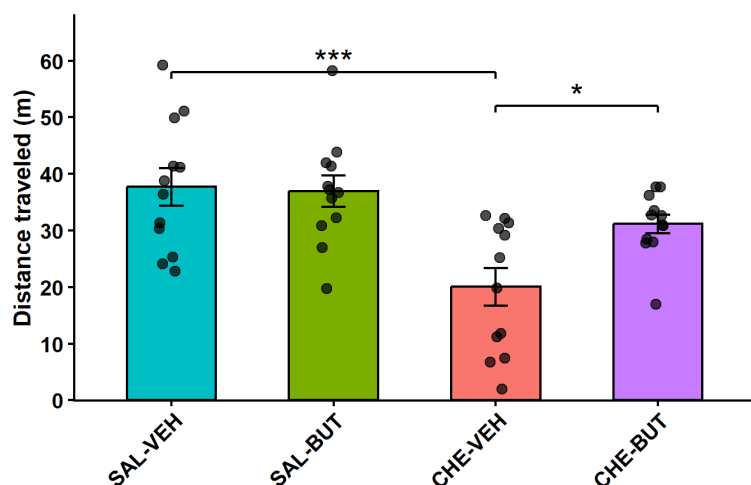
Results Total Beam Crossings per Hour in the 24-Hour Activity Test



Note. Chemotherapy reduced locomotor activity across several hours of the 24-hour recording period. Butyrate showed limited attenuation of this effect. Data are presented as mean $\pm$ SEM.

Open Field

The OF assessed both exploratory and anxiety-like behaviour. Exploratory behaviour was analysed with a two-way ANOVA on total distance travelled (Figure 7). This revealed a significant main effect of treatment ($F(1, 44) = 16.92, p < .001, \eta^2_p = .28$) and a significant treatment $\times$ intervention effect ($F(1, 44) = 4.36, p = .043, \eta^2_p = .09$). These results indicate that chemotherapy reduced exploratory behaviour, and that this effect depended on butyrate intervention. Planned contrasts showed that the CHE-VEH group travelled significantly less distance than the SAL-VEH group ($p < .001$), and that the CHE-BUT group travelled significantly more distance than the CHE-VEH group ($p = .025$). The contrast between CHE-BUT and SAL-VEH was not significant. These findings suggest that chemotherapy negatively affected exploratory behaviour, whereas butyrate attenuated this effect.

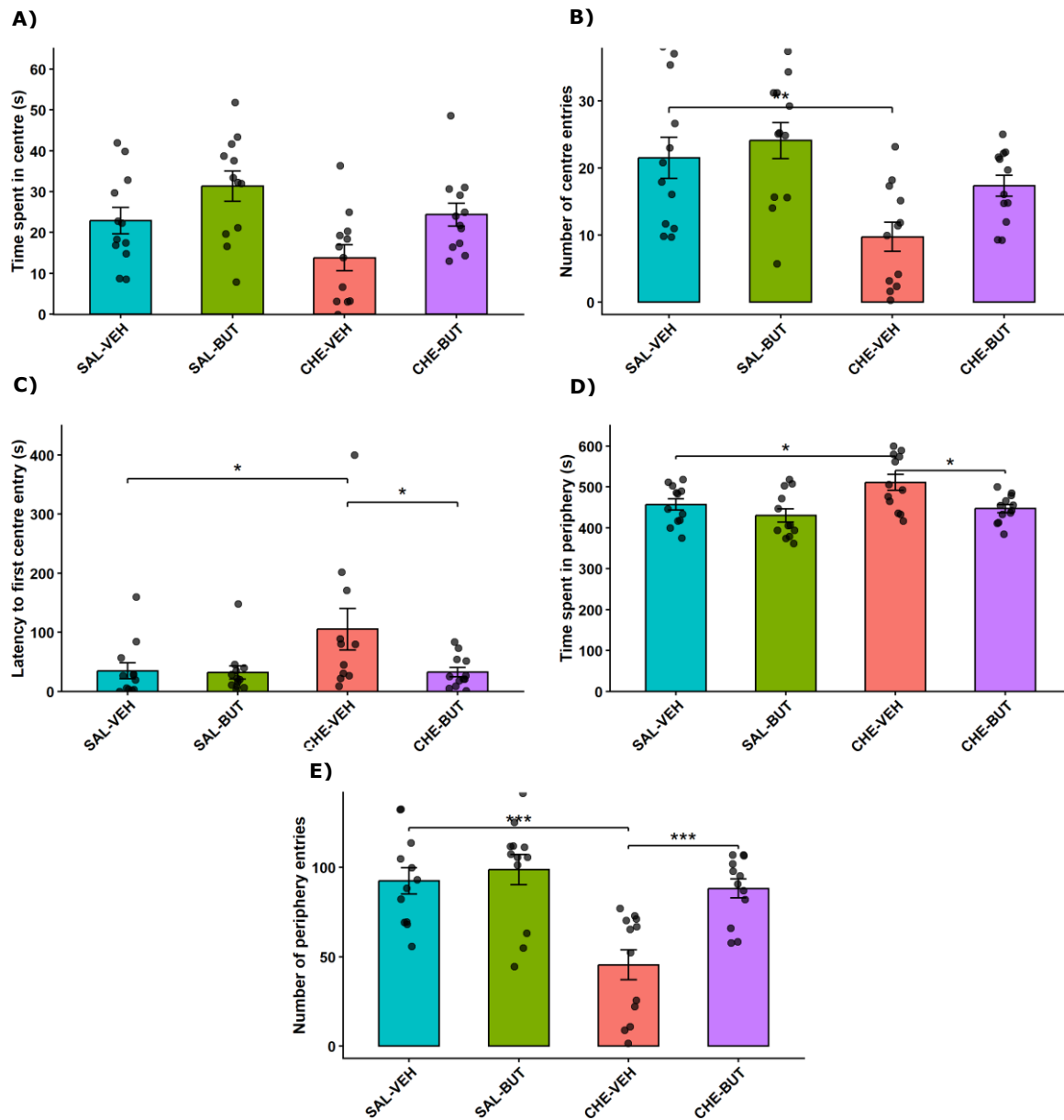
Figure 7*Results Distance Travelled Open Field*

Note. Chemotherapy reduced exploratory behaviour in the Open Field test, as the CHE-VEH group travelled significantly less distance than the SAL-VEH group. Butyrate mitigated this effect, as the CHE-BUT group travelled significantly more distance than the CHE-VEH group and did not differ significantly from the SAL-VEH group. Data are presented as mean $\pm$ SEM.

* $p < .05$, *** $p < .001$.

Anxiety-like behaviour was assessed using two-way ANOVAs on time spent in centre, number of centre entries, latency to first centre entry, time spent in the periphery, and number of periphery entries (Figure 8). A significant main effect of treatment was observed for time spent in centre ($F(1, 44) = 6.16, p = .017, \eta^2_p = .12$), centre entries ($F(1, 44) = 14.43, p < .001, \eta^2_p = .25$), time spent in periphery ($F(1, 44) = 5.37, p = .025, \eta^2_p = .11$), and periphery entries ($F(1, 44) = 14.87, p < .001, \eta^2_p = .25$). A significant main effect of intervention was observed for time spent in centre ($F(1, 44) = 8.96, p = .005, \eta^2_p = .16$), centre entries ($F(1, 44) = 4.36, p = .043, \eta^2_p = .09$), time spent in periphery ($F(1, 44) = 8.86, p = .005, \eta^2_p = .17$), and periphery entries ($F(1, 44) = 10.80, p = .002, \eta^2_p = .20$). A significant treatment $\times$ intervention interaction was only found for periphery entries, $F(1, 44) = 5.98, p = .012, \eta^2_p = .12$. These effects indicate that chemotherapy induced a pattern consistent with increased anxiety-like behaviour, whereas butyrate induced a pattern consistent with reduced anxiety-like behaviour. For periphery entries, the effect of treatment depended on intervention. Latency to first centre entry showed no significant effects in the ANOVA.

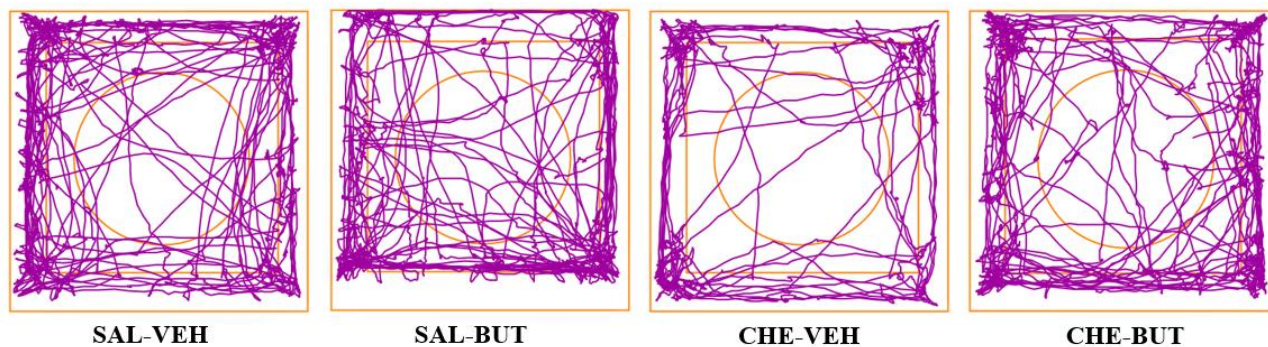
None of the planned contrasts for time spent in the centre were significant after Bonferroni correction, but a trend suggesting that the CHE-VEH group spent less time in the centre than the SAL-VEH group and CHE-BUT group is visible. The SAL-VEH vs. CHE-VEH contrast was significant for centre entries ($p = .004$), latency to first centre entry ($p = .040$), time spent in periphery ($p = .048$), and periphery entries ($p < .001$). The CHE-VEH vs. CHE-BUT contrast was significant for latency to first centre entry ($p = .032$), time spent in periphery ($p = .014$), and periphery entries ($p < .001$). The SAL-VEH vs. CHE-BUT contrast was not significant for any of the anxiety-related open-field measures, suggesting that chemotherapy-treated mice receiving butyrate did not differ significantly from saline-treated controls. Together, these results indicate that chemotherapy increased anxiety-like behaviour in the open field, whereas butyrate seemed to attenuate these anxiogenic effects. This pattern is also illustrated by the representative open-field trajectories shown in Figure 9.

Figure 8*Results Open Field*

Note. Panels show (A) time spent in the centre, (B) number of centre entries, (C) latency to first centre entry, (D) time spent in the periphery, and (E) number of periphery entries.

Chemotherapy promoted a more anxiety-like behavioural profile in the Open Field test: the CHE-VEH group showed fewer centre entries, longer latency to first centre entry, more time in the periphery, and fewer periphery entries than the SAL-VEH group. Butyrate attenuated several of these effects: the CHE-BUT group showed shorter latency to first centre entry, less time in the periphery, and more periphery entries than the CHE-VEH group. No significant group differences were found for time spent in centre. Data are presented as mean $\pm$ SEM.

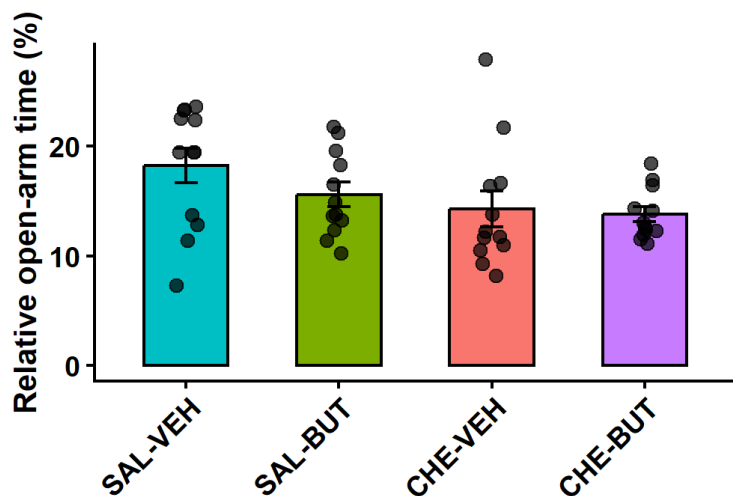
* $p < .05$, ** $p < .01$, *** $p < .001$.

Figure 9*Representative Open Field Trajectories*

Note. Movement trajectories of one representative mouse from each experimental group during the Open Field test.

Elevated Plus Maze

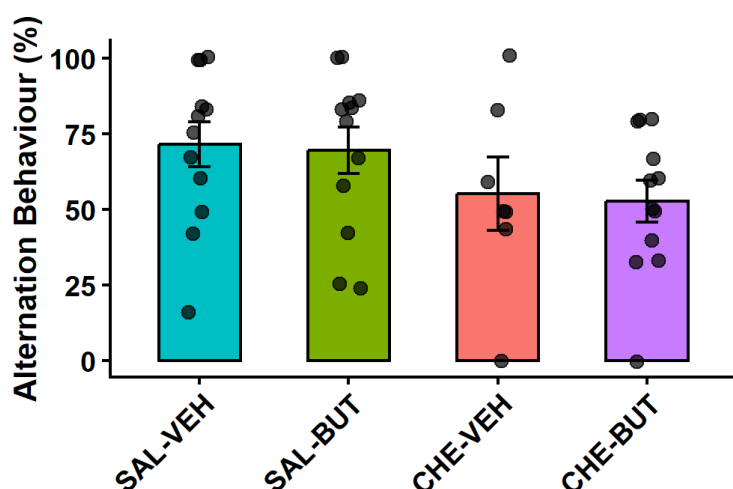
The EPM assessed anxiety-like behaviour using relative open-arm time (Figure 10). A two-way ANOVA demonstrated a significant main effect of treatment, $F(1, 44) = 4.84$, $p = .033$, $\eta^2_p = .10$, with chemotherapy-treated mice (CHE-VEH and CHE-BUT) showing lower relative open-arm time than saline-treated mice (SAL-VEH and SAL-BUT), indicative of an effect on anxiety-like behaviour. There was no significant main effect of intervention, nor a treatment x intervention interaction. Although the Bonferroni-corrected planned contrasts were not significant, exploratory uncorrected versions of the same contrasts showed lower relative open-arm time in both chemotherapy groups compared with the SAL-VEH group. These uncorrected findings should therefore be interpreted cautiously.

Figure 10*Results Elevated Plus Maze*

Note. Chemotherapy-treated mice showed lower relative open-arm time than saline-treated mice, as reflected by a significant main effect of treatment, suggesting increased anxiety-like behaviour in the EPM. No evidence was found for an effect of butyrate. None of the planned contrasts were significant. Data are presented as mean $\pm$ SEM.

T-Maze

The T-maze assessed spatial working memory using the spontaneous alternation test. The measure of interest was the proportion of spontaneous alternations relative to the maximum possible alternations (Figure 11). Mice with fewer than two potential alternations were excluded due to insufficient task engagement/lack of task motivation. Noteworthy, five mice met this criterion, all from the CHE-VEH group, which may reflect reduced task engagement in this group and suggests a possible mitigating effect of butyrate on task engagement in chemotherapy-treated mice. A two-way ANOVA showed no significant effects. None of the planned contrasts were significant.

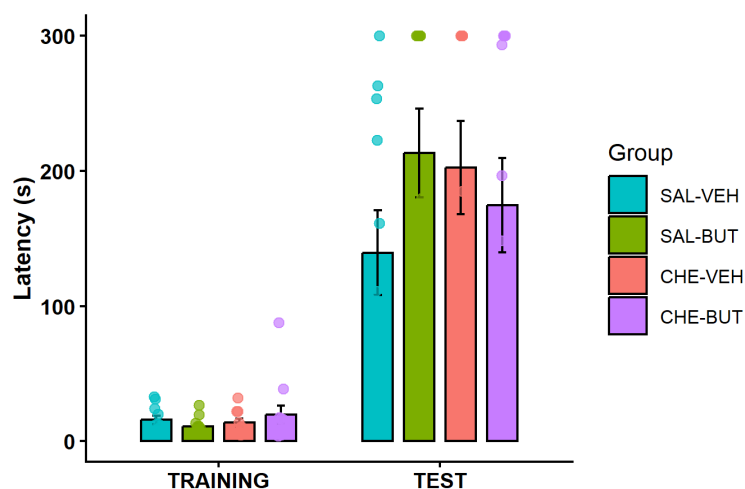
Figure 11*Results T-Maze*

Note. None of the main or interaction effects were significant, nor any of the planned contrasts. Data are presented as mean $\pm$ SEM.

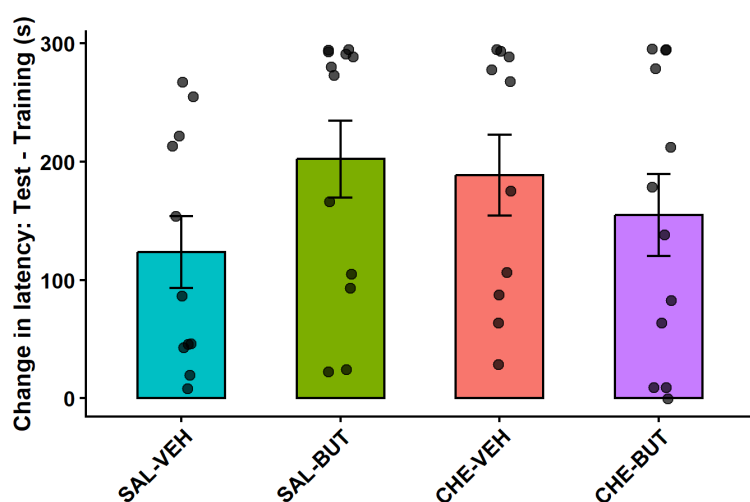
Passive Avoidance

The PA test assessed associative learning and memory, with performance indexed by the latency to enter the dark compartment during the test phase relative to the training phase (Figure 12). Two mice from the CHE-VEH group were excluded because test-phase data were missing, and one mouse from the SAL-VEH group was excluded because it showed a markedly shorter latency during the test phase compared to the training phase and thus formed an extreme outlier. A mixed-design ANOVA showed a significant main effect of phase ($F(1, 41) = 102.23$, $p < .001$, $\eta^2_p = .71$). This main effect showed that latencies were higher during the test phase than during the training phase, indicating retention of the aversive association across groups. No significant main effects of treatment or intervention were observed, and none of the interaction effects were significant. None of the planned contrasts were significant.

To further explore this pattern, a change score was calculated as test-phase latency minus training-phase latency (see Figure 13). A two-way ANOVA on this change score indicated no significant effects. None of the Bonferroni-corrected planned contrasts were significant. Altogether, these findings provide no evidence that chemotherapy or butyrate influenced Passive Avoidance performance.

Figure 12*Results Passive Avoidance Test*

Note. A significant main effect of phase suggested successful learning and memory of the dark room-shock association, as reflected by longer latencies during the test phase compared with the training phase in the PA test. No evidence was found that chemotherapy or butyrate affected performance. None of the planned contrasts were significant. Data are presented as mean $\pm$ SEM.

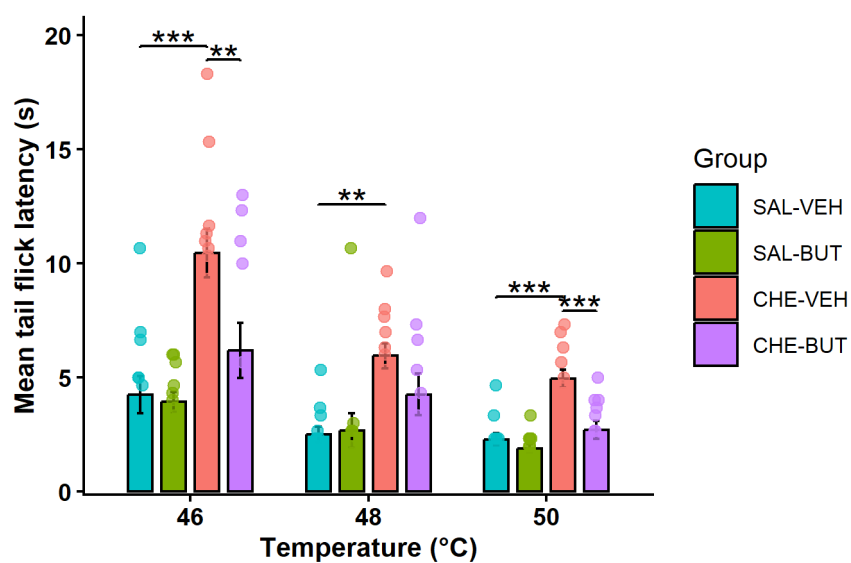
Figure 13*Results Change in Latency Passive Avoidance Test*

Note. No significant main effects, interaction effect, or planned contrasts were found for the Passive Avoidance change score. Data are presented as mean $\pm$ SEM.

Tail Flick

The Tail Flick test assessed thermal nociceptive sensitivity by measuring the latency to withdraw the tail from hot water (Figure 14). A mixed-design ANOVA revealed a significant main effect of treatment ($F(1, 44) = 37.36$, $p < .001$, $\eta^2_p = .46$), a significant main effect of intervention ($F(1, 44) = 10.03$, $p = .003$, $\eta^2_p = .19$), a significant main effect of temperature ($F(1.73, 76.26) = 28.69$, $p < .001$, $\eta^2_p = .39$), a significant treatment x intervention interaction ($F(1, 44) = 7.65$, $p = .008$, $\eta^2_p = .15$), and a significant treatment x temperature interaction ($F(1.73, 76.26) = 4.13$, $p = .025$, $\eta^2_p = .09$). These effects indicate that chemotherapy-treated mice (CHE-VEH and CHE-BUT) showed longer tail flick latencies than saline-treated mice (SAL-VEH and SAL-BUT), consistent with reduced thermal nociceptive sensitivity. Across groups, butyrate-treated mice (SAL-BUT and CHE-BUT) generally showed shorter latencies than vehicle-treated mice (SAL-VEH and CHE-VEH), and higher temperatures elicited shorter latencies. In addition, the significant interaction effects indicate that the effect of chemotherapy varied as a function of both intervention and temperature.

At 46°C, the contrasts between SAL-VEH and CHE-VEH ($t(44) = 4.71$, $p < .001$, 95% CI [2.92, 9.47], $d = 1.42$), and CHE-VEH and CHE-BUT ($t(44) = -3.25$, $p = .007$, 95% CI [-7.55, -1.00], $d = -0.98$) were significant. At 48°C, only the CHE-VEH vs. SAL-VEH contrast was significant, $t(44) = 3.61$, $p = .002$, 95% CI [1.07, 5.82], $d = 1.09$. At 50°C, both the SAL-VEH vs. CHE-VEH ($t(44) = 5.86$, $p < .001$, 95% CI [1.53, 3.80], $d = 1.77$) and the CHE-VEH vs. CHE-BUT contrast ($t(44) = -4.94$, $p < .001$, 95% CI [-3.38, -1.12], $d = -1.49$) were significant. Across all temperatures, CHE-VEH mice consistently showed longer latencies than SAL-VEH mice. While the difference between CHE-VEH and CHE-BUT reached significance only at 46°C and 50°C, a similar pattern was observed at 48°C, suggesting that butyrate may mitigate chemotherapy-induced reductions in thermal nociceptive sensitivity.

Figure 14*Results Tail Flick Test*

Note. Chemotherapy increased tail flick latencies, suggesting reduced thermal nociceptive sensitivity. Butyrate partly attenuated this chemotherapy-related increase in latency, particularly at 46°C and 50°C. Higher temperatures elicited shorter latencies across groups. Data are presented as mean $\pm$ SEM.

** $p < .01$, *** $p < .001$.

Discussion

The present study investigated to what extent the Hyper-CVAD polychemotherapy regimen induces cognitive, behavioural, and sensory alterations in adult female mice, and to what extent concurrent butyrate administration can mitigate these alterations. Several domains were assessed including weight, locomotor activity, exploratory and anxiety-like behaviour, learning and memory, and thermal nociceptive sensitivity, as these outcomes are related to common complaints reported by patients undergoing chemotherapy.

Weight Progression

Changes in body weight are a common side effect in chemotherapy-treated patients (Gadéa et al., 2011). In line with this clinical observation, analysis of body weight progression in the present study showed that chemotherapy-treated mice lost weight over time, whereas saline-treated mice remained relatively stable or slightly increased in body weight. Butyrate did not consistently mitigate this chemotherapy-related weight loss. These findings are consistent with previous rodent studies reporting that chemotherapeutic regimens can reduce body weight. For example, the combination of CYC and DOX has been shown to induce weight loss in both mice and rats (Fremouw et al., 2012; Salas-Ramirez et al., 2015). In contrast to the present findings, other studies have reported clear protective effects of butyrate on chemotherapy-related weight loss. In mouse models, butyrate attenuated weight loss induced by 5-fluorouracil and irinotecan treatment (Cheatham et al., 2026; Zeng et al., 2025). Together, these results suggest that although butyrate did not consistently attenuate chemotherapy-related weight loss in the present study, it may still hold therapeutic potential. Differences in butyrate dosage, route of administration, or treatment context may help explain these divergent findings.

Locomotor Activity

Chemotherapy-related effects are not limited to body weight, but may also extend to patients' daily functioning and activity levels. Research has shown that breast cancer patients receiving chemotherapy report reduced levels of physical activity (Kwan et al., 2012). Moreover, physical activity has been linked to better cognitive functioning in chemotherapy-treated breast cancer patients (Salerno et al., 2021). This makes chemotherapy-related reductions in activity clinically relevant in the context of CICI. The present findings mirror this clinical pattern at the preclinical level, as the Hyper-CVAD regimen reduced locomotor activity in mice. The 24-Hour Activity test showed that chemotherapy reduced spontaneous

locomotor activity. The CHE-BUT group did not differ significantly from either the CHE-VEH or SAL-VEH group, meaning that this test did not provide clear evidence for or against a butyrate effect. In contrast, the OF test showed that chemotherapy reduced total distance travelled, whereas butyrate attenuated this negative effect. Taken together, these findings suggest that chemotherapy negatively affected locomotor activity, whereas butyrate showed potential to mitigate this effect.

Previous rodent studies have also shown that chemotherapeutic agents can reduce locomotor activity, although findings vary across agents and experimental models. For instance, cisplatin and vincristine have been associated with reduced activity or distance travelled, while CYC showed only a non-significant trend towards reduced activity (Shabani et al., 2012; Terry et al., 2023). Studies using DOX or MTX did not observe clear reductions in total distance travelled or locomotor activity, despite signs of oxidative stress, neuroinflammation, or neuronal apoptosis in the hippocampus (Ding et al., 2023; Ramalingayya et al., 2017). Previous literature also points to the potential of butyrate to alleviate locomotor alterations, as NaB improved locomotor outcomes in mouse models of Parkinson's disease, potentially by reducing (neuro)inflammation and restoring gut homeostasis (Avagliano et al., 2025; Guo et al., 2023).

Altogether, these findings suggest that the Hyper-CVAD regimen reduced locomotor activity, even though its individual agents do not always do so when studied separately. This discrepancy between single-agent studies and the present multi-agent study may stem from the cumulative or synergistic burden of several chemotherapeutic agents acting together. Butyrate may have the potential to alleviate these negative effects on locomotor activity by targeting pathophysiological mechanisms relevant to chemotherapy-induced neurotoxicity, such as neuroinflammation and oxidative stress.

Anxiety-Like Behaviour

Beyond changes in body weight and activity levels, psychological symptoms are also common among cancer survivors. Many cancer survivors experience anxiety, with some even reporting increased levels after chemotherapy treatment ended (Konieczny et al., 2025; Yi & Syrjala, 2017). Moreover, higher levels of anxiety are associated with greater comorbidity burden, including fatigue, pain, depressive symptoms, and cognitive difficulties, underlining its clinical relevance (Oppegaard et al., 2021). In the present study, the OF and EPM results indicated that the Hyper-CVAD regimen promoted anxiety-like behaviour, in line with these

previous clinical findings. In the OF, chemotherapy treatment increased anxiety-like behaviour, whereas butyrate attenuated several of these effects. The EPM suggested lower relative open-arm time for chemotherapy-treated animals compared with saline-treated animals, suggesting increased anxiety-like behaviour, without evidence for a mitigating effect of butyrate.

These findings are consistent with previous preclinical studies showing that chemotherapeutic agents induced anxiety-like behaviour. For example, DOX increased anxiety-like behaviour in rats, across OF and EPM outcomes, potentially through oxidative stress (Merzoug et al., 2011). Similarly, a combination of DOX, CYC, and PTX increased anxiety-like behaviour in mice in the OF and was accompanied by microbiome dysbiosis and impaired neurogenesis (Cronin et al., 2026).

Previous literature also supports the potential of butyrate to reduce anxiety-like behaviour. In a mouse model of Parkinson's disease, oral NaB reduced anxiety-like behaviour in the EPM, and was linked to reduced levels of pro-inflammatory cytokines in the brain (Avagliano et al., 2025). In a PTX chemotherapy model, NaB protected against microbiota disturbances, altered gut permeability, and neuroinflammation, thereby alleviating anxiety-like behaviour in the EPM (Cristiano et al., 2022). Together, these findings suggest that the increased anxiety-like behaviour observed in the present study may be related to Hyper-CVAD-induced pathophysiological changes, such as oxidative stress, reduced neurogenesis, and disrupted microbiome functioning. Given the bidirectional communication between the gut and the brain, butyrate may exert its beneficial effects by restoring gut homeostasis and reducing neuroinflammation.

Learning and Memory

Alongside weight loss and activity-related and psychological complaints, many cancer survivors report cognitive difficulties, including problems with learning and memory (Argyriou et al., 2011; Mounier et al., 2020). For example, some patients showed reduced verbal memory capacity shortly after chemotherapy, or even more than two years after treatment had ended (Andrzejak et al., 2018; Kesler & Blayney, 2016). Difficulties in working memory have also been noted, with breast cancer survivors performing worse on working memory tasks than healthy controls even more than ten years after finishing chemotherapy treatment (Yamada et al., 2010). In the present preclinical study, the Hyper-CVAD regimen appeared to have little effect on spatial working memory. Moreover, no direct

evidence was found for a mitigating effect of butyrate on spontaneous alternation performance. However, all excluded animals belonged to the CHE-VEH group, which may point to reduced task engagement or motivation in this group. Since no excluded animals belonged to the CHE-BUT group, this observation may suggest that butyrate helped preserve task engagement in chemotherapy-treated mice, although this should be interpreted cautiously.

The absence of a clear chemotherapy effect on spatial working memory differs from previous research showing that chemotherapy can negatively affect this domain. For example, CYC impaired spatial working memory in mice, and the combination of CYC and DOX induced spatial working memory deficits in rats while also impairing hippocampal cell proliferation (Alhowail et al., 2019; Kitamura et al., 2017). Similarly, the absence of a clear butyrate effect contrasts with previous studies reporting beneficial effects of butyrate on spatial working memory deficits in Alzheimer's disease mouse models. Brossaud et al. (2026) reported that butyrate significantly improved spatial working memory in the Y-maze, likely by counteracting the negative effects of A β on the gut and enteric nervous system and by reducing neuroinflammation. Similarly, Zhou et al. (2023) found that a diet increasing brain acetate and butyrate levels improved spontaneous alternation T-Maze performance. Together, these previous findings suggest that chemotherapy can negatively affect spatial working memory, potentially through mechanisms such as reduced hippocampal proliferation. Previous research also indicates that butyrate may have protective effects in conditions characterised by cognitive decline, possibly through actions on gut functioning and neuroinflammation. However, neither a clear chemotherapy-induced impairment nor a clear protective effect of butyrate was observed in the present study. This discrepancy may be explained by differences in disease model, chemotherapy regimen, route of butyrate administration, or timing of behavioural assessment.

Beyond working memory, associative learning and memory represent another form of memory that may be relevant to CICI. Although this domain has been studied less extensively in chemotherapy-treated patients, de Ruiter et al. (2011) reported that chemotherapy-treated breast cancer survivors performed marginally worse on a paired-associates task than controls ten years after treatment. This cautious clinical evidence is somewhat consistent with the present PA results, which also did not provide strong evidence for a clear chemotherapy-related impairment. Although mice showed increased latencies during the test phase compared with the training phase, consistent with retention of the dark room-shock

association, there was no support for an effect of chemotherapy or butyrate on this evaluation of associative learning and memory.

Previous literature has also yielded mixed findings for associative learning and memory in chemotherapy-treated rodents. Morid et al. (2023) reported that the combination of CYC and DOX impaired associative memory in rats and increased oxidative stress and inflammation, whereas Sieklucka-Dziuba et al. (1998) found impaired PA performance following MTX administration, but not after DOX administration. In contrast, previous preclinical work has shown beneficial effects of butyrate on associative memory, with Wang et al. (2022) reporting that NaB improved PA memory in an induced Alzheimer's disease mouse model. These inconsistent findings may reflect differences in chemotherapy regimens, administration procedures, and PA protocols. Given that the present study did not indicate an effect of chemotherapy or butyrate on PA performance, and that previous findings remain mixed, future research should consider using more sensitive measures of associative learning and memory to further investigate the effects of Hyper-CVAD and butyrate.

Nociceptive and Heat Sensitivity

In addition to the observed effects on weight, behaviour, and cognition, the present study also found chemotherapy-related alterations in sensory responsiveness in the form of altered thermal nociceptive sensitivity, which may be clinically relevant given that chemotherapy-induced peripheral neuropathy (CIPN) is another common complaint among cancer survivors. CIPN involves chemotherapy-induced nerve damage and is often accompanied by tingling, altered sensations, numbness, and pain (Zajęzkowska et al., 2019). In the present study, the Hyper-CVAD regimen altered thermal nociceptive sensitivity in the Tail Flick test. Higher water temperatures elicited shorter withdrawal latencies, confirming that mice responded in a temperature-dependent manner, while Hyper-CVAD-treated mice showed longer tail-flick latencies, suggesting reduced thermal nociceptive sensitivity. Butyrate mostly attenuated this chemotherapy-related effect.

Previous research has similarly shown that chemotherapeutic agents can alter pain sensitivity, although the direction of the effect appears to differ across agents and experimental models. Yılmaz and Bozkurt (2024) reported that MTX administration in rats induced neuropathy accompanied by longer response latencies in the Hot Plate and Tail Flick tests. In contrast, vincristine decreased tail flick latency and induced neuropathic pain in mice (Kamei et al., 2006; Usman et al., 2023). Although these findings differ in direction, they

consistently indicate that chemotherapeutic agents can disrupt peripheral neural sensations, in line with the broader phenomenon of CIPN. Evidence also suggests that butyrate may alleviate altered pain sensitivity. Avagliano et al. (2025) reported that mice in a Parkinson's disease model showed shorter tail flick latencies than controls, while butyrate partly reversed this effect. They attributed these beneficial effects to reduced inflammation in the brain and spinal cord, as well as normalisation of the opioid and endocannabinoid signalling pathways. Together, these findings suggest that the altered thermal nociceptive sensitivity observed in the present study may reflect Hyper-CVAD-induced disruption of nervous system functioning, and that butyrate may mitigate these effects through anti-inflammatory and neuromodulatory mechanisms.

Study Limitations, Strengths and Future Research

The present study contributes to the understanding of chemotherapy-induced cognitive, behavioural and sensory alterations and the potential of butyrate as a mitigating intervention, but several limitations should be acknowledged. First, only female mice were included. Although including both sexes would have been preferable, this was not feasible within the practical constraints of the study, including limitations in sample size, time, and resources. Future research should include both male and female mice to determine whether the Hyper-CVAD regimen exerts sex-specific effects and whether the effects or mechanisms of butyrate differ between sexes. Second, behavioural testing was conducted only immediately after the treatment and intervention phase. Given that many patients report long-term complaints, future studies should include follow-up assessments to examine the longer-term effects of Hyper-CVAD and butyrate. Third, because 72 mice were included, the study had to be conducted in two separate batches to ensure high-quality treatment administration, intervention delivery, and behavioural testing. Although considerable effort was made to standardise procedures across batches, batch-related variation cannot be fully excluded and may have influenced the results. Fourth, while several ANOVAs revealed significant effects, many planned contrasts remained non-significant, suggesting that the behavioural test battery may not have been sufficiently sensitive to detect more subtle group differences consistently. This is in line with patients reporting cognitive difficulties, while performing normally on cognitive assessments. Future research could therefore benefit from including more sensitive behavioural tests, as well as tests assessing social, motivational, or depressive-like behaviour, to provide a broader and more nuanced picture of chemotherapy-induced impairments and the potential effects of butyrate. Fifth, this study did not include immunohistochemical testing to

assess whether Hyper-CVAD or butyrate induced changes at the brain level. Although these analyses were not included in the current thesis, they form part of the broader PhD project in which this work is embedded. Sixth, chemotherapy was administered in the absence of a tumour. Because cancer itself may contribute to cognitive impairment, future studies should consider using tumour-bearing mice to investigate the combined effects of cancer, chemotherapy, and butyrate on behavioural and cognitive outcomes. Finally, butyrate was administered through oral gavage, which may have introduced stress and thereby influenced the results. Alternative routes of administration may prove less stressful and could possibly enhance the intervention's beneficial effects. In addition, only one dose of butyrate was investigated, so it remains possible that a different dose would yield stronger effects. Future research could therefore explore a broader range of butyrate dosages.

This study also has several important strengths. First, by using the Hyper-CVAD regimen rather than a single chemotherapeutic agent, this study more closely reflects clinical chemotherapy exposure, as most patients receive a polychemotherapy regimen. As a result, the findings may be more clinically relevant and more readily translatable to humans. Second, the behavioural test battery assessed multiple cognitive, behavioural, and sensory outcomes, including spontaneous locomotor activity, exploration, anxiety-like behaviour, spatial working memory, associative learning and memory, and thermal nociceptive sensitivity. Whereas many studies focus on only one or a few domains, the present study employed a broader and more comprehensive behavioural assessment. Finally, butyrate represents an intervention with clear translational potential, as it can be administered in the form of dietary intervention or supplementation, making it relatively easy to apply and minimally invasive.

Conclusion

The present study aimed to investigate to what extent the Hyper-CVAD regimen induces cognitive, behavioural, and sensory alterations in adult female mice, and to what extent concurrent oral butyrate administration could alleviate these chemotherapy-induced alterations. It was hypothesised that chemotherapy exposure would worsen performance across the behavioural test battery, and that butyrate administration would attenuate these chemotherapy-induced alterations. Overall, the findings partially support these hypotheses. The Hyper-CVAD regimen affected several outcomes, including body weight, spontaneous locomotor activity in the 24-Hour Activity test, locomotor activity, exploration, and anxiety-like behaviour in the Open Field test, and thermal nociceptive sensitivity in the Tail Flick test. Butyrate showed beneficial effects on several of these chemotherapy-related alterations, particularly in the Open Field and Tail Flick tests, whereas evidence for attenuation of changes in body weight and 24-Hour Activity was more limited. In contrast, no clear chemotherapy-induced impairments or protective effects of butyrate were observed in anxiety-like behaviour in the Elevated Plus Maze, spatial working memory in the T-Maze, or associative learning and memory in the Passive Avoidance test. Together, these findings indicate that the Hyper-CVAD regimen can affect several, but not all, of the assessed cognitive, behavioural, and sensory outcomes in the current preclinical mouse study, and that butyrate may reduce some of these chemotherapy-induced alterations. Therefore, butyrate appears to be a promising agent for further investigation in the context of chemotherapy-induced cognitive, behavioural, and sensory alterations. Given its potential applicability through dietary intervention or supplementation, it may also hold translational relevance. Future research should further clarify the mechanisms through which chemotherapy induces these alterations and through which biological pathways butyrate may exert its beneficial effects.

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