Statement of Translational Relevance
Given the growing number of long-term cancer survivors, it is critical to address the extent, persistence and neuropathological mechanisms underlying chemotherapy-related cognitive decline, or chemobrain. The present study used a rat 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.

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 breast cancer patients, cyclophosphamide and doxorubicin (Adriamycin). Athymic nude rats were given 50mg/kg cyclophosphamide, 2mg/kg doxorubicin or saline injections once per week for 4 weeks. A novel place recognition task and contextual and cued fear conditioning were employed 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 to untreated controls, suggesting disrupted hippocampal-based memory function. Chemotherapy-treated animals showed a significant decline in neurogenesis (80 to 90% drop in BrdU labeled cells expressing NeuN). Activated microglia (ED1 positive) were found after cyclophosphamide, but not doxorubicin treatment.

Conclusions: Our results demonstrate 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.

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 (7). The cognitive domains that are disrupted are diverse and include memory, processing speed, attention and executive function (reviewed in (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 (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, psychological reactions to diagnosis and treatment, 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 (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 two-commonly used cytotoxic agents, cyclophosphamide (CYP), an alkylating agent, and doxorubicin (DOX), a topoisomerase interactive agent, in a rodent model of chemotherapy-induced cognitive decline.

A growing number of studies report chemotherapy-induced changes on cognition and brain function in rodent models (reviewed in (27). Reports on the effects of CYP treatment are equivocal. Two separate mouse studies report transient hippocampal-based memory deficits following a single injection of CYP (28-29). By 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 CYP injection (30), and a recent study reported no change in spatial recognition memory performance 5 days after chronic CYP administration (31). Reports on the cognitive effects of DOX treatment are also mixed. Leidke and colleagues (2009) found a dose- and time-specific effect of chronic treatment on memory acquisition as assessed by an inhibitory avoidance task in male rats (32). By contrast, a study employing a similar hippocampal fear-based task reported no effects of DOX treatment in male and female mice (33).

Thus, while the existing clinical and animal literature suggests that exposure to CYP or DOX 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 CYP or DOX 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 CYP and DOX on hippocampal neurogenesis three 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 (ATN) rats (strain 02N01 Cr:NIH-rnu) were obtained from National Cancer Institute. The ATN rat is a standard model used in xenograft studies that has proven to be a valuable tool in cancer research. ATN rats were used in the present study, as future studies assessing chemotherapy-induced cognitive impairments in human tumor models will require an immunocompromised host. Further, rats (as compared to 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°C ± 1°C; 70% ± 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 DietGeï¿½ Recovery (ClearH2O, Portland, ME) every second day to maintain hydration, stimulate appetite and avoid excessive weight loss. Body weight was measured at baseline and one week after each injection in order to confirm the absence of major peripheral toxicity that might have influenced behavior on subsequent cognitive tests.
Cyclophosphamide (50mg/kg) or doxorubicin (2mg/kg; both from Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline and injected immediately (i.p.). Control animals received 0.9% sterile saline injections (i.p.) of the same volume. Animals in all groups received one injection per week for four consecutive weeks (see Figure 1 for study timeline). Treatment groups were as follows: N=8 saline-treated controls (CON), N=10 cyclophosphamide-treated animals (CYP) and N=9 doxorubicin-treated animals (DOX).

![INSERT FIGURE 1 HERE.]

**Novel place recognition (NPR) task.**

To evaluate the effect of chemotherapy on cognitive performance, the NPR task was administered one week after the 4th 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 × 70 × 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, Leesburg, VA). 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 min before being returned to their home cages. Two toy objects were placed 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 (~8 × 3 × 10 cm 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’), while the other block remained at its former spatial location (‘familiar place’). Rats were allowed to explore the stimuli freely for 3 min. Following the 5-min test phase, rats were returned to their home cages for a delay of 24 h, and then returned to the test arena to explore for 3 min during the 24-h 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 (FC) task.**

A FC task was administered 2 weeks after the final and 4th injection to further characterize the chemotherapy effects on cognitive performance. The FC task consisted of three 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 FC has been shown to rely on intact amygdala function, while contextual FC has been shown to additionally engage the hippocampus (36). For all phases, rats were placed in a clear, acrylic chamber (30 cm × 30 cm × 40 cm high; PhenoTyper 3000, Noldus Information Technology, Leesburg, VA). A video camera centered in the PhenoTyper’s top unit was used to observe the rats during FC 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 FC task began with a 15-minute training phase on day 1. Each rat was placed in the chamber and explored freely for 5 min in order to establish baseline-freezing behavior. A series of 5 tone-shock pairings was administered over the following 5 min. For each pairing, a 2000 Hz, 90 dB was sounded for 30 sec, and a mild (1 mA) footshock was administered concurrent with the final 1 sec 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, Leesburg, VA). Following the final tone-shock pairing, rats were observed for an additional 5 min to assess post-training 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 min, 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 hr later (36).

The cue test phase was administered 1 hr after the context test phase was complete. For this phase, removing the stainless steel grid floor, adding panels to the sides of the FC chamber and cleaning the floor of the chamber using a scented detergent solution all served to change the context. Rats explored freely for 5 min; for the first minute, no tone was played in order to assess pre-cue freezing in the altered context. During the following 3 minutes, the same 2000 Hz, 90 dB tone was played as during training. Cue test freezing behavior was measured during this 3 min period and for an additional minute after the tone was turned off.

**BrdU treatment and immunohistochemistry.**

In order to assess the impact of chemotherapy on hippocampal neurogenesis, 5-bromo-2'-deoxyuridine (BrdU; 100mg/kg i.p.; Sigma-Aldrich, St. Louis, MO) was administered for 6 consecutive days beginning 2 days after the 4th 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 µm-thick sections) through the hippocampus using a cryostat; sections were collected serially (every 20th for DCX and ED-1) in phosphate buffered saline (PBS) with sodium azide.

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% TX-100) for 30 min, then followed by primary antibody incubation (goat-anti-DCX, 1:200, Santa Cruz) overnight at 4°C. Subsequently, biotinylated horse anti-goat IgG (1h, 1:200, Vector Labs) and streptavidin Texas-Red (1h, 1:200, Vector Labs) were used to facilitate color development. The nuclear counterstain was TOTO-3 (1µM for 15min, Sigma-Aldrich).

To identify mature neurons, representative sections were processed using dual immunofluorescence staining for BrdU 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 Tris-buffer saline (TBS, 100 mM, pH 7.6) and subjected to a BrdU-pretreatment protocol using 50% formamide (made in 2X SSC buffer, Sigma-Aldrich) at 68°C for 2 h and 2N HCl (at 37°C for 45 min), followed by serum blocking (10% normal donkey serum, NDS, Sigma Aldrich) and overnight incubation in a rat anti-BrdU solution (1:200, AbD Serotec). The sections were then treated with donkey anti-rat IgG Alexa Fluor 594 (1:200, Invitrogen) for 60 min, 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). BrdU and DCX positive cells were visualized under fluorescence 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, 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 (1µM for 15min, Sigma-Aldrich) to visualize the different hippocampal cell layers.

Confocal analyses were carried out using multiple Z-stacks taken at 1-µm 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 BrdU-positive cells co-expressing the mature neuronal marker, NeuN. At least 50-100 BrdU-positive cells were counted for each animal (6-8 serial sections). The percentage of dual labeled BrdU-NeuN positive cells was derived from 3 individual animals in each group.

For BrdU, DCX and ED-1 enumeration, every 20th section (30 µm 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 µm thick Z-stacks scanned from 3 individual animals in each group. Data for DCX are represented as the mean number of DCX+ neurons in GCL-SGZ region per 30 µm section. ED-1+ and BrdU+ cells were quantified across hippocampal subfields (dentate hilus (DH), granule cell layer-subgranular zone (GCL-SGZ) and CA1 and CA3 layers) and are represented as the mean number of BrdU+ or ED-1+ cells per hippocampal section.

**Statistical analysis.**

All statistical analyses were conducted using PASW Statistics 18 (SPSS, IBM Corporation, Somers, NY). All analyses were 2-tailed, and a value of p ≤ 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’s protected least significant different (FPLSD) post hoc tests to compare the individual groups.

Weight, in grams, was analyzed at five time points (i.e. at baseline and one 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 novel/t novel + t 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 conducting 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 non-specific 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 FC task. For both the baseline and post-training 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 three 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 (BrdU-NeuN and DCX analyses) or repeated measures ANOVA (ED1 analyses). When significant overall group differences were found, post hoc FPLSD tests were conducted, as for analysis of cognitive data.

**Results**

**Chemotherapy-induced weight changes.**

As shown in Figure 2, animals in all groups gained weight over the course of the study, as expected based on their starting age of two-months. Repeated measures ANOVA revealed a significant time x group effect. As can be seen in Figure 2, chemotherapy-treated animals tended to weigh less than controls at 1-week post injection 3 (F(2, 24)=3.35; p=0.052) and 1-week post injection 4 (F(2,24)=3.18; p=0.06). CYP-treated animals weighed approximately 27g (or 8.5%) less than controls one week after the final injection, and DOX-treated animals weighed approximately 33g (or 10.4%) less.

**Effects of chemotherapy on novel place recognition 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 Figure 3A, DOX-treated animals spent significantly more time exploring the stimuli compared to both CYP (p=0.018) and CON (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 (CIs) were as follows: CON (mean=0.816, 95% CI=0.67 – 0.96); CYP (mean=0.577, 95% CI=0.42 – 0.73); DOX (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). CYP-treated animals showed significantly decreased preference for the novel spatial location compared with CON animals (p=0.024). DOX-treated animals did not differ significantly from CON (p=0.119) or CYP animals (p=0.449). One-sample t tests comparing group exploration ratios to chance (i.e. 0.5) revealed that only CON animals showed significant preference for the novel spatial location (t(7)=5.283; p=0.001), while neither chemotherapy-treated group explored the novel place more than chance (CYP, p=0.295; DOX, p=0.084).

Exploration ratios during the 24-hour test did not differ significantly between the groups (Fig. 3D; p=0.586). Further, none of the groups spent significantly more time than expected by chance exploring the novel spatial location (CON, p=0.28; CYP, p=0.53; DOX, p=0.063).

**Effects of chemotherapy on fear conditioning memory performance.**

A significant overall group X 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 FC task only (F(2,24)=11.442, p<0.0001). For the context test, group means and 95% CIs were as follows: CON (mean=64.59; 95% CI=51.58 – 77.6), CYP (mean=34.84; 95% CI=23.93 – 45.75), DOX (mean=34.93; 95% CI=24.68 – 45.17). Post hoc FPLSD tests showed that both CYP (p<0.0001) and DOX (p<0.0001) groups spent significantly decreased percentages of time
freezing compared to the CON group. Groups did not differ significantly in freezing behavior across baseline (p=0.124), post-training (p=0.62), pre-cue test (p=0.395) and cue test (p=0.073) phases.

**INSERT FIGURE 4 HERE.**

**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 (DG). Newly born cells in the subgranular zone (SGZ) and granule cell layer (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 (Figure 5). In contrast to this observation, both chemotherapy groups showed abnormal dendritic development and disorientation of dendritic projections of DCX+ cells into the GCL. Ectopic location of DCX+ cells was also observed frequently in the DH of the hippocampus in DOX-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 CYP (mean=46.28, 95% CI=18.72-73.84) and DOX (mean=41.22, 95% CI=21.8-60.65) groups had significantly fewer DCX+ cells compared to saline-treated controls (mean=86.56, 95% CI=56.49-116.62, both p's≤0.003). By contrast, the number of DCX+ cells did not differ between CYP- and DOX-treated groups (p=0.577). Importantly, the number of newly born DCX+ neurons was reduced by 47% and 53% in CYP and DOX treated groups, respectively. These reductions indicate the marked toxicity of the chemotherapeutic agents on neurogenic cell populations in the hippocampus.

**INSERT FIGURE 5 HERE.**

To further analyze the effects of the drugs on neurogenesis, we quantified the percentage of cells dual-labeled for BrdU and NeuN. In controls, 64% of BrdU-positive cells were also positive for the NeuN marker (Fig. 6J). ANOVA revealed an overall significant effect of group for the percentage of BrdU-NeuN+ cells (F(2,18)=52.97; p=0.0001), and post hoc tests showed that both CYP (mean=7.99; 95% CI=2.53-18.51) and DOX (mean=12.95; 95% CI=5.67-20.24) groups had significantly fewer double-positive labeled cells compared to saline-treated controls (mean=64.04; 95% CI=50.82-77.86, both p's≤0.0001). This represents an 81% drop in CYP-treated animals and 88% drop in DOX-treated animals compared to controls. By contrast, the number of BrdU+ cells per section did not differ between groups (Fig. 6K), suggesting that survival of cells proliferating at the time of BrdU injections was unaffected. Taken together with our findings of reductions in the number of DCX+ neurons and percentage of BrdU-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 CYP or DOX.

**INSERT FIGURE 6 HERE.**

**Effects of chemotherapy on inflammation in the hippocampus.**
Microglia cells are sensors of 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, granule cell layer-subgranular zone, CA1 and CA3 layers) in rats treated with saline, CYP or DOX four weeks after their final injection. 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 (DH, 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 CYP animals had significantly more ED-1+ cells compared to saline-treated controls across all three regions assessed (p's<0.024), while DOX animals did not differ from controls. These results suggest that only CYP treatment resulted in increased inflammation in the hippocampus, as assessed using the ED-1 marker.

**Discussion**

Here we report that chronic exposure to either CYP or DOX 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 CYP and DOX 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 to 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 CYP-treated versus DOX-treated animals, although the two 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 non-specific, 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 FC task (Fig. 4); both CYP and DOX animals spent less time engaged in freezing behavior compared to 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 post-training freezing observed was comparable between drug-treated and control animals, indicating that neither CYP nor DOX 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 that CYP and DOX 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 (BrdU-NeuN positive) were found in drug-treated groups compared to controls. By contrast, the number of BrdU-positive cells in the hippocampus did not differ between treatment groups, suggesting that the survival of those cells proliferating at the time of BrdU injection (i.e. during the week following the one-month treatment regimen) was unaffected. Moreover, the number of DCX-positive immature neurons in the hippocampus was reduced in both CYP- and DOX-treated animals, and cells displayed abnormal dendritic morphology and ectopic migration. Taken together, the present results suggest that the maturation and morphological 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 (38)) and chemotherapy (reviewed in (9)) studies reporting neuroinflammation as a consequence of cytotoxic treatments.

The current results are consistent with two studies conducted in mice in which acute CYP treatment 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 post-treatment (i.e. 24 and 12 hours post-treatment, respectively). However, our results are not consistent with a report in rats in which a chronic CYP 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 post-treatment time of 7 weeks (30) or with a recent study in which chronic CYP treatment resulted in no change in novel place recognition 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 CYP-induced cognitive impairments (28, 30), nor was it as long-term as the study showing CYP-induced improvements (30). Although the dosing schedule used in the latter study followed a schedule similar to that administered to breast cancer patients (i.e. once every 3 weeks for 4 cycles), it did not take into account the physiological time difference between rats and humans. With a protracted inter-injection time of 4 weeks, it is possible that CYP-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 humans (39). The study conducted by Lyons and colleagues (2011) used a dose of 30mg/kg given every 2 days for 14 days (31), compared to 50mg/kg used in the present study. The standard dose of cyclophosphamide used in human breast cancer patients is 600mg/m², which is roughly equivalent to 100mg/kg in a rat. Thus, the dosing schedule and dose used in the present study may have more closely modeled what is used in clinic for breast cancer patients. 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 CYP and DOX (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 post-treatment assessment times. Further, 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 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 non-spatial tasks to assess a broader scope of cognitive abilities.

Several previous reports have established the negative impact of CYP treatment on hippocampal cell proliferation (29, 42-43). Janelsins and colleagues (2009) show a 30% decrease in newly divided neural cells (i.e. BrdU-positive cells) in the subgranular zone of the dentate gyrus 24hr after BrdU 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 CYP exposure (29). Dietrich and colleagues (2006) report increased cell death and decreased cell division of CNS progenitor cells compared to multiple
cancer cell lines in vitro demonstrating the extreme sensitivity of proliferating cells in the CNS to CYP and other chemotherapeutic agents (42). Lyons and colleagues (2011) report no change in Ki-67+ cells, but a significant drop in BrdU+ cells in CYP-treated animals assessed one week after their final CYP injection (31). Our current results extend these previous findings by showing that, following a clinically relevant dosing schedule, the detrimental effects of CYP on hippocampal neurogenesis persist 1 month after the cessation of treatment. Further, our observations that CYP treatment does not significantly impact the number of BrdU+ cells in the hippocampus but does impair the formation of immature (DCX+) neurons suggests that CYP-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 DOX administration (32), but are inconsistent with a report showing no effect of DOX treatment on passive avoidance in mice (33). These inconsistencies may be related to the susceptibility of DOX to blood-brain-barrier (BBB) resistance due to p-glycoprotein efflux mechanisms (44). However, despite decreased DOX brain penetrance compared to CYP, which readily crosses the BBB, hippocampal cell proliferation is disrupted to a similar extent following DOX 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. Further, 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. For example, DOX has been shown to increase peripheral levels of the proinflammatory cytokine tumor necrosis factor (TNFα), which itself can cross the BBB leading to increased oxidative stress and inflammation (45).

In summary, the present results show that clinically-relevant doses of CYP or DOX, administered following a schedule similar to that used in breast cancer patients, causes hippocampal-based memory deficits and severe disruptions of hippocampal neurogenesis in a rodent 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 CYP and DOX, as well as the persistence of cognitive deficits following cessation of drug exposure in order to develop reliable and valid animal models of chemotherapy-induced cognitive decline in which preventative and treatment strategies can be evaluated.

**Figure Legends**

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

**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 one week after each injection. Data are presented as means ± 1 SEM.

**Figure 3.** Treatment with cyclophosphamide (CYP) or doxorubicin (DOX) impairs spatial recognition memory performance on a novel place recognition 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 $\pm$ 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, and * in C indicates a significantly higher proportion of time spent exploring the novel spatial location than expected by chance in saline-treated (CON) animals.

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 ('Post-training'). The context test was administered 24 hour later, and chemotherapy-treated animals showed decreased freezing compared to 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 upon sounding of the tone ('Cue Test'). Data are presented as means $\pm$ 1 SEM. P values are derived from FPLSD post hoc comparisons.

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 (30µm) from Control (A), CYP (B), and DOX (C) treated animals show that drug-treatments (B,C) reduce the number of immature neurons (DCX+, red; nucleus, TOTO-3, blue) and contribute to abnormal dendritic morphology and ectopic migration in the DH (indicated by white arrows). Quantification is presented in panel D (means $\pm$ 1 S.E.M.). DH, dentate hilus and GCL, granule cell layer. Scale bar=50 µm, A-C.

Figure 6. Treatment with doxorubicin (DCX) or cyclophosphamide (CYP) impairs neurogenesis in the dentate gyrus of the hippocampus. Dual immunofluorescence staining for BrdU-positive cells (red) co-expressing NeuN (green) show reduced numbers of BrdU-NeuN+ cells (white arrows) in CYP and DOX treated groups (D - I) compared to saline treated controls (A - C). Orthogonal Z-stack reconstructions of cells of interest are shown in insets in panels C, F and I. Note that dual-labeled BrdU-NeuN+ cells are shown in inserts for Panels C and I only. Quantification of the percentage of BrdU+ cells expressing NeuN is presented in panel J, and panel K shows the average number of BrdU+ cells per section for each of the treatment groups (means $\pm$ 1 S.E.M.). Scale bar=50 µm, A-I, and inserts, 10µm.

Figure 7. Representative coronal sections (30µm) 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 morphological characteristics consistent with resting microglia (A). CYP treatment led to a significant increase in the number of ED1+ 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 panel D (means $\pm$ 1 S.E.M.). DH, dentate hilus; GCL-SGZ, granule cell layer-subgranular zone; CA1&CA3, CA1 and CA3 layers. *’s indicate significant differences versus control animals.

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References


Figure 1
Figure 2

The graph shows the weight (g) over time for different groups: CON, CYP, and DOX. The x-axis represents the time points: baseline, 1 wk post inj 1, 2 wk post inj 2, 3 wk post inj 3, and 4 wk post inj 4. The y-axis represents the weight in grams, ranging from 200 to 340 g. The CON group shows a consistent weight gain, the CYP group has a slight increase, and the DOX group shows a more significant weight gain compared to the other two groups.
Figure 6
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Impaired Cognitive Function and Hippocampal Neurogenesis Following Cancer Chemotherapy


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