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5-Fluorouracil chemotherapy upregulates cytokines and alters hippocampal dendritic complexity in aged mice

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Abstract

5-fluorouracil (5-Fu) is commonly used chemotherapy drug, but it can lead to the impairment of cognitive function. The pathogenesis of this injury is unknown but may involve modifications to dendritic structure and/or alterations in dendritic spine density and morphology. Dendritic spines are sites of excitatory synaptic transmission and changes in spine structure and dendrite morphology are thought to represent a morphological correlate of altered brain functions associated with hippocampal dependent learning and memory. A total of 28 one-year-old C57BL/6J male mice were used in this study; 14 mice received 5-Fu treatment and 14 were given saline injections. One month post treatment, 14 cytokines were measured at the same time Golgi samples were taken. 8 analytes were significantly elevated in mice treated with 5-Fu. 5-Fu significantly compromised the dendritic architecture and reduced spine density throughout the hippocampal tri-synaptic network. The present data provide the evidence that 5-Fu has deleterious effects on mature neurons associated with hippocampal learning and memory.

Graphical abstract

Representative Images of Spine morphological sub types.

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Keywords

5-Fluorouracil; hippocampus; dendritic spines; Cytokines; Chemokines

Introduction

Approximately 1 in 2 adults will be diagnosed with cancer during their lifetime with a median age at diagnosis of 66 years. Improvements in cancer therapy have helped a growing number of patients to survive¹. It is estimated that by year 2020, there will be approximately 70 million cancer survivors worldwide². Due to this survivorship, there has been a greater focus on researching the long-term adverse effects of treatment and the impact this treatment can have on the daily lives of patients³. Chemotherapy is the most commonly implemented treatment, often used in conjunction with local therapies such as surgery and radiotherapy⁴. However, neurotoxicity is a frequent accompaniment of cancer chemotherapy⁵. Chemobrain, also known as “chemofog,” is a cognitive impairment consisting of deficits in attention, executive and motor function, memory, and speed of information processing, following chemotherapy for a multitude of cancers^{6,7}. These deficits, while usually temporary, can persist for several years after completion of drug treatment in a subgroup (17-34%) of patients and can dramatically affect many aspects of daily living, such as employment, social functioning, and community integration⁸.

5-FU, an anti-metabolite and pyrimidine analogue, was developed in 1957 and has been used to treat breast, bowel, prostate, gastrointestinal, vaginal, and cervical cancer^{6,9,10}. 5-FU interacts with nucleic acids within the DNA sequence and interferes with RNA and DNA synthesis. Intracellular activation of 5-FU requires it to be metabolized to one of the following nucleotides: 5-fluorodeoxyuridylate (5-FdUMP), 5-fluorodeoxyuridine triphosphate (5-FdUTP), or fluorouridine triphosphate (5-FUTP). The major cytotoxic effect of 5-FU seen in humans is thought to be from the binding of 5-FdUMP to thymidylate synthase (TS), which results in DNA synthesis inhibition and programmed cell death. The formation of 5-FUTP causes abnormal RNA processing by incorporating itself into the RNA, while converting 5-FU to 5-FdUTP results in DNA strand breaks due to 5-FdUTP being incorporated into the DNA. This incorporation of 5-FdUTP into the DNA causes apoptosis in further rounds of cell division¹¹.

Animal studies that have examined intraperitoneal injections of 5-FU have shown a disruption of learning and memory across multiple tasks, including object recognition, avoidance conditioning, cue-specific and contextual fear condition tasks, and Morris water

maze⁷. 5-FU passes through the blood brain barrier (BBB) by simple diffusion and can affect proliferating cells in neurogenic zones, like the subgranular zone (SGZ) of the hippocampus⁷. 5-FU treatment has been shown to reduce the number of SGZ cells dividing, which are necessary for hippocampal neurogenesis. The SGZ of the dentate gyrus (DG) is one of a few areas where neurogenesis occurs during adulthood. Development of new neurons from proliferating precursor and stem cells within the SGZ is required for spatial memory formation^{9,12}. It is proposed that these altered neurogenic zones may contribute heavily to the cognitive deficits seen in those with chemobrain. However, these cognitive deficits are not solely based on altered neurogenic zones; thiamine (vitamin B1) deficiency and/or 5-FU metabolites may also contribute to neuronal toxicity⁶.

Cytokines and chemokines (chemoattractant cytokines) are secreted signaling proteins derived from many different types of cells that help determine and regulate immune responses¹³. There are two basic immune responses for cytokine production: 1) antigen presenting cells (APCs) take up antigens, process them, and then present them to T-lymphocytes to produce cytokines and 2) APCs, such as monocytes, are activated to produce cytokines through pattern recognition receptors that recognize a foreign pathogen¹⁴. Cytokines predominantly produced by APCs include multiple interleukin (IL) and tumor necrosis factor (TNF) molecules¹⁵. Within the brain, cytokines are mostly produced by microglia, with astrocytes, oligodendrocytes, and neurons contributing smaller amounts. Several clinical studies have demonstrated that administering a standard dose of chemotherapeutic drugs causes an increase in cytokine levels for a variety of cytokines (TNF- α , IL-6, IL-8, IL-10, and monocyte chemoattractant protein-1 (MCP-1)).

Acharya *et al.* (2015) found that cyclophosphamide chemotherapy caused significant reductions of certain dendritic spine types in two different areas: 1) stubby and mushroom spines in the DG and 2) all three spine morphologies in cornu ammonis (CA1). Furthermore, these alterations lead to an overall significant decrease in spine density¹⁶. Cisplatin has also been found to affect dendritic morphology of pyramidal neurons as soon as three days following treatment. Cisplatin reduces dendritic branching and spines in CA1 as well as CA 3 neurons¹⁷.

The present investigation was designed to assess how a relatively low dose of 5-Fu would affect dendritic spine density and morphology. Such data might provide critical information regarding the mechanism of disruption of neural circuitry following chemotherapeutic treatment.

Methods

Animals and study design

Six-month-old male C57Bl6/J wild-type mice (n = 28) purchased from the Jackson Laboratory (Bar Harbor, ME) were used for the experiments. The mice were housed and aged under a constant 12 h light: 12 h dark cycle. Food and water were provided *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at UAMS.

Chemotherapeutic paradigm

All chemotherapeutic agents were purchased from the UAMS inpatient Pharmacy then stored according to manufacturer's instructions until use. All chemotherapeutic agents were diluted in sterile saline. Mice received weekly intraperitoneal injections of saline (0.9% sterile saline; n = 14) or 5-fluorouracil (5-FU) (60 mg/kg; n = 14). The injections occurred over 3 weeks for a total of three injections (days 1, 8, and 15). All animals were injected between 0900 h–1200 h. Doses of drug were chosen based on previously published 5-FU studies in mouse models¹⁸.

Golgi staining and tissue preparation

The brains were carefully extracted and cut in half along the midsagittal plane. The right hemisphere of the brain sham (N = 7) and drug treated mice (N= 7) was immediately subjected to the Bioenno Tech superGolgi kit for Golgi-Cox method of staining. The samples were impregnated with the potassium dichromate and mercuric chloride solution at room temperature for 11 days. After immersion in the post-impregnation buffer for 2 days, brains were serially sectioned at 150 μ m using a vibrating microtome and sections were collected in chilled mounting buffer. The sections were mounted on 0.3% gelatin-coated slides, washed in 0.01 M PBS buffer (pH 7.4) with Triton X-100 (0.3%), and stained with ammonium hydroxide solution (provided in the kit). After staining and washing, slides were gradually dehydrated through a series of increasing alcohol concentrations, then with xylene and finally mounted in Permount™ (Fisher) for analysis.

Spine Density and Spine Morphology

Spine analyses were conducted blind to the experimental conditions on coded Golgi impregnated brain sections containing the dorsal hippocampus. Spines were examined on dendrites of DG granule neurons as well as apical (stratum radiatum) and basal (stratum oriens) dendrites of CA1 and CA3 pyramidal neurons. The neurons that satisfied the following criteria were chosen for analysis in each of the experimental groups: 1) presence of untruncated dendrites; 2) consistent and dark Golgi staining along the entire extent of the dendrites; and 3) relative isolation from neighboring neurons to avoid interference with analysis¹⁹. Three–5 dendritic segments, each at least 20 μ m in length²⁰, were analyzed per neuron, and 6–7 neurons were analyzed per brain. Neurons that met staining criteria were traced using a 60 X oil objective, a computerized stage, and NeuroLucida software (Ver. 11, MicroBrightfield, Inc., Williston, VT).

To acquire images for spine analysis, the dendritic segments were imaged under brightfield illumination on a Zeiss Axioimager microscope with a 100 $\times$ oil immersion objective. Spine analyses were based on the method of Margarinos *et al.*²¹. This method does not assess spine density in a 3 dimensional manner but focuses on spines that are parallel to the plane of section. Although the method may underestimate the total number of spines, it facilitates a direct comparison of treatment groups when they are analyzed in an identical manner²¹. ImageJ software was used to calculate linear spine density²², which was presented as the number of spines per 20 μ m of dendrite length.

Dendritic morphology quantification

The explored morphological characteristics included Sholl analysis, total dendritic length, number of branch points and dendritic complexity index (DCI), performed using the Neuroexplorer component of the NeuroLucida program. First, we collected the Sholl analysis which is used to assess the amount and distribution of the arbor at increasing distances from the cell body²³. The distance between each radius was set to 10 μ m for our experiments. The length of dendritic branch within each progressively larger circle is counted from the soma. This provides information concerning the amount and distribution of dendritic material. Next, we performed a branch point analysis. A branch point represents a bifurcation of the dendrite when a branch divides into two sub-branches. Branch point analysis is based on the number of bifurcations and the order of the points²⁴. Lower branch point orders represent proximal regions of the tree, whereas larger orders characterize distal regions. The branch point analysis was used to determine the complexity of the dendritic arborization. The complexity of the dendritic tree is an important phenotypic component of the branching analysis. DCI was determined by the following equation, $DCI = \sum \text{branch tip orders} + \# \text{ branch tips} \times (\text{total dendritic length} / \text{total number of primary dendrites})$. In the CA1 and CA3 area, apical and basal dendrites were analyzed separately.

Cytokine and Chemokine analysis

Hippocampi were dissected from sham (N = 7) and Drug treated mice (N= 7) then homogenized individually in 200 μ l of PBS (Roche, Switzerland), containing Complete protease inhibitor (Roche, Switzerland) using a Kontes cordless motor and pestle (New Jersey, United States) at 4°C. Homogenized samples were centrifuged at 500 g at 4°C for 10 minutes. Supernatants were transferred to new Eppendorf tubes on ice and centrifuged at 15,000 g at 4°C for 5 minutes. Supernatants were collected and shipped to Quansys Biosciences (Logan, UT) for analysis of the following cytokines using Q-Plex Array kits for Mouse Cytokine: Interleukins (IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-10, IL-12p70, MCP-1, TNF- α), granulocyte macrophage-colony stimulating factor (GMCSF), and the chemokine, regulated on activation, normal T cell expressed and secreted (RANTES). All cytokine data were expressed as pg/mg protein.

Statistical Analysis

Data were expressed as Mean (+/-) SEM. Paired two-tailed t-tests were used to evaluate statistical differences between sham and 5 Fu treated groups. All statistical analyses were conducted using GraphPad Prism 6.0 software (La Jolla, CA), and $p < 0.5$ was considered significant. For measures of dendritic intersections, a mixed factors analysis of variance (ANOVA) tested for the effects of 5 Fu treatment (between subjects variable) and distance from the cell body (Sholl radius, repeated measures variable) followed by Fisher LSD post hoc tests when appropriate.

Results

Changes in Spine Density and Spine Morphology

Quantitative analysis showed that 5-Fu treatment did not affect overall spine density in the Dentate Gyrus (DG) but, there was a trend towards significance ($t = 2.112$, $P = 0.07$; Fig 1a). When we analyzed the density of the various types of spines, we found that the density of mushroom spines was dramatically decreased ($t = 9.54$, $P < 0.0001$; Table 1). However, there was an increase in the proportion of thin spines ($t = 2.42$, $P < 0.05$; Table 1) and in stubby spines ($t = 12.14$, $P < 0.0001$; Table 1).

In the CA1 apical pyramidal dendrites, there was significant decrease in the overall density of spines ($t = 7.68$, $P < 0.01$; Fig 2a). Similar to the DG, when we analyzed spine morphology we found a significant decrease in the density of mushroom spines ($t = 9.94$, $P < 0.001$; Table 2). Furthermore, there was an increase in the density of stubby spines ($t = 10.60$, $P < 0.001$; Table 2), but so significant changes in the thin spines ($t = 1.70$, $P = 0.16$; Table 2). In contrast, 5-Fu treatment did not significantly affect basal spine density ($t = 1.79$, $P = 0.15$; Fig 2b). Nevertheless, there was a reduction in the density of mushroom spines ($t = 3.88$, $P < 0.05$; Table 2), a significant increase in the density of stubby spines ($t = 14.83$, $P < 0.001$; Table 2) and no significant changes in thin spines ($t = 0.63$, $P = 0.56$).

Similar to what was observed in DG and CA 1, 5-Fu treatment significantly decreased spine density in the CA 3 apical neurons ($t = 7.54$, $P < 0.01$; Fig 3a) compared to saline treated controls. In contrast to what was observed in DG and CA 1, 5-Fu treatments did not significantly affect apical area spine morphology (see Table 3). In the CA 3 basal pyramidal dendrites, there was a significant reduction in the overall density of spines ($t = 5.57$, $P < 0.05$; Fig 3b). Our analysis showed a significant decline in the density of thin spine only ($t = 3.92$, $P < 0.05$; Table 3), but there was no effect on stubby ($t = 1.24$, $P = 0.30$) or mushroom spines ($t = 0.20$, $P = 0.85$; Table 3) compared to saline treated controls.

Dendritic Morphology

Dentate Gyrus Granule Neurons—To further investigate the effects of 5-Fu chemotherapy on neuronal morphology, a segmental Sholl analysis was performed to examine changes in dendritic length as a function of radial distance from the cell soma. In the DG, there was a significant interaction between 5-Fu treatment and segmental dendritic length ($F_{(25, 234)} = 5.56$, $P < 0.0001$), indicating that the effect of 5-Fu treatment is associated with a different distribution of dendritic branches over the entire tree. The ANOVA also revealed a significant main effect of treatment ($F_{(1, 234)} = 116.4$, $P < 0.0001$). Post hoc analysis revealed that 5-Fu exposure reduced dendritic arborization compared to saline injected controls. This decrease in arborization was particularly evident between 60 – 140 μm (Fisher's LSD, $P < 0.001$; Fig 1b) plus 40 and 100 μm (Fisher's LSD, $P < 0.05$; Fig 1b) when saline injected mice were compared to 5-Fu treated mice. DG branch order analysis revealed a significant interaction between treatment and branch order ($F_{(8, 36)} = 25.61$, $P < 0.001$). Additional analysis with Fisher's LSD showed 5-Fu treatment decreased length at the 2nd order, 4th – 6th order (Fisher's LSD, $P < 0.001$; Fig 1f). Our analysis also revealed significant decreases in dendritic length ($t = 8.52$, $P < 0.001$; Table 1),

the number of branch points ($t = 10.26$, $P < 0.0001$; Table 1) and overall dendritic complexity ($t = 8.07$, $P < 0.0001$; Table 1). These data suggest that 5-Fu treatment significantly decreases dendritic complexity throughout the DG.

CA 1 Pyramidal Neurons

A similar analysis was performed on the apical and basal region of the CA 1 and CA 3 pyramidal neurons. There was a significant interaction between treatment and segmental dendritic length in the CA1 apical area ($F_{(25, 130)} = 5.20$, $P < 0.001$). Two-way ANOVA revealed a significant main effect of 5-Fu treatment ($F_{(1, 130)} = 27.09$, $P < 0.001$) and an effect of length ($F_{(1, 130)} = 113.8$, $P < 0.001$). Further analysis with Fisher's LSD showed 5-Fu treatment decreased dendritic arborization 40-100 μm (Fisher's LSD, $P < 0.001$; Fig 2c) and 130-160 μm (Fisher's LSD, $P < 0.05$; Fig 2c) away from the soma when compared to saline treated controls. In addition, branch order analysis of the CA 1 apical dendritic length revealed a significant interaction between treatment and branch order ($F_{(18, 95)} = 11.96$, $P < 0.001$). Additional analysis with Fisher's LSD showed 5-Fu treatment decreased length at the 2nd order, 5th – 11th order (Fisher's LSD, $P < 0.001$; Fig 2g) and 12th and 13th order levels (Fisher's LSD, $P < 0.05$; Fig 2g). Our analysis also revealed significant decreases in dendritic length ($t = 10.12$, $P < 0.001$; Table 2), the number of branch points ($t = 6.03$, $P < 0.01$; Table 2) and overall dendritic complexity ($t = 5.74$, $P < 0.01$; Table 2). These data suggests 5-Fu treatment decreased the complexity of dendritic morphology of apical dendrites.

In the basal dendrites of the CA1 pyramidal cells, there was a significant interaction between 5-Fu treatment and segmental dendritic length ($F_{(25, 130)} = 24.07$, $P < 0.001$). The two-way ANOVA also detected a main effect of 5-Fu treatment ($F_{(1, 130)} = 256.40$, $P < 0.001$) and an effect of length ($F_{(25, 130)} = 122.8$, $P < 0.001$). Fisher's LSD showed 5-Fu treatment decreased dendritic arborization 30-80 μm (Fisher's LSD, $P < 0.001$; Fig 2d) and 100-140 μm (Fisher's LSD, $P < 0.05$; Fig 2d) away from the soma when compared to saline treated controls. In addition, branch order analysis of the CA 1 apical dendritic length revealed a significant interaction between treatment and branch order ($F_{(15, 80)} = 9.36$, $P < 0.001$). Additional analysis with Fisher's LSD showed 5-Fu treatment decreased length at the 2nd – 6th level orders (Fisher's LSD, $P < 0.001$; Fig 2h). Similar to the observation in the DG, significant differences were found in the CA 1 apical and basal area for total dendritic length (Apical $t = 10.12$, $P < 0.001$; Basal $t = 18.21$, $P < 0.001$; Table 2), the number of branch points (Apical $t = 6.03$, $P < 0.01$; Basal $t = 13.93$, $P < 0.001$; Table 2) and the branch point complexity (Apical $t = 5.74$, $P < 0.01$; Basal $t = 4.21$, $P < 0.01$; Table 2).

CA3 Pyramidal Neurons

In contrast to what was observed in CA1 spine analysis, there was no significant interaction between 5-Fu and segmental dendritic length in the CA3 Apical area ($F_{(28, 87)} = 0.91$; $P = 0.59$; Fig 3c). However, in the basal dendrites of the CA 3 pyramidal cells, there was significant interaction between treatment and segmental dendritic length ($F_{(25, 78)} = 1.85$; $P < 0.05$). Two-way ANOVA revealed a significant main effect of 5-Fu treatment ($F_{(1, 78)} = 25.27$, $P < 0.001$) and an effect of length ($F_{(1, 78)} = 99.47$, $P < 0.001$). Fisher's LSD showed 5-Fu treatment decreased dendritic arborization 90-100 μm (Fisher's LSD, $P < 0.01$; Fig 3d),

70–80 and 110–120 μm (Fisher's LSD, $P < 0.05$; Fig 3d). No differences were found in the CA3 apical for total dendritic length ($t=0.10$, $P=0.92$), the number of branch points ($t=0.41$, $P = 0.41$) and the branch point complexity (Apical $t=0.83$, $P = 0.46$; Table 3). Conversely, in the basal dendrites of the CA 3 pyramidal cells, a significant difference was found in the number of branch points ($t=4.22$, $P < 0.05$; Table 3) and a trend towards significance for total dendritic length ($t=2.90$, $P < 0.06$) but impact on branch point complexity (Apical $t=0.82$, $P = 0.47$). No difference in branch order were observed in CA3 Apical or basal.

Cytokine/chemokine analysis

Next, effects of 5-Fu on cytokine and chemokine levels in the hippocampus were assessed. As indicated in Fig 4 and 5, levels of IL-1B ($t= 7.55$, $P < .001$; Fig 4a), IL-2 ($t= 8.77$, $P < .001$; Fig 4b), IL-3 ($t= 4.00$, $P < .05$; Fig 4c), IL-4 ($t= 7.81$, $P < .001$; Fig 4d), IL-5 ($t= 5.10$, $P < .01$; Fig 5a), IL-17 ($t= 3.47$, $P < .01$; Fig 5b), GM-CSF ($t= 4.55$, $P < .01$; Fig 5c), RANTES ($t= 7.41$, $P < .001$; Fig 5d), were significantly elevated compared saline treated group. The rest of the cytokine/chemokine levels examined were comparable between 5-Fu treated and saline treated controls.

Discussion

Cognitive impairment has been observed following the use of chemotherapeutic agents in humans and rodents^{25,26}. Mechanisms underlying this cognitive and neurobehavioral toxicity have not yet been clearly elucidated. Multiple candidate mechanisms for chemobrain have been proposed, including indirect chemical toxicity and oxidative damage, direct injury to neurons, inflammation, or induction of autoimmune responses²⁷. 5-Fluorouracil (5-FU), one of the most important agents for the treatment of colorectal, head and neck, pancreatic and breast carcinomas, has been associated with cognitive impairment in^{28,29}. Its ability to cross the blood-brain barrier by passive diffusion enables it to affect the brain when given systemically³⁰. Past work by Mustafa *et al*/has shown that 5-Fu chemotherapy affects spatial working memory while disrupting neurotrophin levels and neurogenesis in the murine hippocampus⁶. To establish the impact of 5-Fu on mature neuronal morphology, brains from mice exposed to 5-Fu were Golgi stained and analyzed for structural changes in hippocampal neurons. The Golgi-Cox method has been described as a useful tool in a variety of human diseases and animal models, where data from gross inspection or histology was not consistent with the expected behavioral/neurologic alteration^{31,32}.

In the current study, dendritic branch points (indicated by bifurcation), dendritic length and complexity were reduced significantly by 5-Fu treatment in the DG and CA1. This is in agreement with a previously reported study where reductions in DG and CA1 dendritic structure were observed after chronic Cyclophosphamide (CYP) treatment. In the study by Acharya *et al*⁶, significant changes in the number of dendritic length and dendritic complexity were observed 30 days post-treatment, indicating a reduction in dendritic complexity. However, CYP had little impact on the number of dendritic branch points in CA area. In contrast, 5-Fu significantly decreased the number of dendritic branch points within CA1. Another study by Andres *et al*¹⁷ has shown low doses of Cisplatin also causes a decrease in dendritic branches and reduces dendritic complexity throughout the CA 1 area.

To measure dendritic arborization, the three-dimensional dendritic tree, including all branches, were reconstructed in a two-dimensional plane and the dendritic tracing was quantified by Sholl analysis²³. In the current study, 5-Fu treatment significantly modulated dendritic arborization in the DG and CA1 areas. This data is in agreement with Acharya *et al*⁶, where a reduction in DG and CA1 arborization was associated with deficits in hippocampal-dependent learning and memory.

In an attempt to understand the potential role of 5-Fu in mediating cytokine responses after treatment, 14 cytokines were measured at the same time Golgi samples were taken. Interestingly, 8 analytes were significantly elevated in mice treated with 5-Fu. Our current results showed that the levels of pro-inflammatory cytokines, IL-1 β , IL-17, and GM-CSF were significantly increased in 5-Fu treated mice. IL-1 β is synthesized during injury, infection, and disease, mediating inflammatory responses^{33,34}. Several studies demonstrate a critical role for IL-1 β in the formation of hippocampal dependent memory. IL-17 is also well known for its pro-inflammatory effects³⁵. IL-17 acts synergistically with TNF and IL-1 to shape CNS inflammation. IL-17 has been found to play a pivotal role in the pathogenesis of numerous inflammatory diseases in the CNS, such as multiple sclerosis and stroke^{36,37}. In the central nervous system, GM-CSF can activate resident macrophage-like microglia and promote neuroinflammation through the up regulation of toll-like receptor 4 and CD14³⁸.

Multiple clinical studies have demonstrated that administration of a standard dose of chemotherapy causes increases in cytokine levels such as IL-1 β , IL-6, and GM-CSF in cancer patients and that these changes are more prominent in patients who experienced cognitive dysfunction^{39,40}. Persistent activation of microglia and the release of inflammatory cytokines such as IL-1 β could cause neuronal and glial cell damage⁷. Cytokines and chemokines are secreted proteins that determine the outcome of immune responses, the cellular organization of immune organs, and immune cell trafficking¹⁵. Chemotherapeutic agents can induce an inflammatory response in the hippocampus of rats and the release of cytokines throughout the body. These cytokines can then cross the BBB and lead to further cytokine release within the brain, which is then associated with cognitive dysfunction.

Our current results showed that the levels of anti-inflammatory cytokines IL-2, IL-3, IL-4 and IL-5 significantly increased in 5-Fu treated mice. IL-2 effects are mediated by binding to its specific receptor IL-2R^{41,42}. The IL-2R is expressed in cerebral areas related to cognition, like the neocortex, cerebellum and hippocampus. IL-2 has been reported to play a role in neuronal proliferation, survival and neuroprotection. IL-3 promotes the process extension of cultured cholinergic and prevents neuronal death in the hippocampus after ischemia^{43,44}. It also promotes the survival of sensory neurons and protects against neuronal death induced by A β ⁴⁵. IL-4 is an anti-inflammatory cytokine; it is involved in the resolution of brain inflammation by the regulation of lipopolysaccharide (LPS) – induced production of TNF- α , IL-1 β promoting the survival of hippocampal neurons^{46,47}. IL-4, the key factor in decreasing microglial activation, reduces MCP-1 production by activated microglia *in vivo* and *in vitro*^{48,49}. The anti-inflammatory cytokines IL-4 and IL-5 belong to the group of Th2 cytokines and may act as immunosuppressors. In case of IL-4, the upregulation might be linked to inflammation by antagonizing the proinflammatory activity of IL-1 β ⁵⁰.

There was one chemokine upregulated in our study that may be relevant in a range of inflammatory disorders: RANTES (regulated upon activation normal T cell expressed and secreted), a powerful leukocyte activator. RANTES, now given the immunologic designation CCL5, is one of the principal chemotactic agents that induce recruitment of the immune cells to the CNS via interaction with its corresponding receptor CCR5⁵¹. RANTES receptors are expressed by hippocampal neurons where it modulates calcium signaling and glutamate release⁵². RANTES has been proposed to be neuroprotective. The addition of RANTES to a culture medium slows the rate of apoptotic death of hippocampal neurons⁵³. In addition, RANTES induces the expression of genes involved in neuronal survival, neurite outgrowth, and synaptogenesis⁵⁴.

In conclusion, to the best of our knowledge the present report provides the first evidence that in adult mice, 5-Fu causes alteration in spine density and dendritic morphology in the hippocampus in a region-specific manner. In addition, we show that cytokines associated with both improved cognition as well as impaired cognition were expressed in concentrations above control in mice exposed to 5-Fu. The functional implications of the observed cytokine effects on dendritic spines at the hippocampal sub regions are not yet clear. Additionally, if or how these chemotherapeutic induced alterations may relate to the behavioral, cellular, and molecular changes remain to be determined.

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Figure 1. Spine density, Sholl and Branch point analysis of neurons in the dentate gyrus
(a) In the DG granule dendrites, there were no significant changes in the overall density of spines. **(b)** Dendritic length measured by Sholl analysis revealed a decrease in arborization particularly evident at distances between 40 – 150 μm from the soma. **(e-f)** Representative tracings of DG granule neurons superimposed over concentric rings (10 μm) used for Sholl analyse. **(g)** Length per branch order in pyramidal neurons of the CA1 hippocampal region. Average $\pm$ SEM (n=6)

Figure 2. Spine density, Sholl and Branch Point analyses of CA1 pyramidal neurons

(a) In the CA1 apical pyramidal dendrites, 5-Fu significantly decreased spine density. (b) In the basal dendrite, there were no significant changes in the overall density of spines. (c) Sholl analysis revealed 5-Fu treatment significantly decreased dendritic arborization 40-100 μm and 130-160 μm away from the soma in the CA1 apical pyramidal dendrites. (d) In the basal dendrites 5-Fu treatment decreased dendritic arborization 30-80 μm and 100-140 μm away from the soma. (e-f) Representative tracings of CA1 pyramidal neurons superimposed over concentric rings (10 μm) used for Sholl analyses show typical basal and apical branching patterns. (g-h) Length per branch order in pyramidal neurons of the CA1 hippocampal region. Average $\pm$ SEM (n=6)

Figure 3. Spine density and Sholl analyses of CA3 pyramidal neurons

(a) In the CA3 apical pyramidal dendrites, 5 Fu significantly decreased spine density. (b) In the basal dendrite, 5 Fu significantly decreased overall density of spines. (c) There was no significant effect of 5-Fu treatment in the CA 3 apical area. (d) In the basal dendrites, 5-Fu treatment decreased dendritic arborization 70 –120µm away from the soma. Average ± SEM (n=6).

Figure 4. Effects of 5-Fu on hippocampal cytokine and chemokine levels

There were effects of treatment on levels of IL-1B ($t = 7.55$, $P < .001$; Fig 4a), IL-2 ($t = 8.77$, $P < .001$; Fig 4b), IL-3 ($t = 4.00$, $P < .05$; Fig 4c), IL-4 ($t = 7.81$, $P < .001$; Fig 4d). Compared with saline treated mice, the levels of IL-1B, IL-2, IL-3 and IL-4 were significantly higher in mice receiving 5-Fu treatment. Average $\pm$ SEM ($n=6$)

Figure 5. Effects of 5-Fu on hippocampal cytokine and chemokine levels

There were effects of treatment on levels of IL-5 ($t = 5.10$, $P < .01$; Fig 5a), IL-17 ($t = 3.47$, $P < .01$; Fig 5b), GM-CSF ($t = 4.55$, $P < .01$; Fig 5c), RANTES ($t = 7.41$, $P < .001$; Fig 5d).

Compared with saline treated mice, the levels of IL-5, IL-17, GM-CSF and RANTES were significantly higher in mice receiving 5-Fu treatment. Average $\pm$ SEM ($n=6$)

Table 1

Effects of 5-Fu on spine morphology and dendritic complexity in hippocampal dentate gyrus

Cell Type and Measurement	Sham (mean $\pm$ SEM)	5-Fu (mean $\pm$ SEM)	<i>p</i> -value
Dentate Gyrus			
Thin Spines	20.60 $\pm$ 0.77	27.35 $\pm$ 1.74	p < .05
Stubby Spines	26.17 $\pm$ 0.68	47.62 $\pm$ 1.55	p < .001
Mushroom Spines	55.22 $\pm$ 2.70	23.95 $\pm$ 1.33	p < .001
Total Dendritic Length (μ m)	974.1 $\pm$ 47.59	424.70 $\pm$ 29.83	p = 0.001
Total # Branch Points	7.95 $\pm$ 0.38	3.21 $\pm$ 0.20	p = 0.001
Branch Complexity	36818.00 $\pm$ 3676	5753.00 $\pm$ 723.80	p = 0.001

* Bold figures represent significant values compared to sham treated mice

Table 2

Morphological analysis of apical and basal dendrites in CA1

Cell Type and Measurement	Sham (mean $\pm$ SEM)	5 Fu (mean $\pm$ SEM)	<i>p</i> -value
CA1 Apical			
Thin Spines	27.29 $\pm$ 7.33	30.06 $\pm$ 3.80	<i>p</i> =0.56
Stubby Spines	27.42 $\pm$ 1.50	49.37 $\pm$ 1.87	<i>p</i> < 0.001
Mushroom Spines	51.14 $\pm$ 1.75	20.56 $\pm$ 2.35	<i>p</i> < 0.001
Total Dendritic Length (μ m)	879.50 $\pm$ 34.12	390.50 $\pm$ 23.13	<i>p</i> < 0.001
Total #Branch Points	15.33 $\pm$ 1.61	4.28 $\pm$ 0.28	<i>p</i> < 0.01
Branch Complexity	109,431 $\pm$ 16,767	8876 $\pm$ 1369	<i>p</i> < 0.001
CA1 Basal			
Thin Spines	27.29 $\pm$ 7.33	32.11 $\pm$ 2.65	<i>p</i> = 0.37
Stubby Spines	27.58 $\pm$ 1.03	50.10 $\pm$ 2.35	<i>p</i> < 0.001
Mushroom Spines	45.50 $\pm$ 7.94	17.79 $\pm$ 1.42	<i>p</i> < 0.05
Total Dendritic Length (μ m)	1281 $\pm$ 52.12	489.3 $\pm$ 36.62	<i>p</i> < 0.001
Total #Branch Points	16.80 $\pm$ 1.00	4.32 $\pm$ 0.24	<i>p</i> < 0.001
Branch Complexity	30,576 $\pm$ 6744.00	3744 $\pm$ 458	<i>p</i> < 0.01

Table 3

Effects of 5-Fu on morphologic parameters of hippocampal CA3 pyramidal cells

Cell Type and Measurement	Sham (mean $\pm$ SEM)	5 Fu (mean $\pm$ SEM)	<i>p</i> -value
CA3 Apical			
Thin Spines	27.48 $\pm$.90	24.88 $\pm$ 2.06	<i>p</i> = 0.37
Stubby Spines	36.37 $\pm$ 1.80	40.86 $\pm$ 1.91	<i>p</i> = 0.31
Mushroom Spines	36.38 $\pm$ 1.02	34.27 $\pm$ 2.34	<i>p</i> = 0.54
Total Dendritic Length (μ m)	1179 $\pm$ 214.5	1151 $\pm$ 50.89	<i>p</i> = 0.92
Total # Branch Points	10.10 $\pm$ 1.36	8.50 $\pm$ 0.38	<i>p</i> = 0.41
Branch Complexity	68,634 $\pm$ 22,183	45,400 $\pm$ 8897	<i>p</i> = 0.46
CA3 Basal			
Thin Spines	28.20 $\pm$ 1.32	21.05 $\pm$ 1.75	<i>p</i> < 0.05
Stubby Spines	35.05 $\pm$ 2.34	41.42 $\pm$ 2.7	<i>p</i> = 0.30
Mushroom Spines	36.74 $\pm$ 1.69	37.54 $\pm$ 3.15	<i>p</i> = 0.85
Total Dendritic Length (μ m)	1429 $\pm$ 89.59	1151 $\pm$ 50.89	<i>p</i> = 0.06
Total # Branch Points	13.90 $\pm$ 0.73	9.0 $\pm$ 0.71	<i>p</i> < 0.05
Branch Complexity	38,073 $\pm$ 7469	29,270 $\pm$ 3210	<i>p</i> = 0.47