

Impaired Cognitive Function and Hippocampal Neurogenesis following Cancer Chemotherapy

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Abstract

Purpose: A substantial proportion of breast cancer survivors report significant, long-lasting impairments in cognitive function, often referred to as "chemobrain." Advances in detection and treatment mean that many more patients are surviving long-term following diagnosis of invasive breast cancer. Thus, it is important to define the types, extent, and persistence of cognitive impairments following treatment with cytotoxic cancer drugs.

Experimental Design: We examined the effects of chronic treatment with two agents commonly used in patients with breast cancer, cyclophosphamide and doxorubicin (Adriamycin). Athymic nude rats were given 50 mg/kg cyclophosphamide, 2 mg/kg doxorubicin, or saline injections once per week for 4 weeks. A novel place recognition task and contextual and cued fear conditioning were used to characterize learning and memory ability. Immunofluorescence staining for immature and mature neurons and activated microglia was used to assess changes in neurogenesis and neuroinflammation.

Results: Cyclophosphamide- and doxorubicin-treated rats showed significantly impaired performance on the novel place recognition task and the contextual fear conditioning task compared with untreated controls, suggesting disrupted hippocampal-based memory function. Chemotherapy-treated animals showed a significant decline in neurogenesis [80%–90% drop in bromodeoxyuridine (BrdUrd)-labeled cells expressing NeuN]. Activated microglia (ED1-positive) were found after cyclophosphamide but not doxorubicin treatment.

Conclusions: Our results show that chronic treatment with either of two commonly used chemotherapeutic agents impairs cognitive ability and suggest that strategies to prevent or repair disrupted hippocampal neurogenesis may be effective in ameliorating this serious side effect in cancer survivors. *Clin Cancer Res*; 18(7); 1954–65. ©2012 AACR.

Introduction

Moderate cognitive impairments are reported in 15% to 50% of breast cancer survivors following chemotherapy (1–5) and recent reports suggest that many more (up to 75%) may experience more subtle cognitive changes that nonetheless make post-cancer, day-to-day management of work and family responsibilities very difficult (6 and reviewed in ref. 7). The cognitive domains that are disrupted are diverse and include memory, processing speed, attention, and executive function (reviewed in refs. 8, 9). Currently, there are multiple hypotheses for the brain changes that might underlie these cognitive impairments, including disruption of hippocampal cell proliferation and neurogenesis (reviewed in ref. 10), chronic increases in inflammation

(11, 12), increased oxidative stress (11), white matter disruption (13, 14), and long-term changes in cerebral blood flow and metabolism (15).

Determination of a causal connection between chemotherapy and cognitive impairment is difficult in clinical studies due to patient-to-patient differences in disease status, treatment regimen, psychologic reactions to diagnosis and treatment, and baseline cognitive reserve, as well as differences in test administration (16–19). The exact nature and extent of impairments reported are difficult to quantify, and often, patient complaints are more frequent and more severe than what is detected in the clinic (20–22). However, it is clear that the effects on mood, memory, concentration, and executive functions reported (2–4, 23, 24 and reviewed in ref. 8), whether mild or severe, are lasting and have a major negative impact on quality of life (4, 25, 26). In an attempt to better control some of these factors, we evaluated the effects of 2 commonly used cytotoxic agents, cyclophosphamide, an alkylating agent, and doxorubicin, a topoisomerase interactive agent, in a rat model of chemotherapy-induced cognitive decline.

A growing number of studies report chemotherapy-induced changes on cognition and brain function in rodent

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Translational Relevance

Given the growing number of long-term cancer survivors, it is critical to address the extent, persistence, and neuropathologic mechanisms underlying chemotherapy-related cognitive decline or chemobrain. The present study used a rodent model to delineate the effects of two widely used cytotoxic agents, cyclophosphamide and doxorubicin, on cognitive function. The experiments also evaluated the hypothesis that unintended disruption of hippocampal neurogenesis plays a causal role in the development of chemobrain. Following chronic exposure to clinically relevant doses, rats were impaired on two memory tasks known to rely on intact hippocampal function and exhibited disrupted neurogenesis. The development of animal models is needed to delineate the underlying mechanisms of chemobrain and will help identify potential therapeutic targets for preventing and/or treating this serious side effect of chemotherapy.

models (reviewed in ref. 27). Reports on the effects of cyclophosphamide treatment are equivocal. Two separate mouse studies report transient hippocampal-based memory deficits following a single injection of cyclophosphamide (28, 29). In contrast, a study conducted in young and old female rats reported a transient *improvement* in hippocampal learning and memory when animals were assessed 8 weeks or more after their final cyclophosphamide injection (30), and a recent study reported no change in spatial recognition memory performance 5 days after chronic cyclophosphamide administration (31). Reports on the cognitive effects of doxorubicin treatment are also mixed. Liedke and colleagues found a dose- and time-specific effect of chronic treatment on memory acquisition as assessed by an inhibitory avoidance task in male rats (32). In contrast, a study using a similar hippocampal fear-based task reported no effects of doxorubicin treatment in male and female mice (33).

Thus, while the existing clinical and animal literature suggests that exposure to cyclophosphamide or doxorubicin causes alterations in cognition, further systematic studies are clearly needed. To address these discrepancies and to uncover the potential mechanisms underlying chemobrain, we evaluated the effects of chronic treatment with clinically relevant doses of cyclophosphamide or doxorubicin in the absence of disease, thereby avoiding some of the confounding factors that typically complicate related clinical studies. We assessed cognitive changes using paradigms known to engage the hippocampus. To further evaluate the hypothesis that unintended hippocampal damage may play a causal role in the development of chemotherapy-induced cognitive dysfunction, we evaluated the effects of cyclophosphamide and doxorubicin on hippocampal neurogenesis 3 weeks after the cessation of treatment.

Materials and Methods

Subjects and drug treatments

All animal procedures were carried out in accordance with NIH and Institutional Animal Care and Use Committee guidelines. Two-month-old, male athymic nude rats (strain 02N01 Cr:NIH-*rmu*) were obtained from National Cancer Institute (Bethesda, MD). The athymic nude rat is a standard model used in xenograft studies that has proven to be a valuable tool in cancer research. Athymic nude rats were used in the present study, as future studies assessing chemotherapy-induced cognitive impairments in human tumor models will require an immunocompromised host. Furthermore, rats (as compared with mice) are a more suitable species for assessing cognitive outcomes following exposure to cytotoxic agents. Animals were group-housed in a specific pathogen-free room under controlled conditions ($20^{\circ}\text{C} \pm 1^{\circ}\text{C}$; $70\% \pm 10\%$ humidity). Artificial lighting was maintained on a 12:12 hour light:dark cycle, and animals had free access to a conventional diet and water. On weeks of chemotherapy administration, all rats also received DietGel Recovery (ClearH₂O) every second day to maintain hydration, stimulate appetite, and avoid excessive weight loss. Body weight was measured at baseline and 1 week after each injection to confirm the absence of major peripheral toxicity that might have influenced behavior on subsequent cognitive tests.

Cyclophosphamide (50 mg/kg) or doxorubicin (2 mg/kg; both from Sigma-Aldrich) was dissolved in sterile saline and injected immediately (intraperitoneally). Control animals received 0.9% sterile saline injections (intraperitoneally) of the same volume. Animals in all groups received one injection per week for 4 consecutive weeks (see Fig. 1 for study time line). Treatment groups were as follows: N = 8 saline-treated controls (CON), N = 10 cyclophosphamide-treated animals (cyclophosphamide; CYP), and N = 9 doxorubicin-treated animals (DOX).

Novel place recognition task

To evaluate the effect of chemotherapy on cognitive performance, the novel place recognition (NPR) task was administered one week after the fourth and final injection. The NPR task uses spontaneous exploration as a means of assessing spatial recognition memory, which has been shown previously to rely on intact hippocampal function (34, 35). Two open-field white acrylic arenas, each measuring 45 high \times 70 \times 70 cm were used for NPR testing. The arenas were placed next to each other on the floor of a brightly lit, dedicated behavioral testing room. A video camera was centered on the ceiling above each arena, and live tracking of the animals was achieved using Noldus Ethovision XT (version 7.0; Noldus Information Technology). Various large, high-contrast posters placed on the walls of the testing rooms served as extramaze spatial cues.

The NPR task began with 2 days of habituation. Each day, rats were individually placed in the open-field arenas and allowed to explore freely for 20 minutes before being returned to their home cages. Two toy objects were placed

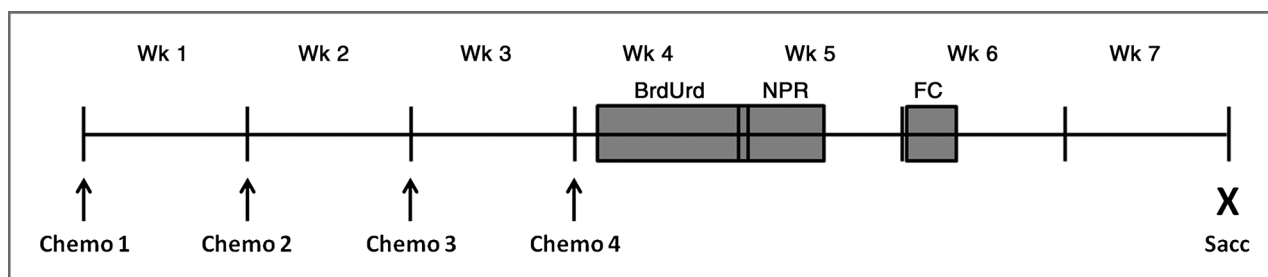


Figure 1. Study time line. Rats received 1 injection per week for 4 consecutive weeks (arrows), followed 2 days later by 6 consecutive daily injections of BrdUrd. This was followed immediately by 4 days of NPR testing. One week after the start of NPR testing, 2 days of fear conditioning (FC) testing were administered. Rats were euthanized (Sacc) 3 weeks after the first BrdUrd injection.

in each arena during the habituation sessions to further acclimatize them to the test situation; these objects were not used in the subsequent experiment trials. For all phases, arenas and objects were cleaned with 70% ethanol between trials to minimize odor cues.

The familiarization phase and 5-minute test phase were administered the following day. The NPR objects used were identical plastic blocks ($\sim 8 \times 3 \times 10 \text{ cm}^3$ high), which were placed 27 cm from opposing corners of the open field. Small pieces of white Velcro placed on the undersides of the objects and on the arena floor were used to secure the objects in place during testing. Rats were placed in the arenas and allowed to explore freely for 5 min. Rats were then returned to a holding cage for a 5-minute retention interval. Following this delay, one of these blocks was moved to an open corner at a distance of 18 cm from the arena wall ("novel place"), whereas the other block remained at its former spatial location ("familiar place"). Rats were allowed to explore the stimuli freely for 3 minutes. Following the 5-minute test phase, rats were returned to their home cages for a delay of 24 hours and then returned to the test arena to explore for 3 minutes during the 24-hour test phase. For this phase, the "novel place" block was again moved, this time to the remaining open corner and the "familiar place" block remained in the same location. For all phases, the "head direction to zone" function in Ethovision XT was used to track exploration of the blocks. A rat was considered to be exploring a block when its head was oriented toward it and its nose was within a 4-cm radius.

Contextual and cued fear conditioning task

A fear conditioning task was administered 2 weeks after the final and fourth injection to further characterize chemotherapy effects on cognitive performance. The fear conditioning task consisted of 3 distinct phases: a training phase, a context test phase, and a cue test phase. The neural circuitry underlying cue-specific and contextual conditioned fear memory is well established; cued fear conditioning has been shown to rely on intact amygdala function, whereas contextual fear conditioning has been shown to additionally engage the hippocampus (36). For all phases, rats were placed in a clear, acrylic chamber ($30 \times 30 \times 40 \text{ cm}^3$ high; PhenoTyper 3000, Noldus Information Technology). A video camera centered in the PhenoTyper's top unit

was used to observe the rats during fear conditioning trials. For the training and context test phases, the floor of the PhenoTyper contained a stainless steel grid, with an inter-bar separation of 0.9 cm. For all phases, freezing behavior was scored by an observer blind to treatment status and was defined as complete immobility, except for breathing movements.

The fear conditioning task began with a 15-minute training phase on day 1. Each rat was placed in the chamber and explored freely for 5 minutes to establish baseline-freezing behavior. A series of 5 tone-shock pairings was administered over the following 5 minutes. For each pairing, a 2,000 Hz, 90 dB was played for 30 seconds, and a mild (1 mA) footshock was administered concurrent with the final 1 second of the tone via the stainless steel grid floor. Administration of the tone and shock was automated using Ethovision XT software (version 7.0) plus additional trial-control software (Noldus Information Technology). Following the final tone-shock pairing, rats were observed for an additional 5 minutes to assess posttraining freezing levels.

The following day, rats were returned to same context for the context test phase. During this phase, rats were allowed to explore freely for 5 minutes, and neither the tone nor shock stimuli were administered. If memory for the context-shock conditioned association is intact, rats will spend a significant proportion of the trial engaged in freezing behavior in response to be returned to the identical environment 24 hours later (36).

The cue test phase was administered 1 hour after the context test phase was complete. For this phase, removing the stainless steel grid floor, adding panels to the sides of the fear conditioning chamber, and cleaning the floor of the chamber using a scented detergent solution all served to change the context. Rats explored freely for 5 minutes; for the first minute, no tone was played to assess pre-cue freezing in the altered context. During the following 3 minutes, the same 2,000 Hz, 90 dB tone was played as during training. Cue test freezing behavior was measured during this 3-minute period and for an additional minute after the tone was turned off.

BrdUrd treatment and immunohistochemistry

To assess the impact of chemotherapy on hippocampal neurogenesis, 5-bromo-2'-deoxyuridine (BrdUrd; 100 mg/kg

intraperitoneally; Sigma-Aldrich) was administered for 6 consecutive days beginning 2 days after the fourth and final drug injection. Three weeks later, animals were euthanized by intracardiac perfusion with 4% paraformaldehyde. Brains were processed in a sucrose gradient (10%–30%) and sectioned coronally (30- μ m thick sections) through the hippocampus using a cryostat; sections were collected serially [every 20th for doublecortin (DCX) and ED-1] in PBS with sodium azide (0.02%).

Immunohistochemical studies used both monoclonal and polyclonal primary antibodies. DCX staining was carried out to identify immature neurons. Sections were washed with PBS, blocked with normal horse serum (NHS; 10% with 0.1% Triton X-100) for 30 minutes, then followed by primary antibody incubation (goat-anti-DCX, 1:200; Santa Cruz) overnight at 4°C. Subsequently, biotinylated horse anti-goat IgG (1 hour, 1:200; Vector Labs) and streptavidin Texas-Red (1 hour, 1:200; Vector Labs) were used to facilitate color development. The nuclear counterstain was TOTO-3 (1 μ mol/L for 15 minutes, Sigma-Aldrich).

To identify mature neurons, representative sections were processed using dual immunofluorescence staining for BrdUrd and neuron-specific nuclear antigen (NeuN). Serial sections taken through the middle of hippocampus were selected for staining and stored in PBS overnight. Free-floating sections were first rinsed in TBS (100 mmol/L, pH 7.6) and subjected to a BrdUrd pretreatment protocol using 50% formamide [made in 2 \times saline-sodium citrate (SSC) buffer; Sigma-Aldrich] at 68°C for 2 hours and 2N HCl (at 37°C for 45 minutes), followed by serum blocking (10% normal donkey serum, NDS; Sigma-Aldrich) and overnight incubation in a rat anti-BrdUrd solution (1:200; AbD Serotec). The sections were then treated with donkey anti-rat IgG Alexa Fluor 594 (1:200; Invitrogen) for 60 minutes, rinsed in PBS, then blocked in serum, and incubated overnight with primary antibodies (mouse anti-NeuN, 1:200; Millipore). The following day, sections were washed with PBS and treated with biotinylated secondary antibody (horse anti-mouse, 1:200; Vector Labs). Color development was facilitated by fluorescein (1:200 in PBS; Vector Labs). Immunostained sections were rinsed in PBS and mounted on clean Vectabond (Vector Labs)-treated slides using Slow Fade anti-fade mounting medium (Invitrogen). BrdUrd and DCX-positive cells were visualized using fluorescence microscopy as red and NeuN-positive cells were visualized as green.

To identify activated microglia (ED-1⁺ cells), sections were washed with PBS several times, blocked with 10% NDS in PBS containing 0.1% Triton X-100 for 30 minutes, and then incubated overnight in anti-ED-1 (mouse, 1:200; AbD Serotec) prepared in PBS containing 2% NDS and 0.1% Triton X-100. On the second day, sections were washed and incubated for 1 hour in secondary antibody (donkey anti-mouse IgG, 1:200; Invitrogen). Sections were then washed thoroughly and counterstained with TOTO-3 (1 μ mol/L for 15 minutes; Sigma-Aldrich) to visualize the different hippocampal cell layers.

Confocal analyses were carried out using multiple Z-stacks taken at 1- μ m intervals using a confocal laser scanning microscope (Nikon Eclipse TE2000-U, EZ-C1 interface). Individual Z-sections were then analyzed using Nikon Elements software (version 3.0). The main determinant for the assessment of chemotherapy effects on hippocampal neurogenesis was the percentage of BrdUrd-positive cells coexpressing the mature neuronal marker, NeuN. At least 50 to 100 BrdUrd-positive cells were counted for each animal (6–8 serial sections). The percentage of dual-labeled BrdUrd-NeuN-positive cells was derived from 3 individual animals in each group.

For BrdUrd, DCX and ED-1 enumeration, every 20th section (30 μ m thickness) through the rostrocaudal span of the hippocampus (3–6 sections per animal) was included in the analysis, and positive cells were counted from 1- μ m-thick Z-stacks scanned from 3 individual animals in each group. Data for DCX are represented as the mean number of DCX⁺ neurons in granule cell layer-subgranular zone (GCL-SGZ) region per 30- μ m section. ED-1⁺ and BrdUrd⁺ cells were quantified across hippocampal subfields [dentate hilus, GCL-SGZ, and CA1 and CA3 layers] and are represented as the mean number of BrdUrd⁺ or ED-1⁺ cells per hippocampal section.

Statistical analysis

All statistical analyses were conducted using PASW Statistics 18 (SPSS, IBM Corporation). All analyses were 2-tailed, and a value of $P \leq 0.05$ was considered statistically significant. In all cases, normal distribution of the data (Kolmogorov–Smirnov test) and homogeneity of variance (Levene's test of equality of error variances) were confirmed. When a statistically significant overall group effect was found, multiple comparisons were made using Fisher protected least significant different (FPLSD) *post hoc* tests to compare the individual groups.

Weight, in grams, was analyzed at 5 time points (i.e., at baseline and 1 week after each injection) by repeated measures ANOVA with group as the between-subjects factor.

Exploration ratio, or the proportion of total time spent exploring the novel spatial location ($t_{\text{novel}}/t_{\text{novel}} + t_{\text{familiar}}$), was used as the main dependent measure for the NPR task. We analyzed the behavior of the animals during minute 1 of the 5-minute and 24-hour test phases; previous research has shown that preference for the novel place diminishes after the first minute, as the spatial locations become equally familiar to the animals (34). Exploration ratio data were analyzed using one-way ANOVAs for the 5-minute and 24-hour test phases. Additional analyses of recognition memory were conducted using 1-sample *t* tests to determine whether the mean proportion of time spent exploring the novel spatial location for each group differed significantly from chance (i.e., 0.5). To establish baseline exploratory behavior and assess any nonspecific toxicity effects of the drug treatments, we also analyzed time spent exploring the stimuli and velocity of locomotion during the initial familiarization phase.

Percentage of time spent freezing was used as the main dependent measure for the fear conditioning task. For both the baseline and posttraining phases, freezing was assessed during the final minute of the 5-minute interval. For the context test, freezing was assessed over the entire 5-minute trial. For the pre-cue test, freezing was assessed during the first minute, in which no tone was sounded, and for the cue test, freezing was assessed across the 3-minute interval that the tone was sounded and for the final minute of the trial in which no tone was sounded. Repeated measures ANOVA was used to assess group (between-subjects factor) and phase (within-subjects factor) effects on freezing behavior.

For analysis of immunohistochemical images, group differences were assessed by one-way ANOVA (BrdUrd-NeuN and DCX analyses) or repeated measures ANOVA (ED1 analyses). When significant overall group differences were found, FPLSD *post hoc* tests were conducted, as for analysis of cognitive data.

Results

Chemotherapy-induced weight changes

As shown in Fig. 2, animals in all groups gained weight over the course of the study, as expected on the basis of their starting age of 2 months. Repeated measures ANOVA revealed a significant time \times group effect. As can be seen in Fig. 2, chemotherapy-treated animals tended to weigh less than controls at 1-week postinjection 3 [$F(2,24) = 3.35$; $P = 0.052$] and 1-week postinjection 4 [$F(2,24) = 3.18$; $P = 0.06$]. Cyclophosphamide-treated animals weighed approximately 27 g (or 8.5%) less than controls 1 week after the final injection and doxorubicin-treated animals weighed approximately 33 g (or 10.4%) less.

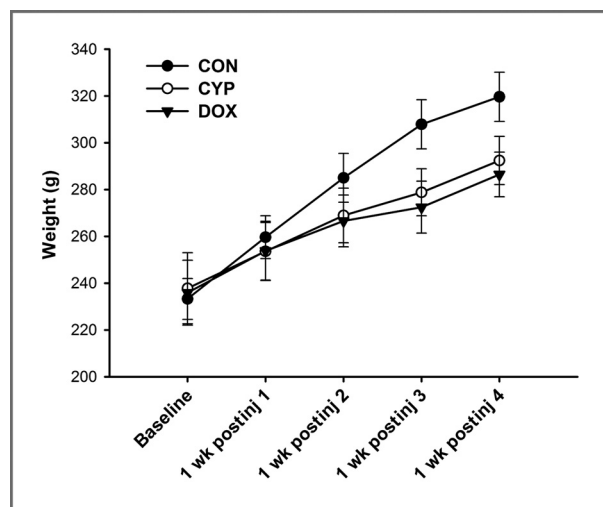


Figure 2. Rats treated with doxorubicin (DOX) or cyclophosphamide (CYP) tended to gain less weight over the course of the study compared to saline-treated (CON) animals. Body weight was measured at baseline and 1 week after each injection (postinj). Data are presented as means \pm 1 SEM.

Effects of chemotherapy on NPR performance

A significant overall group effect was found [$F(2,24) = 3.902$; $P = 0.034$] during the initial familiarization phase, when the spatial locations of the NPR stimuli were equally unfamiliar to the animals. As can be in Fig. 3A, doxorubicin-treated animals spent significantly more time exploring the stimuli than both cyclophosphamide ($P = 0.018$) and control ($P = 0.032$) animals. Velocity of locomotion during the initial familiarization phase did not differ between the groups (Fig. 3B, $P = 0.702$).

For the 5-minute test (Fig. 3C), group means and 95% confidence intervals (CI) were as follows: control (mean, 0.816; 95% CI, 0.67–0.96); cyclophosphamide (mean, 0.577; 95% CI, 0.42–0.73); and doxorubicin (mean, 0.651; 95% CI, 0.58–0.76). There was a trend for exploration ratio to differ between the groups following the short, 5-minute retention interval [$F(2,24) = 2.958$; $P = 0.071$]. Cyclophosphamide-treated animals showed significantly decreased preference for the novel spatial location compared with control animals ($P = 0.024$). Doxorubicin-treated animals did not differ significantly from control ($P = 0.119$) or cyclophosphamide ($P = 0.449$) animals. One-sample *t* tests comparing group exploration ratios to chance (i.e., 0.5) revealed that only control animals showed significant preference for the novel spatial location [$t(7) = 5.283$; $P = 0.001$], whereas neither chemotherapy-treated group explored the novel place more than chance (cyclophosphamide, $P = 0.295$; doxorubicin, $P = 0.084$).

Exploration ratios during the 24-hour test did not differ significantly between the groups (Fig. 3D, $P = 0.586$). Furthermore, none of the groups spent significantly more time than expected by chance exploring the novel spatial location (control, $P = 0.28$; cyclophosphamide, $P = 0.53$; and doxorubicin, $P = 0.063$).

Effects of chemotherapy on fear conditioning memory performance

A significant overall group \times phase interaction effect was found by repeated measures ANOVA for percentage of time spent freezing during the fear conditioning task [Fig. 4; $F(8,96) = 4.292$; $P = 0.001$]. Subsequent individual one-way ANOVAs conducted for each phase of the task revealed a significant group effect for the context test phase of the fear conditioning task only [$F(2,24) = 11.442$; $P < 0.0001$]. For the context test, group means and 95% CIs were as follows: control (mean, 64.59; 95% CI, 51.58–77.6), cyclophosphamide (mean, 34.84; 95% CI, 23.93–45.75), and doxorubicin (mean, 34.93; 95% CI, 24.68–45.17). FPLSD *Post hoc* tests showed that both cyclophosphamide ($P < 0.0001$) and doxorubicin ($P < 0.0001$) groups spent significantly decreased percentages of time freezing compared with the control group. Groups did not differ significantly in freezing behavior across baseline ($P = 0.124$), posttraining ($P = 0.62$), pre-cue test ($P = 0.395$), and cue test ($P = 0.073$) phases.

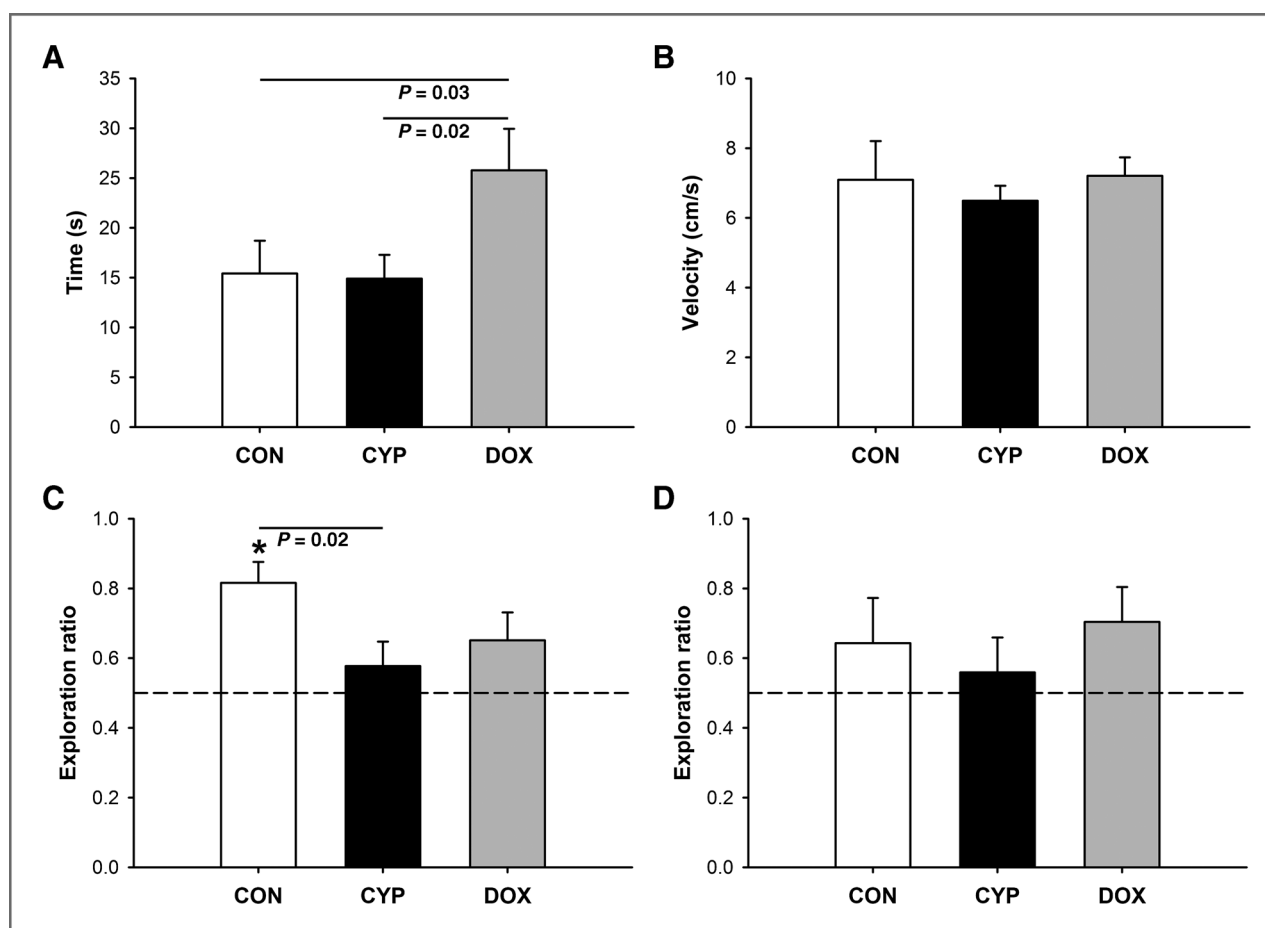


Figure 3. Treatment with cyclophosphamide (CYP) or doxorubicin (DOX) impairs spatial recognition memory performance on an NPR task. A and B, time spent exploring both objects and mean velocity of locomotion during the initial familiarization phase, respectively. C and D, exploration ratios (i.e., $t_{\text{novel}}/t_{\text{novel}} + t_{\text{familiar}}$) for the first minute of the 5-minute and 24-hour test sessions, respectively. Data are present as means + 1 SEM, and the dashed lines (in C and D) indicate chance performance (i.e., 0.5). *P* values are derived from FPLSD *post hoc* comparisons. *, a significantly higher proportion of time spent exploring the novel spatial location than expected by chance in saline-treated (CON) animals.

Effects of chemotherapy on hippocampal neurogenesis

The impact of chemotherapy on neurogenesis was assessed using immature (DCX) and mature (NeuN) neuronal markers. DCX is widely used as a marker of immature neurons in the dentate gyrus. Newly born cells in the SGZ and GCL express DCX within 3 hours after commitment to a neuronal lineage and remain stable from approximately 12 to 14 days (37). The number of DCX⁺ neurons per hippocampus was assessed in each group. In controls, DCX⁺ neurons exhibited morphology expected of differentiated granule cells with projections of developing dendrites into the GCL (Fig. 5). In contrast to this observation, both chemotherapy groups showed abnormal dendritic development and disorientation of dendritic projections of DCX⁺ cells into the GCLs. Ectopic location of DCX⁺ cells was also observed frequently in the dentate hilus of the hippocampus in doxorubicin-treated animals. ANOVA revealed an overall significant effect of group on the number of DCX⁺ cells [$F(2,6) = 16.8$; $P = 0.003$], and *post hoc* tests showed that both cyclophosphamide (mean, 46.28;

95% CI, 18.72–73.84) and doxorubicin (mean, 41.22, 95% CI, 21.8–60.65) groups had significantly fewer DCX⁺ cells than saline-treated controls (mean, 86.56; 95% CI, 56.49–116.62; both $P \leq 0.003$). In contrast, the number of DCX⁺ cells did not differ between cyclophosphamide- and doxorubicin-treated groups ($P = 0.577$). Importantly, the number of newly born DCX⁺ neurons was reduced by 47% and 53% in cyclophosphamide- and doxorubicin-treated groups, respectively. These reductions indicate the marked toxicity of the chemotherapeutic agents on neurogenic cell populations in the hippocampus.

To further analyze the effects of the drugs on neurogenesis, we quantified the percentage of cells dual-labeled for BrdUrd and NeuN. In controls, 64% of BrdUrd-positive cells were also positive for the NeuN marker (Fig. 6J). ANOVA revealed an overall significant effect of group for the percentage of BrdUrd-NeuN⁺ cells [$F(2,18) = 52.97$; $P < 0.0001$], and *post hoc* tests showed that both cyclophosphamide (mean, 7.99; 95% CI, 2.53–18.51) and doxorubicin (mean, 12.95; 95% CI, 5.67–20.24) groups

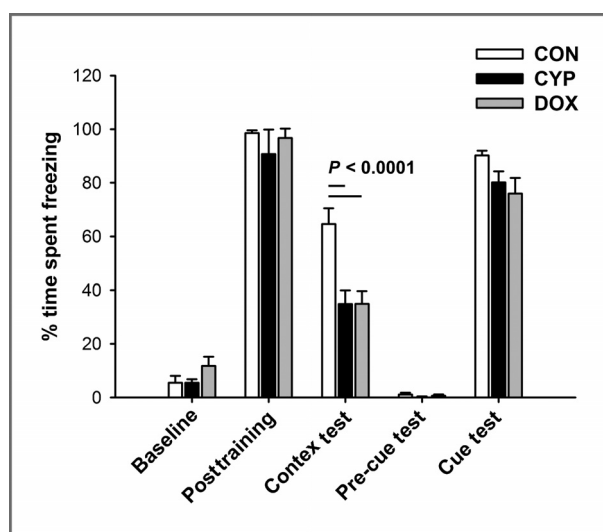


Figure 4. Treatment with cyclophosphamide (CYP) or doxorubicin (DOX) impairs performance on a contextual fear conditioning task. Baseline freezing levels were equivalent across groups, and all groups showed increased freezing behavior, as expected, following a series of 5 tone-shock pairings ("Posttraining"). The context test was administered 24 hours later, and chemotherapy-treated animals showed decreased freezing behavior compared with saline-treated controls (CON). One hour later, the context was changed, which resulted in a substantial reduction in freezing behavior in all groups ("Pre-cue Test"). Freezing levels were subsequently restored in all groups when the tone was played ("Cue Test"). Data are presented as means + 1 SEM. *P* values are derived from FPLSD *post hoc* comparisons.

had significantly fewer double-positive labeled cells than saline-treated controls (mean, 64.04; 95% CI, 50.82–77.86; both $P < 0.0001$). This represents an 81% drop in cyclophosphamide-treated animals and 88% drop in doxorubicin-treated animals compared with controls. In contrast, the number of BrdUrd⁺ cells per section did not differ between groups (Fig. 6K), suggesting that survival of cells proliferating at the time of BrdUrd injections was unaffected. Taken together with our findings of reductions in the number of DCX⁺ neurons and percentage of BrdUrd-NeuN⁺ cells in treated animals, this suggests that the maturation of neurons in the neurogenic zones of the hippocampus was adversely affected by chronic exposure to cyclophosphamide or doxorubicin.

Effects of chemotherapy on inflammation in the hippocampus

Microglia cells are sensors of central nervous system (CNS) pathology and the first cells of the CNS parenchyma that become activated in response to inflammation, infection and trauma. Using ED-1 immunostaining, a specific marker of activated microglia, we determined the number of activated microglia in the hippocampus (dentate hilus, GCL-SGZ, CA1 and CA3 layers) in rats treated with saline, cyclophosphamide, or doxorubicin 4 weeks after their final injection (Fig. 7). Repeated measures ANOVA revealed an overall significant effect of region [$F(2,12) = 6.53$; $P = 0.01$] and group [$F(2,6) = 22.34$; $P = 0.002$] for the number of

ED-1⁺ cells. Individual ANOVAs for each region revealed overall group differences [dentate hilus: $F(2,8) = 16.64$; $P = 0.004$; GCL-SGZ: $F(2,8) = 14.21$; $P = 0.005$; CA1&CA3: $F(2,8) = 7.07$; $P = 0.026$]. *Post hoc* tests showed that cyclophosphamide animals had significantly more ED-1⁺ cells than saline-treated controls across all 3 regions assessed ($P < 0.024$), whereas doxorubicin animals did not differ from controls. These results suggest that only cyclophosphamide treatment resulted in increased inflammation in the hippocampus, as assessed using the ED-1 marker.

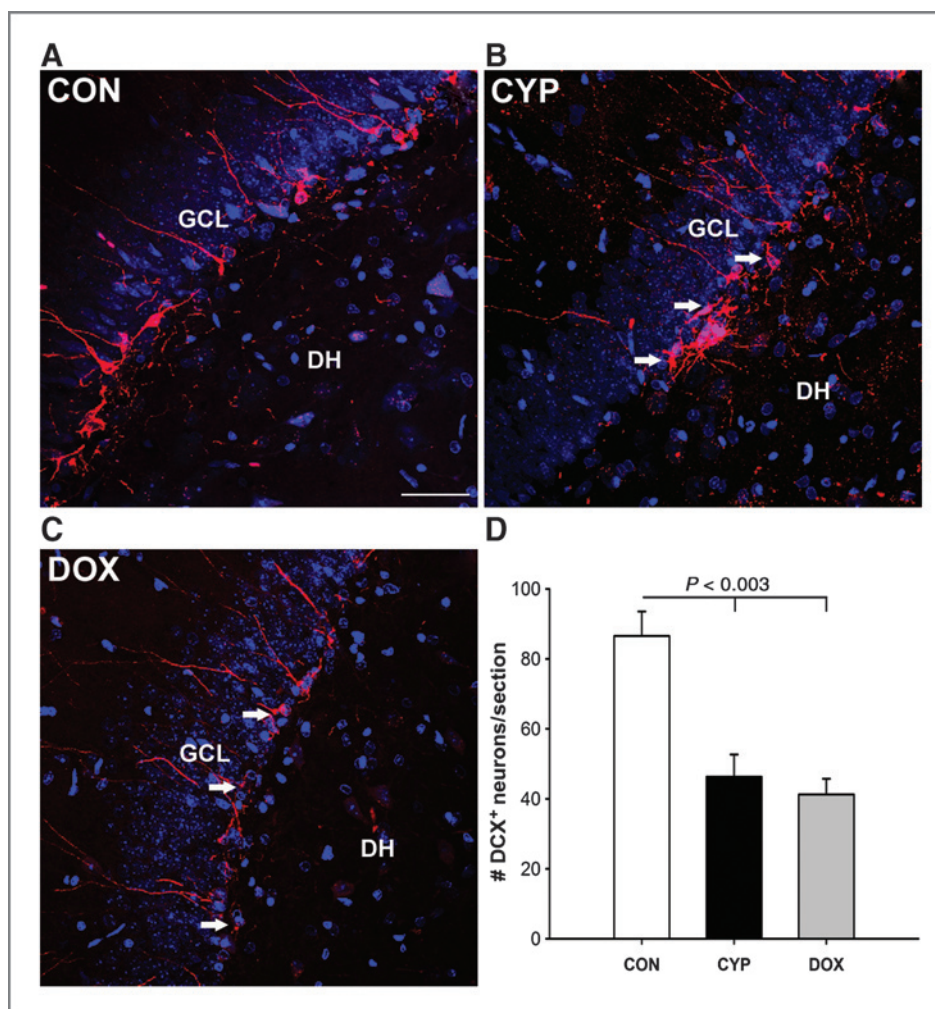
Discussion

Here, we report that chronic exposure to either cyclophosphamide or doxorubicin impairs spatial recognition memory and contextual memory for a learned fear association. Both of these tasks are well known to engage the hippocampus (34–36), and consistent with functional deficits, we observed significant disruptions in hippocampal neurogenesis in treated animals.

The effects of cyclophosphamide and doxorubicin on NPR performance were similar; both cytotoxic agents resulted in reduced exploration of the novel spatial position following a short, 5-minute retention interval compared with saline-treated controls (Fig. 3C). This suggests that memory for the initial spatial configuration of the objects was disrupted by drug treatment (34). There was a trend for this effect to be more pronounced in cyclophosphamide-treated versus doxorubicin-treated animals, although the 2 groups did not differ significantly from each other. Long-term, 24-hour memory for the NPR task did not differ between the groups (Fig. 3D), however, even control animals did not show robust memory at this time point, and so it is difficult to interpret these results. Importantly, initial exploration of the stimuli during the familiarization phase was not reduced in drug-treated animals (Fig. 3A), and speed of locomotion did not differ between the groups (Fig. 3B). These observations suggest that nonspecific, peripheral toxicity effects, such as fatigue and malaise, which might have affected motivation to explore or ambulate, were not major contributing factors to the observed deficits.

A specific impairment was detected with the fear conditioning task (Fig. 4); both cyclophosphamide and doxorubicin animals spent less time engaged in freezing behavior than controls during the context phase of the task. This finding suggests that both chemotherapeutic agents disrupted 24-hour memory for the shock-context association, which has been shown to rely on intact hippocampal function (36). The amount of posttraining freezing observed was comparable between drug-treated and control animals, indicating that neither cyclophosphamide nor doxorubicin treatment affected initial acquisition of the conditioned freezing response. Similarly, drug treatment did not affect freezing behavior during the cue test phase, indicating intact acquisition and memory for the conditioned tone stimulus, which has been shown to rely on intact amygdala function (36). The specific deficits in contextual fear memory observed in the present study suggest

Figure 5. Treatment with doxorubicin (DOX) or cyclophosphamide (CYP) decreases the number of immature neurons (DCX⁺) in the dentate gyrus of the hippocampus. Coronal sections (30 μ m) from control (CON; A), CYP- (B), and DOX (C)-treated animals show that drug treatments (B and C) reduce the number of immature neurons (DCX⁺, red; nucleus, TOTO-3, blue) and contribute to abnormal dendritic morphology and ectopic migration in the dentate hilus (DH; indicated by white arrows). Quantification is presented in D (means \pm 1 SEM). GCL, granule cell layer. Scale bar, 50 μ m (A–C).



that cyclophosphamide and doxorubicin selectively disrupt hippocampal function, which is consistent with the NPR deficits and disruptions in hippocampal neurogenesis that we observed.

To explain these functional decrements, we hypothesized that chemotherapy would have an adverse impact on hippocampal neurogenesis. Significant reductions in the number of mature neurons (BrdUrd-NeuN positive) were found in drug-treated groups compared with controls. In contrast, the number of BrdUrd-positive cells in the hippocampus did not differ between treatment groups, suggesting that the survival of those cells proliferating at the time of BrdUrd injection (i.e., during the week following the 1-month treatment regimen) was unaffected. Moreover, the number of DCX-positive immature neurons in the hippocampus was reduced in both cyclophosphamide- and doxorubicin-treated animals, and cells displayed abnormal dendritic morphology and ectopic migration. Taken together, the present results suggest that the maturation and morphologic development of newly born neurons is severely disrupted under the chronic drug treatment administered. Nonetheless, further studies are needed to clarify whether

treatment-induced reductions in proliferation and/or survival and/or maturation underlie the cognitive deficits observed.

It is also probable that additional microenvironmental factors such as oxidative stress, inflammation, and microvasculature alterations serve to perturb normal physiology and the regulation of endogenous neurogenesis. To address this possibility, we conducted immunohistochemical analysis of a marker of microglia activation, ED-1. Interestingly, we found an increased number of ED-1⁺ cells in the hippocampus of cyclophosphamide- but not doxorubicin-treated animals. While the reason for these group differences are presently uncertain, these data suggest that chemotherapy-induced cognitive impairments may not solely be the result of stem cell depletion but may also be caused by persistent inflammatory signatures. This finding is consistent with previous radiation (reviewed in ref. 38) and chemotherapy (reviewed in ref. 9) studies reporting neuroinflammation as a consequence of cytotoxic treatments.

The current results are consistent with 2 studies conducted in mice in which acute cyclophosphamide treatment

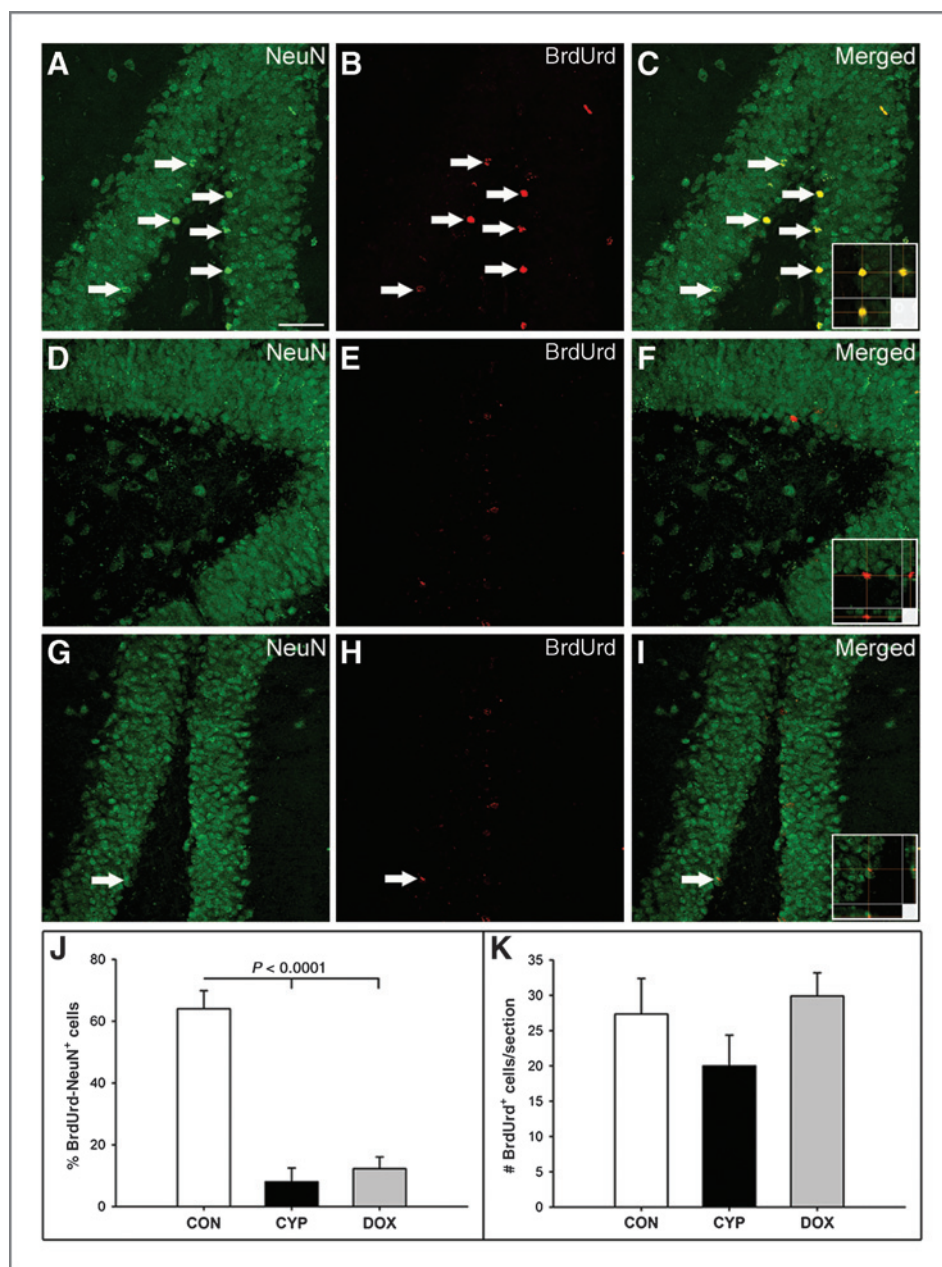
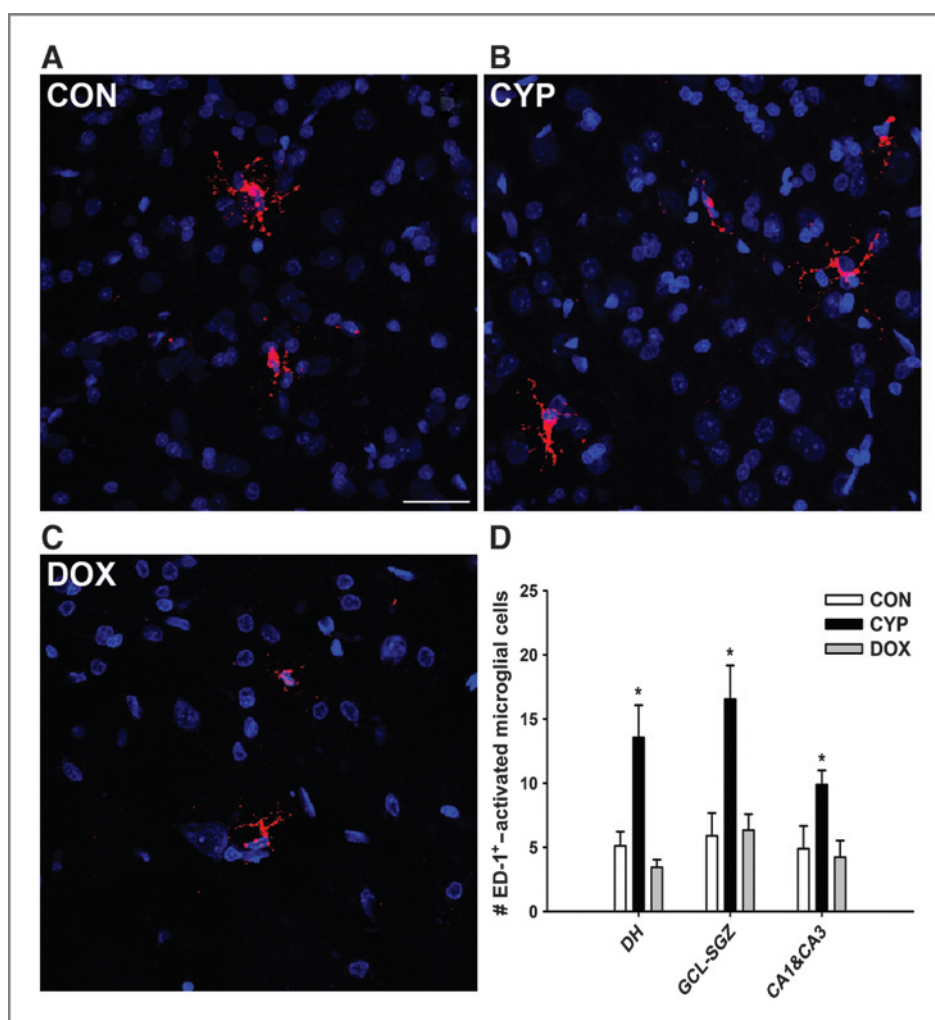


Figure 6. Treatment with doxorubicin (DOX) or cyclophosphamide (CYP) impairs neurogenesis in the dentate gyrus of the hippocampus. Dual immunofluorescence staining for BrdUrd-positive cells (red) coexpressing NeuN (green) show reduced numbers of BrdUrd-NeuN⁺ cells (white arrows) in CYP (D–F) and DOX (G–I) treated groups compared with saline-treated controls (A–C). Orthogonal Z-stack reconstructions of cells of interest are shown in insets in C, F, and I. Note that dual-labeled BrdUrd-NeuN⁺ cells are shown in inserts for C and I only. Quantification of the percentage of BrdUrd⁺ cells expressing NeuN is presented in J, and K shows the average number of BrdUrd⁺ cells per section for each of the treatment groups (means + 1 SEM). Scale bar, 50 μ m (A–I), and inserts, 10 μ m.

resulted in impaired performance on a step-down inhibitory avoidance task (28), a passive avoidance task (29), and a novel object recognition task (29), when animals were assessed at acute time points posttreatment (i.e., 24 and 12 hours posttreatment, respectively). However, our results are not consistent with a report in rats in which a chronic cyclophosphamide treatment paradigm (i.e., one injection every 4 weeks for 16 or 18 weeks) produced a transient *improvement* in spatial learning and memory when animals were assessed at a posttreatment time of 7 weeks (30) or with a recent study in which chronic cyclophosphamide treatment resulted in no change in NPR performance (31). Important differences between the current study and these

previous studies exist. First, our treatment paradigm was not acute as in the former reports showing cyclophosphamide-induced cognitive impairments (28, 30), nor was it as long-term as the study showing cyclophosphamide-induced improvements (30). Although the dosing schedule used in the latter study followed a schedule similar to that administered to patients with breast cancer (i.e., once every 3 weeks for 4 cycles), it did not take into account the physiologic time difference between rats and humans. With a protracted interinjection time of 4 weeks, it is possible that cyclophosphamide-treated animals were able to recover from any direct or indirect neurotoxic effects, as plasma clearance in rats is approximately 7 times faster than in

Figure 7. Representative coronal sections (30 μm) from control (CON; A), cyclophosphamide- (CYP; B), and doxorubicin (DOX; C)-treated animals show that CYP treatment increased the number of ED-1⁺ cells (red; nucleus, TOTO-3, blue) in the hippocampus. Microglia in saline-treated animals show morphologic characteristics consistent with resting microglia (A). CYP treatment led to a significant increase in the number of ED-1⁺ cells with swollen and enlarged cell bodies. Activated microglia in CYP-treated animals were characterized by thicker and longer processes consistent with persistent activation (B). Microglia in DOX-treated animals show normal morphology, and their numbers were not increased. Scale bar, 50 μm (A–C). Quantification is presented in D (means \pm 1 SEM). DH, dentate hilus; CA1&CA3, CA1 and CA3 layers. *, significant differences versus control animals.



humans (39). The study conducted by Lyons and colleagues used a dose of 30 mg/kg given every 2 days for 14 days (31), compared with 50 mg/kg used in the present study. The standard dose of cyclophosphamide used in human patients with breast cancer is 600 mg/m², which is roughly equivalent to 100 mg/kg in a rat. Thus, the dosing schedule and dose used in the present study may have more closely modeled what is used in the clinic for patients with breast cancer. Consistent with this interpretation, a recent study following the same dosing schedule as used in the present study reported a selective deficit in contextual fear conditioning in rats treated with combined cyclophosphamide and doxorubicin (40). Differences in cognitive outcomes between studies highlight the need for additional studies that compare the effects of single and combined chemotherapy agents while controlling dosing schedules and posttreatment assessment times. Furthermore, additional studies evaluating multiple cognitive endpoints will be necessary to clarify conflicting results that may be related to the use of neurogenesis-dependent versus neurogenesis-independent tasks. In support of the current findings,

ablation of hippocampal neurogenesis using focal X-ray irradiation impairs contextual, but not cued, fear conditioning and does not impair water maze or Y-maze performance (41). Thus, if disruption of hippocampal neurogenesis is a major mechanism underlying chemobrain, neurogenesis-dependent tasks may show the greatest sensitivity. Future studies should address this possibility by administering several spatial and nonspatial tasks to assess a broader scope of cognitive abilities.

Several previous reports have established the negative impact of cyclophosphamide treatment on hippocampal cell proliferation (29, 42, 43). Janelins and colleagues showed a 30% decrease in newly divided neural cells (i.e., BrdUrd-positive cells) in the SGZ of the dentate gyrus 24 hours after BrdUrd injections (43). Yang and colleagues report a decrease in proliferating cells (i.e., Ki-67⁺) and immature neurons (DCX⁺) in the dentate gyrus following acute cyclophosphamide exposure (29). Dietrich and colleagues report increased cell death and decreased cell division of CNS progenitor cells compared with multiple cancer cell lines *in vitro* showing the extreme sensitivity of

proliferating cells in the CNS to cyclophosphamide and other chemotherapeutic agents (42). Lyons and colleagues report no change in Ki-67⁺ cells, but a significant drop in BrdUrd⁺ cells in cyclophosphamide-treated animals assessed 1 week after their final cyclophosphamide injection (31). Our current results extend these previous findings by showing that, following a clinically relevant dosing schedule, the detrimental effects of cyclophosphamide on hippocampal neurogenesis persist 1 month after the cessation of treatment. Furthermore, our observations that cyclophosphamide treatment does not significantly impact the number of BrdUrd⁺ cells in the hippocampus but does impair the formation of immature (DCX⁺) neurons suggests that cyclophosphamide-induced disruption of hippocampal cell maturation is a major contributor to the functional deficits observed.

The doxorubicin-induced cognitive deficits observed in the current study are consistent with a report in rats showing dose-specific impairments on an inhibitory avoidance task 24 hours and 7 days after acute doxorubicin administration (32) but are inconsistent with a report showing no effect of doxorubicin treatment on passive avoidance in mice (33). These inconsistencies may be related to the susceptibility of doxorubicin to blood-brain barrier (BBB) resistance due to p-glycoprotein efflux mechanisms (44). However, despite decreased doxorubicin brain penetrance compared with cyclophosphamide, which readily crosses the BBB, hippocampal cell proliferation is disrupted to a similar extent following doxorubicin treatment, as shown in the present study and by others (43). These studies, along with the present results, suggest that low levels of drug penetration in the brain may be sufficient to disrupt sensitive, rapidly dividing cells in the neurogenic regions. This is not altogether unexpected, as many chemotherapeutic agents were developed to target proliferating cancer cells and cause DNA damage that elicits cell kill. Furthermore, BBB damage may also occur as a result of chemotherapeutic treatment, leading to elevated permeability or "leakage," facilitating the entry of neurotoxic agents capable of disrupting neurogenesis and cognition. Further, doxorubicin has been shown to increase peripheral levels of the proinflammatory cytokine TNF α , which itself can cross the BBB leading to increased oxidative stress and inflammation (45).

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In summary, the present results show that clinically relevant doses of cyclophosphamide or doxorubicin, administered following a schedule similar to that used in patients with breast cancer, cause hippocampal-based memory deficits and severe disruptions of hippocampal neurogenesis in a rat model of chemobrain. These results suggest that strategies to prevent or repair unintended disruption of hippocampal neurogenesis may be effective in ameliorating this serious, currently untreated side effect in cancer survivors. Future studies will assess the effects of combined treatment with cyclophosphamide and doxorubicin, as well as the persistence of cognitive deficits following cessation of drug exposure to develop reliable and valid animal models in which preventative and treatment strategies can be evaluated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.-A. Christie, V.K. Parihar, V. Martirosian, A. Nguyen, C.L. Limoli
Writing, review, and/or revision of the manuscript: L.-A. Christie, M.M. Acharya, V.K. Parihar, C.L. Limoli
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