Transient Improvement in Cognitive Function and Synaptic Plasticity in Rats Following Cancer Chemotherapy

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Abstract

Background: Cancer chemotherapy has been associated with cognitive impairment. Several issues complicate such findings including the patients' health, use of multiple chemotherapeutic agents, and proper assessment of cognition. To control these factors, we conducted cognitive studies in female rats receiving cyclophosphamide or 5-fluorouracil (5FU).

Methods: Young (7 months) female Fischer-344 rats received five injections of cyclophosphamide (100 mg/kg), 5FU (150 mg/kg), or saline i.p. every 4 weeks for a total of 18 weeks. Aged (18 months) female Fischer-344 rats were treated with cyclophosphamide (80 mg/kg i.p.) for 16 weeks. After 8 to 10 weeks of recovery, rats were tested in two maze learning tasks, the Morris water maze and the Stone 14-unit T-maze. Neuronal synaptic function was assessed by examining long-term potentiation (LTP) in hippocampal slices obtained from young cyclophosphamide-treated rats.

Results: Despite the toxic effects induced by chemotherapy, cyclophosphamide- and 5FU-treated rats showed significantly better maze performance compared with controls. Following 29 to 42 weeks of recovery from chemotherapy, no significant effects were observed on maze performance. In aged rats, cyclophosphamide treatment for 14 weeks also produced toxicity, but no impairment in Stone maze learning after 16 weeks of recovery. When assessed during cyclophosphamide treatment, evidence of impaired LTP emerged; however, with 8 weeks of recovery following five cyclophosphamide treatments, we observed enhanced LTP.

Conclusion: Despite toxicity accompanying chemotherapy, no evidence of impaired cognitive performance emerged after recovery. Indeed, following 7 to 9 weeks of recovery, we noted evidence of improved learning and LTP.

Clinical studies of cancer patients have revealed evidence of long-term cognitive impairment associated with chemotherapy (1–3). For example, breast carcinoma patients receiving adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil (5FU) chemotherapy exhibited a significantly higher risk of cognitive impairment (20–30% versus 3–6%) compared with controls (1). van Dam et al. (4) assessed cognitive function in three groups of patients with breast cancer who had received either high-dose chemotherapy, 2 years of standard-dose chemotherapy, or control patients. High-dose chemotherapy comprised four cycles of 5FU, epirubicin, and cyclophosphamide, followed by a single dose of high-dose cyclophosphamide, thiopeta, and carboplatin. The standard-dose chemotherapy group received four cycles of 5FU, epirubicin, and cyclophosphamide. The risk of cognitive impairment in high-dose chemotherapy patients was 8.2 times higher than control risk and was 3.5 times higher than for standard-dose chemotherapy patients. Waber et al. treated children for lymphoblastic leukemia or Wilms's tumor with prednisone, doxorubicin, vincristine, and methotrexate plus radiotherapy and noted that females seemed more susceptible than males to chemotherapy-induced cognitive impairment (5).

Several mechanisms have been suggested to explain cognitive impairment associated with chemotherapy, including indirect chemical toxicity and oxidative damage, direct injury to neurons, inflammation, or induction of autoimmune responses (6). Studies of cyclophosphamide, methotrexate, and 5FU indicated that toxicity resulted from metabolites of cyclophosphamide, such as acrolein or phosphoramide mustard (7), which induce lipid peroxidation by reducing antioxidant activity in erythrocytes, or 2-chloroacetaldehyde, which can deplete cerebral glutathione (8). Direct neurotoxicity of 5FU might occur via its monofluorinated metabolites (9). The overall effect is to reduce cellular resistance to oxidative stress which can damage the blood-brain barrier and thus allow the entry of possible neurotoxic molecules into the brain (10).
Establishing a causal connection between cognitive impairment and cancer chemotherapy can be complicated by factors affecting past clinical studies as follows: (a) poor control over possible health problems, including those resulting from depression and the cancer itself; (b) use of multiple chemotherapeutic agents, which hinders identification of specific routes of neurotoxicity; (c) proper cognitive assessments; and (d) persistent effects of having had cancer and living with the risk of recurrence. To provide better experimental control over these factors than attempted previously in clinical studies, we evaluated the effects of two chemotherapeutic agents, cyclophosphamide and 5FU, on the performance of female rats in two complex learning tasks (Morris water maze and Stone 14-unit T-maze) and on hippocampal long-term potentiation (LTP) as an electrophysiologic measure of neuronal synaptic strength. The cytotoxic effects of cyclophosphamide are directly related to DNA alkylation, which causes a major disruption in nucleic acid function. The cytotoxic effects of 5FU are largely produced by inhibiting thymidylate synthase, thereby reducing TTP and causing DNA strand breakage. Both agents are widely and successfully used in cancer chemotherapeutic protocols. Our objective was to simulate long-term (4 months) adjuvant therapy at doses that would produce minimal systemic toxicity. To our knowledge, this was the first attempt to examine the effects on cognition of such chemotherapy in an animal model.

Materials and Methods

Subjects. Young (7 months) or aged (18 months) female Fischer-344 rats were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The rats were housed in a specific pathogen–free vivarium under controlled conditions (22 ± 1°C, 70 ± 10% humidity) and under artificial light (12 hour light/dark cycle) at the Gerontology Research Center (Baltimore, MD). A conventional diet (NIH-07) was provided ad libitum, as was access to water through an automated and filtered system. All treatment and testing occurred during the light cycle (6 a.m.-6 p.m.). Because of body weight loss and dental problems observed during chemotherapy, rats received special feeding with water-softened food pellets, the same type of food that control rats received, but moistened, every day.

Drug treatments. Cyclophosphamide (100 mg/kg) or 5FU (150 mg/kg, both from Sigma-Aldrich, St. Louis, MO) were dissolved in sterilized saline. The drugs were injected i.p., and the solution was kept in a 60°C water bath periodically until all animals received injection. The control group was injected with 0.9% saline.

We used two different age groups of rats for this investigation. First, we evaluated a young group starting treatment at 7 months and injected with either cyclophosphamide (100 mg/kg, n = 25), 5FU (150 mg/kg, n = 25), or saline (n = 23) as control every 4 weeks for 12 weeks, and then with the last injection occurring after 6 weeks because of toxicity. Second, we examined an aged group starting treatment at 18 months and injected with only cyclophosphamide at a lower dose than provided to young animals (80 mg/kg, n = 17) or saline (n = 16) as control every 4 weeks for 12 weeks, and then with the last injection again occurring after 6 weeks because of toxicity. The rationale for using an aged group was that we wanted to assess whether there would be an age-related increase in behavioral toxicity of the chemotherapy.

Hematology tests. After the first injection of either cyclophosphamide (100 mg/kg) or 5FU (150 mg/kg) on days 4, 6, and during the 4th, 6th, 15th, and 23rd weeks (first recovery period), and 60 weeks (second recovery period), under isoflurane anesthesia (Abbott Laboratories, North Chicago, IL), venous blood samples were taken from the right ventricle of the rats. The blood samples were collected in EDTA for red cell function tests.

Hematocrit. Hematocrit was determined using standard procedures (12,000 rpm for 5 minutes), in a microhematocrit microcapillary centrifuge (IBC model MB), and the percentage of packed cells was estimated.

Mean cell volume. Red cell volumes were determined using a model ZM Coulter counter (Beckman Coulter, Inc., Miami, FL) with a 1,000 channelizer and a Coulter multisizer. Washed, packed red cells were diluted 200,000-fold in PBS-EDTA (pH 7.4) to measure cell volumes of normal biconcave cells (11).

Cell transit analyzer. The transit time of washed red cells was assessed using a Cell transit analyzer (AbX, Montpeliere, France) that measures rate of flow through oligopore filter pores (11). In brief, for each determination, at least 2,000 cells were measured to obtain a mean cell transit time.

Spatial learning analysis. To evaluate the effect of chemotherapy on cognitive performance, saline, cyclophosphamide, and 5FU groups (n = 8) were assessed for performance in a Morris water maze test and/or a Stone 14-unit T-maze. Both of these behavioral paradigms have been used to assess the effects of specific brain injuries on learning performance, and are particularly sensitive to damage of the hippocampal formation and its connections (12, 13). In the study of young rats, the first behavioral testing was initiated 7 weeks after the animals received the last injection of either cyclophosphamide or 5FU. In a separate group, behavioral testing was applied after chemotherapy was halted for 29 weeks (>7 months). In the study of aged rats, the behavioral test was applied 16 weeks after the last treatment with cyclophosphamide (at 24 months of age), and the animals received only Stone maze testing. Each maze test lasted for 1 week, and there was a 1- to 2-week break between the two maze learning tasks when applied in the young groups.

Morris water maze test. This task was conducted with a modified Morris’ protocol (14). Briefly, rats were trained in a 210 cm diameter open-field water maze tank filled with water at 21°C and made opaque with a nontoxic washable white powder (Palmer Paint Products, Inc., Troy, MI). Prominent extra-maze visual cues around the room remained in fixed positions throughout the experiment. A video tracking system (Water Maze Version 3.44 for the Videomex-V; Columbus Instruments, Columbus OH) was used to track the movement of the rats in the water tank. Behavioral data were collected in the following sequence: (a) Visible platform training: rats were first required to locate a square platform (11 × 11 cm), positioned 2.5 cm above the water surface, and they were allowed to rest on the platform for 20 seconds within each trial. Swim time to the platform was recorded as the performance measure. If a rat failed to find the platform within 90 seconds, it would be manually guided to the platform and allowed to rest for 20 seconds. Training involved four trials per session per day for 2 to 3 consecutive days. (b) Hidden platform training: four equally spaced points (north, south, east, and west) around the edge of the pool were used as randomized starting positions, and the rats were given four trials per day for 5 consecutive days (for the 7-week recovery group) or 4 days (for the 29-week recovery group). The rats were first required to locate the same platform, which was positioned 2.5 cm below the water surface, and allowed to rest for 20 seconds. If the rat had not found the platform within 90 seconds, it would be guided manually to the platform. After a 20-second rest, the rat was immediately returned to the pool at a different starting position. The total distance and time each animal swam before reaching the platform on each trial was recorded as the measure of learning. (c) Probe trial of spatial memory: 24 hours after the last hidden platform training, the platform was removed, and each rat was tested within a 90-second probe trial. Performance was assessed using a measure of proximity, which is the cumulative score of the rat from the center of the platform as recorded every second during the probe test, a unique method developed by Gallagher and associates for analysis of performance in this task (15).

Stone 14-unit T-maze. All young rats that had been evaluated in the water maze were evaluated in this task which consisted of two phases. First, they received pretraining for shock avoidance conducted in a...
straight runway (~2 m in length) as described in detail previously (11), which was equivalent to evaluating performance in the visible platform task in the water maze. Successful completion of pretraining in the Stone maze was determined by reaching a criterion of eightavoidances during 10 trials within a maximum of 30 trials.

Training was then conducted in a clear acrylic 14-unit T-maze that has been described previously (11). Briefly, the maze was separated into five distinct sections. To avoid a mild foot shock delivered through a stainless steel grid floor, rats were trained to make 14 correct left-right discriminations in order to negotiate the maze from start box to goal box. The rat had 10 seconds to move through each of the five maze segments to avoid the onset of the foot shock, which was terminated when the rat moved to the next segment. The dependent measure of maze learning was errors made (entries into incorrect arms of the maze or retracing their movements) and run time from the start box to the goal box. A total of 15 trials were conducted during one session. Each rat was trained continuously in the first eight trials, and then allowed to rest in their transfer cages with sufficient water supply for more than 2 hours, after which time they received the final seven trials for that day. Rats from each treatment group (n = 8) were tested at two intervals after the fifth injection, either the ninth week or the 42nd week.

Slice preparation and electrophysiology. To examine the effects of the chemotherapeutic regimens of synaptic plasticity, LTP was evaluated both during chemotherapy and after 7 and 53 weeks of recovery from the last treatment. Standard procedures for preparing and maintaining transverse rat hippocampal slices (350 μm) were used as described (16). Slices were allowed to recover for at least 1 hour, and kept for up to 6 hours, and then transferred to a submerged recording chamber and was continuously perfused at 2 mL/min with artificial cerebrospinal fluid with the temperature of the medium maintained at 30°C to 32°C. (16). All solutions for LTP studies contained 50 μmol/L picrotoxin to block γ-aminobutyric acid–mediated activity. Extracellular field excitatory postsynaptic potentials were recorded in CA1 stratum radiatum. The stimuli (30 μs duration at a frequency of 0.033 Hz) were delivered through fine bipolar tungsten electrodes to activate Schaffer collateral/commissural afferents using a S48K stimulator (Grass Instrument, West Warwick, RI; ref. 17). This experiment was conducted on rats at three time points after the chemotherapy: 15 weeks after the first cyclophosphamide treatment (before the fifth cyclophosphamide injection), then 8 or 53 weeks after the last cyclophosphamide treatment. The LTP-inducing stimulus consisted of one train of 100 stimuli at 100 Hz. Data were collected and analyzed offline using an Axopatch 1D Patch Clamp and pCLAMP 8 software (Axon Instruments, Foster City, CA).

Results

Toxicity induced by chemotherapy

Beginning at 7 months of age, female rats receiving saline exhibited steady weight gains (Fig. 1A). Rats receiving cyclophosphamide or 5FU treatments exhibited a pattern of body weight loss and recovery after each injection. After the third injection, cyclophosphamide-treated rats began to display additional signs of toxicity including aberrant growth of teeth, overgrowth of claws, and poor coat quality.

Cyclophosphamide treatment resulted in the deaths of two young rats following the fourth injection (Table 1); thus, a decision was made to postpone the fifth injection by 2 weeks. Two cyclophosphamide-treated rats also showed transient impairment in locomotion. After the fifth injection, two more young rats died. Due to this unexpected toxicity, chemotherapy was terminated after the fifth injection. No deaths were noted in the 5FU group despite evidence of toxicity.

When cyclophosphamide treatment was initiated in aged (18 months) rats at a lower dose than provided to young rats,
they experienced toxic effects on body weight and physical appearance as observed in younger rats, including poor coat quality and overgrowth of teeth (Table 1). Although only one saline-treated rat was removed from the experiment due to a skin problem, two cyclophosphamide-treated rats died 2 to 4 weeks after the fourth injection and three more rats died after the fifth injection, whereas there were no deaths in the saline-treated group. As seen in Fig. 1B, rats lost body weight after every cyclophosphamide injection, and they failed to return to their baseline body weights after the last treatment. Thus, as observed in young rats, cyclophosphamide treatment was clearly toxic to aged rats despite the lower dose administered.

Selected hematologic measures of toxicity in both young and aged rats, including hematocrit, mean cell volume (MCV), and flow properties as measured by a cell transit analyzer, were evident but inconsistent across treatment intervals and groups. A summary of results are listed in Table 1. In general, there were transient reductions in hematocrit and increases in MCV.

**Table 1. Record of toxicity in both death and hematology test after cyclophosphamide and 5FU treatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline</th>
<th>Cyclophosphamide</th>
<th>5FU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Aged</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young</td>
<td>Aged</td>
<td></td>
</tr>
<tr>
<td>Toxicity record</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Death before the fourth injection</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Death during the fourth to fifth injection</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Death after the fifth injection</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Dental overgrowth</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Hematology record</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematocrit 6th day after the first injection</td>
<td>46.9 ± 1.8</td>
<td>N/A</td>
<td>36.7 ± 2.3*</td>
</tr>
<tr>
<td>Hematocrit 4 weeks after the first injection</td>
<td>45.7 ± 1.0</td>
<td>N/A</td>
<td>48.4 ± 1.2</td>
</tr>
<tr>
<td>Hematocrit 10 weeks after termination of chemotherapy</td>
<td>45.0 ± 1.2</td>
<td>43.0 ± 1.2</td>
<td>45.0 ± 0.6</td>
</tr>
<tr>
<td>MCV 8 weeks after the first injection</td>
<td>53.8 ± 0.7</td>
<td>N/A</td>
<td>53.9 ± 0.3</td>
</tr>
<tr>
<td>MCV 14 weeks after the first injection</td>
<td>50.8 ± 0.2</td>
<td>N/A</td>
<td>54.9 ± 0.3*</td>
</tr>
<tr>
<td>MCV 10 weeks after termination of chemotherapy</td>
<td>51.8 ± 0.2</td>
<td>53.7 ± 0.3</td>
<td>54.4 ± 0.2*</td>
</tr>
<tr>
<td>Cell transit analyzer 8 weeks after the first injection</td>
<td>2.2 ± 0.04</td>
<td>N/A</td>
<td>2.2 ± 0.03</td>
</tr>
<tr>
<td>Cell transit analyzer 12 weeks after termination of chemotherapy</td>
<td>2.1 ± 0.03</td>
<td>N/A</td>
<td>2.0 ± 0.03</td>
</tr>
</tbody>
</table>

NOTE: Toxicity record: in the young group, after the fourth injection, two cyclophosphamide-treated rats were found dead in their cages. Another two rats in the cyclophosphamide group showed transient impairment in walking performance, but recovered before the last treatment. After the fifth injection, two more rats died. Rats that experienced long-term cyclophosphamide treatment (around 14 weeks after the first injection) showed dental problems. Hematology record (mean and SE): hematocrit measurement indicated that acute cyclophosphamide reduced hematocrit. After recovery from chemotherapy, rats also showed an increase in the MCV of RBC, which may indicate an enhanced regeneration of RBC. However, there was no evidence of chemotherapy-induced changes in deformability of RBC as measured by cell transit time.

*Indicates a significant difference from saline controls ($P < 0.05$).

**Effects of chemotherapy on Morris water maze performance**

**Visible platform.** After 7 or 29 weeks of recovery from multiple injections of either cyclophosphamide or 5FU, rats were trained in the Morris water maze. To assess the ability to swim and use visual cues for navigation, the first phase of training involved swimming to a visible platform. As depicted in Fig. 2A, swim time to the visible platform was significantly shorter for both cyclophosphamide and 5FU groups compared with saline controls in both sessions. The same analysis of swim time for rats following 29 weeks of recovery did not reveal significant group effects during either session (Fig. 2B).

**Hidden platform.** After visible platform training, spatial learning was assessed using the hidden platform task. Performance was measured as the distance swum to the submerged platform on each trial. Rats could use visual cues surrounding the pool to orient in space and locate the hidden platform. As observed in Fig. 3A, all groups declined in distance to platform as a function of sessions. However, 7 weeks after the last treatment, the performance of rats receiving either cyclophosphamide or 5FU treatment was clearly superior to that of saline controls. Notably, after 29 weeks of recovery from chemotherapy, performance in the hidden platform task was not significantly different among the treatment groups (Fig. 3B).
memory performance in the water maze task, we found no significant differences among the three treatments after 29 weeks of recovery from chemotherapy (29 weeks, Fig. 3C).

Effects of chemotherapy on Stone maze performance

For assessing performance in the Stone maze, we used the same groups of young rats that were tested in the water maze 2 weeks previously. As observed in Fig. 4A, no significant group differences among young rats were noted during pretraining in the straight runway task following either 9 or 42 weeks of recovery. Similarly, after 16 weeks of recovery for aged rats, there were no significant differences between the saline and cyclophosphamide groups in trails to criterion. Analysis of errors made by young rats during Stone maze training following 9 weeks of recovery paralleled the results in the Morris water maze. As depicted in Fig. 4C, saline-treated animals showed rapid learning in the maze as evidenced by a steady decline in errors across trials; however, both cyclophosphamide- and 5FU-treated groups showed significantly superior performance compared with controls. Similar findings were noted in the analysis of runtime in the Stone maze (results not shown).

As documented for the water maze task with longer recovery from chemotherapy, analysis of Stone maze performance in young rats after 42 weeks of recovery from chemotherapy failed to show a significant group effect (Fig. 4D). Again, the analysis of runtime in the maze yielded the same observations as the analysis of errors—no treatment effects (results not shown). Thus, with longer recovery periods, the enhanced performance of rats that had received chemotherapy was no longer observed, but these treatments also had no significant negative effects despite evidence of toxicity.

As observed in Fig. 4B, the learning performance of aged rats in the Stone maze was also unaffected by cyclophosphamide treatment after allowing 16 weeks of recovery from 16 weeks of chemotherapy. Runtime during acquisition in the Stone maze did not differ significantly among groups (\( F_{1,14} = 0.83, P > 0.2 \); data not shown).

Effects of chemotherapy on LTP

To investigate the possible long-term consequences of cyclophosphamide exposure on synaptic plasticity, analysis of LTP in hippocampal slices was conducted in vitro. When slices were obtained from rats that had received the first cyclophosphamide treatment for 15 weeks (four injections), LTP was not induced (\( P > 0.05 \)), as compared with control slices where LTP was clearly observed (\( P < 0.05 \), Fig. 5A). However, 8 weeks after the last cyclophosphamide treatment, LTP in the cyclophosphamide group was induced, and when comparing responses to the saline rats, were significantly greater during the last 10-minute interval of recording (\( P < 0.05 \); Fig. 5B). After 53 weeks of recovery from chemotherapy, LTP in slices from the cyclophosphamide group was still slightly higher than that recorded in the saline group during the first 10-minute interval poststimulation (\( P < 0.05 \); Fig. 5C). Therefore, induction of LTP was impaired during cyclophosphamide treatment, but was enhanced after recovery from chemotherapy, even after an interval at which no significant effects on learning performance were observed.

After acquisition testing, spatial memory performance was assessed in a probe test in which the platform was removed from the pool. Rats could show spatial memory by swimming in proximity to the previous location of the platform. Analysis of proximity measure after 7 weeks of recovery from chemotherapy further confirmed the superior performance of cyclophosphamide- and 5FU-treated rats compared with saline controls (7 weeks, Fig. 3C). Regarding these measures of spatial...
Discussion

To our knowledge, the current study provides the first systematic investigation in a rodent model assessing the effects of conventional chemotherapeutic agents, cyclophosphamide and 5FU, on cognition. Treatment of young female rats with cyclophosphamide or 5FU, and treatment of aged female rats with cyclophosphamide produced clear toxic effects. During a 4-month regimen, treated rats exhibited significant weight loss, poor coat quality, damaged dentition and nails, and mortality. Effects were more obvious in cyclophosphamide-treated rats compared with 5FU-treated rats. Cyclophosphamide-treated rats showed unrecoverable weight loss, impaired locomotion, altered physical appearance, and mortality.

The original objective was to identify and use doses of these agents with minimal acute toxicity for long-term studies, such that any observed cognitive impairment could not be attributed to systemic toxicity. In view of this objective, chemotherapy was terminated earlier than the projected six cycles because of the toxicity and mortality observed. We allowed a minimum of 7 weeks recovery from chemotherapy before behavioral testing. Unexpectedly, rather than adversely affecting maze performance, the experience of chemotherapy actually enhanced performance after treatment compared with controls in both the Morris water maze and the Stone 14-unit T-maze. The enhanced learning performance of young female rats following cyclophosphamide or 5FU treatment proved to be transient. After 42 weeks of recovery from chemotherapy, we noted no significant performance differences in the two tasks among treatment groups. Thus, it was clear that with sufficient recovery after chronic cyclophosphamide or 5FU treatment at doses producing systemic toxicity, young female rats did not exhibit impaired cognitive performance in two behavioral tasks sensitive to damage in neural systems critical to spatial learning and memory.

To determine if chemotherapy might have a greater cognitive effect on older rats than younger counterparts, we evaluated the Stone maze performance of 24-month-old female rats undergoing cyclophosphamide treatment for 16 weeks and recovery for 16 weeks. Although the dose was less than that administered to young rats (80 versus 100 mg/kg), evidence of toxicity remained, including loss of body weight and mortality. Nonetheless, we observed no significant effects of cyclophosphamide treatment on learning performance.

In addition to analyzing behavioral function, we assessed LTP in hippocampal slices from young female rats obtained at different time points of cyclophosphamide treatment. The results indicated that when slices were obtained from rats undergoing current cyclophosphamide treatment, impaired LTP induction was observed in these rats compared with

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**Fig. 4.** Performance in Stone maze tests. Pretraining: number of trials required for rat to reach 8 of 10 correct avoidances (A). Columns, mean number of trials for each session of each treatment group; bars, ± SE. No significant treatment group differences were found in young rats after 9 or 42 weeks recovery from chemotherapy, or in the aged group after 16 weeks for recovery (9 weeks: $F_{2,22} = 0.40, P > 0.5$; 42 weeks: $F_{2,22} = 1.75, P > 0.1$). Additionally, there were no significant group differences among the aged rats ($F_{1,13} = 3.11, P > 0.1$). Stone maze: errors (± SE) committed in the Stone maze across trial blocks for young rats treated with saline, cyclophosphamide, or 5FU and allowed to recover for aged (B) and young of either 9 (C) or 42 (D) weeks after recovery from chemotherapy. B, maze performance in aged animals that had recovered from cyclophosphamide treatment for 9 weeks did not differ significantly in error performance from saline controls, nor were there significant group differences in errors committed ($F_{2,14} = 0.17, P > 0.5$). C, with 9 weeks of recovery, rats treated with cyclophosphamide or 5FU showed superior learning performance compared with saline controls. Results of a two-way ANOVA with repeated measures across five blocks confirmed the significant effect of group ($F_{2,19} = 3.53, P < 0.05$) and day ($F_{4,38} = 60.21, P < 0.001$) without significant interaction. D, with 42 weeks of recovery from chemotherapy, the cyclophosphamide-treated and 5FU-treated rats did not differ significantly from the saline group in errors committed. Although the results of a two-way ANOVA yielded a significant block effect ($F_{4,12} = 32.83, P < 0.001$), there was no significant group effect or group by block interaction.
controls. However, after 9 weeks of recovery, LTP in cyclophosphamide-treated rats was more readily produced and could be sustained longer compared with controls, which was consistent with the improved learning performance observed within this recovery period. A modest improvement in LTP was maintained even in slices from rats recovered from cyclophosphamide treatment for 53 weeks. This observation was noted even though no differences in maze learning were found after the same recovery period. Thus, whereas enhancement of LTP was observed to be long-lasting after chemotherapy, enhanced cognitive performance was transient.

Regarding the unexpected behavioral results, we can first consider the selection of agents. Cyclophosphamide and 5FU were chosen because of their extensive use in the clinical treatment of several types of cancer, including breast cancer. Although neither drug was expected to produce central effects directly because of their modes of action, we hypothesized that neurotoxicity might occur through other indirect mechanisms, such as oxidized hemoglobin impairing oxygen supply to the brain or damaging endothelial cells to cause a leaky blood-brain barrier or even microhemorrhages (18). Treatment with cyclophosphamide, but not 5FU, did reduce the hematocrit, but the effect was transient. Evidence of altered erythropoiesis emerged 2 months after recovery from cyclophosphamide or 5FU treatment in young rats when elevated MCVs were noted, which had not been observed at previous time points. No significant effects on hematocrit or MCV were observed in cyclophosphamide-treated older rats. Previously, it was noted that chronic treatment with erythropoietin could improve Morris maze performance of young mice (19). During 32 weeks of erythropoietin treatment, the hematocrit initially increased significantly but eventually returned to baseline levels at the end of the treatment, whereas the size (MCV) and density of RBC were still significantly higher than in controls. Results from another study of chronic erythropoietin treatment in aged male F344 rats revealed impaired Stone maze performance, presumably due to reduced blood flow or damage caused by increased blood viscosity associated with an elevated hematocrit (11). In the current study, we found no significant effects of cyclophosphamide treatment on MCV in aged female F344 rats.

Most clinical studies assessing the effects of chemotherapy on cognition use multiple rather than single agent regimens as applied in the current rodent study. We elected to focus on a single agent to permit specific analysis of its effects. However, we cannot evaluate whether the cognitive problems in patients subjected to multiple agents in clinical studies were due to the interactive effects of such treatments. Moreover, these patients have cancer; thus, it is possible that the susceptibility to neurotoxicity is influenced by the tumor-bearing state. In a recent study of 84 patients with breast cancer, 36% were determined to have cognitive problems prior to chemotherapy (20). Further animal studies using such drug combinations in tumor-bearing animals would be needed to assess this possibility experimentally.

Although we embarked on these studies with the hypothesis that adverse effects of chemotherapy on cognition would be detected, some clinical studies have reported improvement in cognitive performance in patients with sufficient recovery from chemotherapy. Specifically, Schagen et al. (21) evaluated the risk for cognitive impairment >2 years after therapy in breast cancer patients who received either adjuvant high-dose cyclophosphamide; cyclophosphamide, thiotepa, and carboplatin; 5FU, epirubicin, and cyclophosphamide; or cyclophosphamide, methotrexate, and 5FU chemotherapy which were related to the highest risk of cognitive impairment associated with adjuvant chemotherapy. With at least a 2-year recovery (<4 years), the results did not support any of the previously observed differences in cognitive functioning between patients treated

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**Fig. 5.** Summary plots of normalized initial slope measurements of field excitatory postsynaptic potential (fEPSP) evoked in hippocampal CA1. Each data point represents averaged values of 1 minute, which consists of three consecutive sweeps with an interval of 20 seconds. Points, mean; bars, ± SE. Traces on top were recorded before (~1 minute) and 30 minutes after high-frequency stimulation:

A, LTP was not induced during cyclophosphamide treatment (16 weeks after first cyclophosphamide injection: cyclophosphamide, 102.6 ± 11.21%, n = 6 from four rats, P > 0.05 versus saline, 128.9 ± 10.49%, n = 6 from four rats, P < 0.05). B, after 8 weeks of recovery from the last cyclophosphamide treatment, a greater LTP was induced compared with saline controls: cyclophosphamide, 158.98 ± 8.28%, n = 5 from three rats versus saline, 138.99 ± 24.79%, P < 0.05, n = 6 from three rats over the last 10 minutes of recording. C, LTP was still stronger in the cyclophosphamide group compared with saline controls after 53 weeks of recovery: cyclophosphamide, 126.4 ± 13.44%, n = 6 from four rats, P < 0.05 for the first 10 minutes.
with either high-dose chemotherapy or standard-dose chemotherapy (21). Furthermore, these studies indicated improved performance in all chemotherapy groups; whereas, in the control group a slight deterioration in performance was observed. This result indicated that cognitive dysfunction following adjuvant chemotherapy in cancer patients might be transient.

One factor to consider regarding the transient improvement of female rats in spatial learning following chemotherapy are the known detrimental effects of cyclophosphamide on estrogen production (22, 23). Because previous results have shown that reduced plasma estradiol levels may facilitate learning in the Morris maze (23, 24) as well as enhance LTP (25), it might be possible that the transient nature of our observations was due to alterations in estrous cycle that could return to normal function with the longer periods of recovery. Similarly, the failure to observe enhanced performance in the young group of female rats following 42 weeks of recovery from cyclophosphamide treatment may have been due to their already low estrous cycling at age 20 months. Further assessment of estrogen levels following chemotherapy would be needed to evaluate this hypothesis.

The issue of diet restriction should also be considered regarding the transient effects of chemotherapy on cognition. Cyclophosphamide and 5FU treatment produced hypophagia and weight loss. Studies have shown that diet restriction (30-40% less food than ad libitum) in rodents, even for a few months, induces neuroprotection against various neurotoxins and various strokes (26). The concept of homeosis has been posited to explain the enhanced stress protection associated with diet restriction (27).

In summary, using a rat model, we found no evidence of significant cognitive deficits following chemotherapy despite evidence of systemic toxicity. Indeed, we documented evidence of transiently improved performance in two tasks as well as sustained improvement in LTP as a measure of synaptic plasticity. Regarding our failure to observe impaired performance in these tasks associated with cyclophosphamide and 5FU treatment, we have offered alternative hypotheses as described above. However, it is possible that the tasks selected for rats might be insensitive to cognitive domains most affected in chemotherapy patients. For example, Schagen noted that more patients treated with a cyclophosphamide, methotrexate, and 5FU course reported problems in concentration (31%) versus memory (21%) following 2 years of recovery (3). Thus, future rodent studies might focus on neurotoxicity assessed in behavioral paradigms making greater demands on attentional processes, such as prepulse inhibition (28). Conversely, the possibility that the cognitive deficits associated with chemotherapy in patients with cancer do not in fact result directly from the chemotherapeutic agents should also be considered. Comorbidity, especially with depression, might be an underlying cause of the cognitive problems reported. It is estimated that 20% to 25% of cancer patients suffer often unrecognized and untreated long-term depression (29). Further studies using other animal models and modes of chemotherapy might be useful in untangling these complex problems.

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References

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