Published in final edited form as:

Behav Brain Res. 2015 October 1; 292: 133–141. doi:10.1016/j.bbr.2015.06.028.

Doxorubicin and Cyclophosphamide Induce Cognitive Dysfunction and Activate the ERK and AKT Signaling Pathways

Kaliris Y Salas-Ramirez^a, Ciara Bagnall^b, Leslie Frias^b, Syed A Abdali^a, Tim A. Ahles^c, and Karen Hubbard^b

^aDepartment of Physiology, Pharmacology and Neuroscience, The Sophie Davis School of Biomedical Education, New York, NY 10031

^bDepartment of Biology, The City College of New York, New York, NY 10031

^cDepartment of Psychiatry and Behavioral Science, Memorial Sloan-Kettering Cancer Center, New York, NY, 10022

Abstract

Chemotherapy is associated with long-term cognitive deficits in breast cancer survivors. Studies suggest that these impairments result in the loss of cognitive reserve and/or induce a premature aging of the brain. This study has been aimed to determine the potential underlying mechanisms that induce cognitive impairments by chemotherapeutic agents commonly used in breast cancer. Intact and ovariectomized (OVX) female rats were treated intravenously with either saline or a combination of cyclophosphamide (40mg/kg) and doxorubicin (4 mg/kg). All subjects were tested for anxiety, locomotor activity, working, visual and spatial memory consecutively. Although anxiety and visual memory were not affected, chemotherapy significantly decreased locomotor activity and impaired working and spatial memory in female rats, independent of their hormonal status. The cognitive deficits observed are hippocampal dependent. Therefore, as a first step to identify the potential signaling pathways involved in this cognitive dysfunction, the protein levels of extracellular signal-regulated kinase 1/2 (Erk1/2), Akt (neuroprotectant) BDNF and (structural protein) PSD95 in hippocampal lysates were measured. Erk1/2 and Akt pathways are known to modulate synaptic plasticity, neuronal survival, aging and cancer. We found an increased activation of Erk1/2 and Akt as well as an increase in the protein levels of PSD95 in OVX female rodents. However, OVX females had a higher overall BDNF level, independent of chemotherapy. These studies provide additional evidence that commonly used chemotherapeutic agents affect cognitive function and impact synaptic plasticity/aging molecules which may be part of the underlying biology explaining cognitive change and can be potential therapeutic targets.

Corresponding Author: Karen Hubbard, Ph.D., Department of Biology, The City College of New York, New York, NY 10031, khubbard@sci.cuny.cuny.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest

The authors declare no competing conflicts of interest.

Keywords

ERK signaling; AKT signaling; Cognitive dysfunction; Behavior; Rat; aging

1. Introduction

Cognitive impairment has been observed following the use of chemotherapeutic agents for a number of cancers in humans and rodents [1, 2] and has emerged as an important concern among survivors [1]. Although the etiology of this condition remains largely undefined, evidence for a direct effect of chemotherapy on brain structure and function has been reported in human imaging studies and animal studies demonstrating decreased hippocampal neurogenesis and enhanced inflammation following administration of chemotherapy [2–4]. Although, several of the antineoplastic agents used in cancer therapy including the anthracycline, doxorubicin (DOX), have limited diffusion across the blood brain barrier into the CNS, these agents induce systemic-wide inflammation and oxidative stress in several organ systems including the brain [5] which could induce changes directly in the brain.

Only a handful of studies have been performed using animals models that elucidate how chemotherapy can impact an array of emotional and cognitive behaviors, in addition to potential mechanisms by which dysfunction can occur. An example of a common chemotherapeutic agent used in breast cancer, methotrexate, impairs spatial memory and performance on object recognition tasks in male rats, which points to a possible adverse effect of the drug on hippocampal function [6]. Another study using only cyclophosphamide showed a decline in hippocampal-dependent learning and memory tasks in rats treated with the drug, suggesting a suppression of hippocampal neurogenesis following chemotherapy [7]. A classic fear conditioning task on ovariectomized (OVX) female rodents showed treated with cyclophosphamide and DOX decreased freezing in a context test, which suggests a specific deficit in hippocampal-related learning and memory [8]. However, this combination of chemotherapeutic agents has not been tested on several cognitive and emotional modalities and related back to potential molecular mechanisms associated to synaptic plasticity.

Several authors have proposed that chemotherapy may indirectly influence cognitive function by accelerating aging at a molecular level [9, 10]. Recently, Sanoff et al [11] demonstrated that anthracycline-based chemotherapy used to treat breast cancer increased biomarkers of cell senescence which they interpreted as evidence for acceleration of molecular aging. These are not mutually exclusive hypotheses and significant overlap exists between pathways that regulate neuronal plasticity, aging, and cognitive function [12, 13]. In the current studies, we investigated chemotherapy cognitive dysfunction induced by a doxorubicin and cyclophosphamide drug combination in female Sprague Dawley rats as a potential model for breast cancer survivors and evaluated the roles of the Erk1/2, Akt, PSD-95, and BDNF pathways, as these pathways have been implicated in neuronal plasticity and survival, aging and cognitive function [12–14]. Further, most animal studies used OVX females to emulate the human condition of hormonal suppression in breast cancer patients;

however, we measured behavioral effects of chemotherapeutics in intact normal and OVX female rats.

2. Methods

2.1. Animals

Intact (n=20) and ovariectomized (OVX; n=20) females Sprague-Dawley rats, eight to ten weeks of age (Charles River Laboratories) were housed in groups of three. Rats were housed in a temperature controlled colony room (± 21 – 22°C), under a 12:12 light dark cycle (lights on at 7:00am) with water and food (Harlan Teklab Rodent Chow) available ad libitum, in accordance with IACUC approval. Forty-eight hours upon arrival, animals were handled daily for at least five minutes. One week upon arrival, each rat in the chemotherapy group (n = 24; n=12/intact or OVX) was treated once a week for 3 weeks with a combination of cyclophosphamide (40 mg/kg; LKT Laboratories) and doxorubicin (DOX; 4 mg/kg; LKT Laboratories). Injections were given intravenous in the rat's tail vein. Rats were anesthetized with isoflurane (2–4% in oxygen) and warm saline (1 ml) was flushed through polypropylene tubing connected to a 25 gauge, 3/4-in. butterfly needle. A 1 cc syringe containing the appropriate dose of doxorubicin was then attached to the tubing and slowly administered, followed by 1ml saline in a 3 cc syringe. Cyclophosphamide was then administered in the same manner; saline-treated rats (n = 16) received 3.5 ml using a 5 cc syringe. Rats were given HydroGels, 98% sterile gel water (Clear H₂O; Portland, ME), from week 2 of treatment and weight was monitored to ensure the well-being of the animals. Rats were allowed 1 week to recover before beginning the battery of behavioral assessments or subjected to molecular assays. This was treatment dosage and schedule was established in other studies [8].

2.3. Behavioral Assays

Anxiety—Elevated Plus Maze (EPM): Animals were tested on an EPM [15] which consists of two open arms and two closed arms elevated 40 cm above the floor. The walls extend from a central neutral area. Subjects will be placed in the neutral area, facing one of the open arms, and given five minutes to explore the maze. The number and duration of entries into open and closed arms was recorded. Animals that spend significantly more time in the closed arms as compared to the open arms are considered to be more anxious.

Open Field (OF)—Locomotor activity was assessed using an open field (OF) paradigm [16]. The open field is a black, plexiglass, open box, with the floor divided into a grid. Each animal was placed in the center square of the box and allowed to explore the field. The number of outer sector visits (movements across squares and a measure of general locomotor activity), inner sector visits (an anxiety-related behavior), total activity (combined outer and inner crossings), wall climbs, rears and grooms were scored for six minutes.

Non-spatial, visual memory - Object recognition (OR)—The object recognition (OR) task is a visual memory task known to be less hippocampal-dependent and requires prefrontal cortex activity [17–19]. Trials consist of an exploration trial (T1) and a recognition trial (T2). The two trials were separated by an interval of varying lengths.

Exploration was defined by when the subject sniffs, whisks, or looks at the object from no more than 2 cm away. The objects used for the trials are different bottles, cans, and containers (such as soda cans and candlesticks). The position of the objects and the objects replaced were counterbalanced across treatment groups. All animals had acclimation sessions with ITDs of 1, 10, and 60 minutes, and were then tested for object recognition with a 2-h and 4-h delay. Animals that explore the old object for a longer period of time or equivalent time to the new object show declined visual memory.

Spatial Memory - Object Placement (OP)—Spatial memory was assessed using an object placement (OP) task, a spatial memory task known to be hippocampal and fornix dependent [17, 20, 21]. This task is identical to the OR procedure, except that during T2 (the recognition trial), one object was moved to a novel location within the field (instead of replacing one object with a new object). Time spent by each subject exploring objects at the old versus new location was recorded during T2. The new location was counterbalanced across treatment groups. All animals had acclimation sessions with inter-trial delays (ITD) of 15, 40 and 60 minutes, and were tested for object recognition with a 2-h and 4-h delay.

Working Memory Y-Maze Spontaneous Alternation—T/ Y-maze behavior measures various aspects of working memory performance and is hippocampal-dependent [22]. Spatial learning in the T-maze (continuous delayed alternation and reversal learning) requires that the animal choose the arm opposite to the one selected on the previous trial. Spatial alternation responses are learned by relying on egocentric information. The Y-maze spontaneous alternation paradigm is based on the natural tendency of rodents to explore a novel environment. The sequence of arms visited was recorded for a total of 5 minutes. Animals with an impaired working memory cannot remember which arm was just visited, and thus show decreased spontaneous alternation [23, 24].

2.4. Tissue collection and protein isolation

Rats were sacrificed by decapitation and brains were rapidly removed. The whole hippocampus from each hemisphere was dissected (isolated) on ice, flash frozen in liquid nitrogen and subsequently stored at -80°C until further processing. The hippocampus from one hemisphere of each animal was homogenized using a disposable Biomasher (Bio Express) in a protein extraction buffer containing 40 mM Tris-HCl (pH 7.5), 274 mM NaCl, 2mM EGTA, 20% v/v glycerol, 50 mM NaF, 1 mM Na_3VO_4 , 1 mM β -glycerol phosphate, 1 mM PMSF, 2.5 mM sodium pyrophosphate, 1% NP-40, and 1 $\times$ complete protease inhibitor cocktail (Pierce). Each sample was process according to the Biomasher's protocol. Briefly, tissue was placed into the BioMasher apparatus with 200 μl of ice-cold homogenization buffer and centrifuged at $14,000 \times g$ for 15 sec. Following the initial homogenization, additional protein extraction buffer was added to each sample to obtain a ratio of 1:4 (w/v, tissue: buffer) and further centrifuged at $14,000 \times g$ for 15 min at 4°C . The supernatants were collected and the total protein content was determined by the bicinchoninic acid method (BCA) protein assay kit [Pierce; 25]. Tissue lysates were stored at -80°C for subsequent biochemical analyses.

2.5. Enzyme linked immunosorbent assays (ELISA)

Protein levels of BDNF in the hippocampus were quantified using the ELISA method kit according the manufacturer's instructions (BDNF ELISA Kit, Abnova). Standards and samples were analyzed at least in duplicate. Samples were read at 450 nm using an automated plate reader (Titertek Multiscan Bichromatic Plate Reader). BDNF levels were normalized to total amount of protein in each sample.

2.6. Immuno-blot analysis

Protein lysates were mixed with Laemmli sample buffer containing β -mercaptoethanol and boiled for 5 min at 95°C followed by standard Western blotting protocols [26]. Equivalent amounts of protein for each condition were separated by SDS-PAGE using the Mini Protean II system (BioRad) and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk in TBS-T for 1 hour at room temperature. The PVDF membranes were then immunoblotted overnight at 4°C with specific monoclonal rabbit primary antibodies: phosphor-Akt (Ser 473, 1:1000); Akt (1:2000); phosphor-Erk1/2 (Thr202/Tyr204, 1:5000); ERK1/2 (1:5000); and PSD95 (1:5000), all from Cell Signaling USA. After incubation with the primary antibody, blots were probed for 1 hour with horseradish peroxidase-conjugated anti-rabbit secondary antibodies. Protein signals were visualized using enhanced chemiluminescence (GE Healthcare Life Sciences) and quantification relative to β -actin was performed by densitometric analysis using the ImageJ software.

2.7. Statistical Analysis

Two-way ANOVAs were used to analyze statistical differences in behavioral performance for control and treatment groups for the EPM, OF and Y-maze. Where appropriate, post-hoc comparisons were assessed by student's t-tests to determine differences between chemotherapy and control subjects within each hormonal status. For OP and OR repeated measures, three-way ANOVAs were used to determine if any interactions existed between gonadectomy, treatment and inter-trial delay times. Two-way ANOVAs were used to determine treatment interactions within each inter-trial delay time, followed by post-hoc simple planned comparisons using a student's t-test to determine significant differences between chemotherapy and control subjects. A repeated measure three-way ANOVA was also used to determine changes in patterns of locomotion throughout the OF test as well as the period time of tested, hormonal status and treatment. Once again, where appropriate, post-hoc simple planned comparisons were done to determine differences between chemotherapy and control within each intact or ovariectomized group. Statistical significance was considered when $p < 0.05$.

Results for the biomarker analysis (Western and ELISA assays) represent the mean $\pm$ SEM values, and unpaired Student's t-tests were used to compare differences between saline and chemotherapy treatment groups.

3. Results

Overall, treated animals appeared to be healthy except a slight decrease in body weight and mild alopecia. OVX animals were slightly lighter than intact animals upon arrival.

Chemotherapy decreased body weight during exposure, however in the following weeks, during the behavioral assessments, the subjects consistently gained weight. Their body weights were not significantly different than when they began chemotherapy as shown in Figure 1. Interestingly, although the OVX females weighed more than the intact females upon arrival, the OVX chemo-treated females suffered from the most weight loss while the intact chemo group did not suffer from any weight fluctuations. Two females from the OVX-chemo group were eliminated from the analysis because they did not perform the behavioral tasks due to lethargy and slow movement. The chemotherapeutic drugs induced alopecia or hair loss is consistent to that seen in human patients [27].

A battery of behavioral assays were then employed to determine the effects of doxorubicin and cyclophosphamide in combination directly on cognitive function in female rodents.

3.1. Elevated plus maze (EPM) – measure of anxiety

A two-way ANOVA was performed to reveal the effects of chemotherapy and OVX on anxiety related behaviors. Overall, there were no differences found between intact and OVX females in any of the anxiety behaviors measured: time spent in open arms, time spent in closed arms, open arm visits and closed arm visits. However, there was a significant decrease in the number of closed arm visits made by females that were treated with chemotherapy ($F_{1, 34} = 46$, $p = 0.01$; Fig 2A). Fisher's PLSD post-hoc analysis revealed that the chemotherapeutic drugs had the significant main effect (mean diff. = 2.25; $p < 0.01$) on reducing the number of closed arm visits. In order to assess whether this was really an effect of chemotherapy on anxiety or locomotion, a two way ANOVA showed that chemotherapy did not affect time spent in the closed arms ($F_{1, 34} = 10.6$, $p = .93$; Fig 2B) in the elevated plus maze task. These finding suggest that chemotherapeutic drugs did not affect anxiety in female rats.

3.2. Locomotor activity

In an open field, two-way ANOVAs were performed in order to investigate any interactions between chemotherapy and OVX for total activity. A significant decrease in locomotor activity was observed for both intact and OVX animals ($F_{1, 34} = 3825.2$, $p = 0.02$; Fig 3A) and chemotherapy ($F_{1, 34} = 18651$, $p < 0.001$; Fig 3A). Fisher's PLSD post-hoc analysis revealed that this is a highly significant effect of treatment (mean diff = 44.4; $p < 0.0001$) in both groups. In order to assess if time was a factor on the decrease in locomotor activity, a three-way repeated measures ANOVA was used to look at interactions between treatment, OVX and length of test (total was 6 mins, the test was divided into three minute intervals). The visits made to the inner sectors and outer sectors were quantified independently. Chemotherapy had an effect on the outer sector visits independent of OVX or the time interval of the test ($F_{1, 34} = 20829.4$, $p = 0.009$; Fig 3B). There was also a significant decrease of inner sector visits by animals treated with chemotherapy ($F_{1, 34} = 31.2$, $p = 0.03$; Fig 3B); however, this effect was driven by the length of time used to measure visits in the open field test ($F_{1, 34} = 41.1$, $p = 0.001$; Fig 3B). The first three minutes, saline and chemo-treated females, independent of OVX, showed no significant differences in inner sector visits. The last three minutes, chemo-treated females had significantly less inner

sector visits. A reduction in inner sector visits suggests anxiety-like tendencies; however, this was not supported by the EPM task.

3.3. Working Memory – Y-maze

A two-way ANOVA revealed that chemo-treated females, independent of OVX, had significantly lower percentages of spontaneous alternations in the Y-maze task ($F_{1, 34} = 13,168.9$, $p = 0.005$; Fig 3). Fisher's PLSD post-hoc analysis revealed significant effects of treatment (mean diff = 37.05; $p = .006$; Fig 4). The number of total arm visits did not differ between treatment groups.

3.4. Visual Memory - Object Recognition

Chemotherapy did not affect visual memory. A three-way repeated measure ANOVA was used to determine if any interactions exist between hormonal status, drug treatment and inter-trial delay (ITD) time. There were no significant differences in any of the treatment groups, whether intact or OVX. In addition, there were no significant differences by inter-trial delay in any of the treatment groups (Fig. 5A).

3.5. Spatial Memory - Object Placement

In contrast, chemotherapy impaired working memory in female subjects. A three-way repeated measures ANOVA revealed a decrease in behavior for this measure ($F_{1, 34} = 1.14$, $p < 0.001$; Fig 5B). There was a decreased recognition ratio (time spent exploring the new location of the object/total time spent exploring the objects) on the object placement task. Using two-way ANOVAs, the main effect of chemotherapy on spatial memory was determined to be persistent at both a two-hour ($F_{1, 34} = 0.4$, $p = 0.003$; Fig 4B) and four-hour inter-trial delay time ($F_{1, 34} = 0.67$, $p < 0.0001$; Fig 5B). Fisher's PLSD post-hoc analysis revealed significant differences between saline and chemo-treated females at the two-hour (mean diff = 0.2; $p < 0.003$) and four-hour time measurements (mean diff = 0.27; $p < 0.001$), respectively. Time spent exploring the objects did not differ between treatment groups.

We performed repeated measures ANOVAs looking at the total exploration time both in the exploration trials (T1) and recognition trials (T2) for the 2 and 4 hour inter-trial delays for the object tasks and there were no significant differences in the time spent exploring the objects, without taking into account OLD versus NEW. As described in the methods section, the few females that did perform the task were excluded from our statistical analysis. In addition, females seemed to gain more mobility by two weeks after the last chemotherapy injection; therefore, their ability to perform the cognitive tasks were comparable.

3.6. Doxorubicin and cyclophosphamide treatment did not increase BDNF

We found that in hippocampal lysates, the neuroprotectant, BDNF, did not exhibit significantly altered levels between control and chemo-treated groups (Fig 6). However, there was an increase in hippocampal BDNF levels in OVX rats compared to intact rats (Fig 6) regardless of treatment.

3.7. Doxorubicin and cyclophosphamide activated the ERK and Akt Signaling Pathways

The ERK and Akt signaling pathways are also induced to mediate neuroprotection during oxidative stress responses [28]. Doxorubicin has been shown by others to increase oxidative stress and induce DNA damage [29], therefore we have initially evaluated these pathways with respect to their contributions to chemotherapy-induced cognitive dysfunction. As a first step, we have measured activation of ERK1/2 and Akt pathways in doxorubicin and cyclophosphamide treated intact and OVX Sprague-Dawley rats that have not been subjected to any behavioral assays. However, these animals received chemotherapy treatment at the same time and in a similar manner as animals that underwent behavioral analysis. We took this approach because behavioral assays may complicate gene expression profiles, especially if small changes are observed. Phosphorylation of Erk1/2 and Akt indicates that these signaling proteins are activated. Western analysis showed that the activated forms (phosphorylated forms) of both Erk1/2 and Akt were significantly increased in chemotherapy treated OVX female rats (Fig 7). However, there was no apparent activation of these pathways in treated intact female rats (Fig 7). PSD95 is a neuronal structural protein important for scaffolding in post-synaptic dendrites [14]. Furthermore the post-synaptic distribution of PSD-95 is regulated by pathways downstream of BDNF-TrkB signaling [30]. We found similar results for PSD95 that we observed for Akt and ERK1/2 in OVX chemotherapy treated rats (Fig 7). The PSD95 protein levels were significantly increased in treated OVX female rats compared to OVX controls but not in treated intact female rats as compared to intact controls.

4. Discussion

The results of the behavioral testing demonstrated that chemotherapy can impair cognitive function, independent of hormonal status or condition. In addition, molecules responsible for synaptic plasticity and aging were upregulated. Our behavioral results are consistent with others [8, 31] which have shown that chemotherapy induces cognitive dysfunction that is hippocampal-dependent. Our studies are significantly different in that we have shown that the cognitive impairment observed in our behavioral assays was independent of hormonal status, in addition to assessing different aspects of cognition and emotional regulation. Nevertheless, hormonal status had a significant effect on the gene expression profiles in our biochemical studies and is an important finding as most studies use only ovariectomized females or male rats to compare the effects of chemotherapy on brain and behavior. We found that the Akt and ERK1/2 pathways were involved in doxorubicin and cyclophosphamide induced cognitive changes and need to be further studied. Our finding that both ERK1/2 and Akt pathways are activated are similar to those recently of Briones et al [32], however, these investigators did not use intact female rats in their study as a comparison.

Changes in gene expression were found in OVX animals but not in intact animals. This may be in part due to a lack of sensitivity in the detection of smaller expression changes in intact animals. Ovariectomy may induce a global stress response that makes female subjects more sensitive to chemotherapy. This idea is underscored by our BDNF results (Fig 6) where a dramatic increase in BDNF is observed in OVX rats as compared to intact rats, independent

of chemotherapy exposure. An induction of synaptic proteins like PSD95 by chemotherapy may be a response to correct impairment in neuronal plasticity. The loss of ovarian hormones may induce a compensatory stress response that activates neuroprotectant proteins, such as BDNF and needs further examination [33].

Doxorubicin and cyclophosphamide are known to be cytotoxic agents. Weekly treatments of these chemotherapeutic drugs for three weeks result in impaired working and spatial memory, independent of hormonal status in female rats. A previous study in OVX females showed that this chemotherapy regimen impairs contextual fear memory [8]. In addition, spatial and nonspatial memory is impacted in intact female mice [34]. This same combination of chemotherapeutic agents impaired visual memory and contextual fear memory in male athymic rats [35]. Similarly, even one exposure to each of these agents in male mice significantly impaired cognitive function [36]. These studies all support that chemotherapeutic drugs result in cognitive decline independent of hormonal manipulations, including these findings. Interestingly, visual memory was not impaired in our study, suggesting that not all cognitive modalities are affected. While spatial and working memory are hippocampal-dependent tasks, visual memory also relies on prefrontal cortex, which may result in intact function. This also supports that cognitive dysfunction occurs as a result of chemotherapy and is not due to lethargy, since the test were run consecutively. In breast cancer patients, effects of chemotherapeutic drugs on memory and executive function have been documented during, immediately after and up to 20 years after chemo exposure [2, 37].

Doxorubicin and cyclophosphamide did not affect anxiety, but did reduce locomotor activity throughout the six-minute test. Even though rodents were given a week to recover from the chemo exposure, some subjects still had reduced body weight and slow movements, which could have impacted locomotor activity. Since the females were able to perform the visual memory task, the chemotherapeutic effects on locomotor activity did not seem to be a factor on cognitive performance. Fortunately, by the time the cognitive assessments began, their body weight was stable and no significant changes were observed in exploration times of objects or movement in the mazes; hence, although chemotherapy results in some locomotor deficiencies, at the time cognitive testing was performed, subjects seemed to have recovered from any of the physiological consequences of chemotherapy. Our studies support many that stress that chemotherapy results in cognitive impairments. Nevertheless, others have shown that long after chemotherapy females rodents either show no impairments in cognitive performance, increased synaptic plasticity molecules and improved long term potentiation (LTP) [38]. One study using intact C57CL/6J female mice did not show any effects on chemotherapy on contextual fear conditioning and novel object recognition [39]; however, C57CL/6J mice may be resistant to the effects of chemotherapy, unlike rats and humans.

Our finding that the ERK and Akt signaling pathways were activated following doxorubicin and cyclophosphamide treatment is intriguing and consistent with the possibility that these agents cause an accelerated aging of the brain. Both pathways have been implicated in aging and the induction of premature senescence [reviewed in 13]. Akt is especially interesting as it induces the mTOR pathway which then antagonizes autophagy and has been shown to be a factor that regulates Alzheimer's Disease [13, 40]. Inhibition of the mTOR pathway by rapamycin increases longevity and mitigates age-related diseases including Alzheimer's [41,

42]. Rapamycin has been shown to inhibit age-related decreases in age-related memory and open-field tasks suggesting that mTOR plays a pivotal role in normal aging [43].

In addition to being up-regulated during aging, the ERK pathway is required for normal neuronal development and synaptic plasticity [12]. However, hyperactivation of ERK signaling may lead to detrimental effects on cognition consistent with our results that ERK 1/2 is activated in OVX rats treated with chemotherapeutic agents. A recent study found that doxorubicin exposure in both rat cortical neurons and Aplysia sensory neurons induced the activation of ERK and p38 MAPK [44]. It is also possible that the differential activation of these MAPK isoforms may ultimately influence synaptic plasticity through the regulation of the transcriptional repressor CREB2 [44]. Both pathways have sites where they can intersect. It is also of interest to determine where induction of PSD95 fits in these signaling pathways following chemotherapy as potential therapeutic target.

Although differences in BDNF levels between intact and OVX groups did not with drug treatment, we cannot rule out the effects of treatment on the activation of the TrkB receptor. BDNF-TrkB signaling mediates the N-methyl-D-aspartate receptor (NMDAR) activity-dependent synaptic localization of PSD-95 through PI3K-Akt [45] and ERK/MAPK [30]. The potential modulation of NMDAR crosstalk to neurotrophin receptor activity may serve as a mechanism by which chemotherapy could exert its effects on cognition. Interestingly, the apoptotic effects of doxorubicin in neuronal cultures were found to be attenuated by the NMDAR antagonist Memantine [46, 47]. Further studies investigating the regulation of Trk B as a possible upstream mechanism are of interest.

Conclusion

The results of this study support the model of examining molecular mechanisms of chemotherapy-induced cognitive change at the interface of neuronal plasticity and survival, aging and cognitive function. The activation of the ERK and Akt biomarkers associated with the biology of aging provide for a novel mechanistic model to explain chemotherapy-induced cognitive change and to evaluate agents and behavioral interventions known to increase longevity and/or slow the process of aging as possible therapeutic interventions to attenuate cognitive dysfunction caused by chemotherapeutic drugs. The pattern of behavioral but not biological changes in intact animals may suggest that younger women treated with chemotherapy for breast cancer who recover ovarian function may be more likely to recover cognitively from the acute effects of chemotherapy. Taken together, these results suggest multiple avenues of research that could lead to an understanding of the mechanisms of chemotherapy-induced cognitive dysfunction and to the identification of new therapeutic strategies to prevent or reduce the negative cognitive impact of chemotherapy.

Acknowledgements

The authors wish to thank funding: NIH/NCI grant U54CA132378/ U54CA137788 to TA, KSR and KH; NIH/RISE GM08168 grant to CB; NIH/RCMI grant 5G12MD007603 to KH and KSR. The authors also wish to thank Cherease Street, Dainn Woo, Nawar Talukder, Mohammad Qadri, Kevin Uribe and Silvia Perez for their help with the behavioral assessments and tissue collection.

References

1. Ahles TA, Root JC, Ryan EL. Cancer and cancer treatment associated cognitive changes: An update on the state of the science. *J Clin Oncol*. 2012; 30:3675–3686. [PubMed: 23008308]
2. Seigers R, Fardell JE. Neurobiological basis of chemotherapy-induced cognitive impairment: a review of rodent research. *Neurosci Biobehav Rev*. 2011; 35(3):729–741. [PubMed: 20869395]
3. Wigmore P. The effect of systemic chemotherapy on neurogenesis, plasticity and memory. *Curr Top Behav Neurosci*. 2013; 15:211–240. [PubMed: 23239468]
4. Ahles TA, Saykin AJ. Candidate mechanisms for chemotherapy-induced cognitive changes. *Nature Rev. Cancer*. 2007; 7:192–201. [PubMed: 17318212]
5. Janelins MC, Mustian KM, Palesh OG, Mohile SG, Peppone LJ, Sprod LK, Heckler CE, Roscoe JA, Katz AW, Williams JP, Morrow GR. Differential expression of cytokines in breast cancer patients receiving different chemotherapies: implications for cognitive impairment research. *Support Care Cancer*. 2012; 20(4):831–839. [PubMed: 21533812]
6. Seigers R, et al. Long-lasting suppression of hippocampal cell proliferation and impaired cognitive performance by methotrexate in the rat. *Behav Brain Res*. 2008; 186(2):168–175. [PubMed: 17854921]
7. Yang M1, Kim JS, Song MS, Kim SH, Kang SS, Bae CS, Kim JC, Wang H, Shin T, Moon C. Cyclophosphamide impairs hippocampus-dependent learning and memory in adult mice: Possible involvement of hippocampal neurogenesis in chemotherapy-induced memory deficits. *Neurobiol Learn Mem*. 2010; 93(4):487–494. [PubMed: 20109567]
8. MacLeod JE, DeLeo JA, Hickey WF, Ahles TA, Saykin AJ, Bucci DJ. Cancer chemotherapy impairs contextual but not cue-specific fear memory. *Behav Brain Res*. 2007; 181:168–172. [PubMed: 17509697]
9. Ahles TA. Brain vulnerability to chemotherapy toxicities. *Psychooncology*. 2012; 21:1141–1148. [PubMed: 23023994]
10. Mandelblatt JS, Jacobsen PB, Ahles TA. Cognitive effects of cancer systemic therapy: Implications for the care of older patients and survivors. *J Clin Oncol*. 2014; 20:2617–2626. [PubMed: 25071135]
11. Sanoff HA, et al. Effect of Cytotoxic Chemotherapy on Markers of Molecular Age in Patients With Breast Cancer. *J Natl Cancer Inst*. 2014; 106(4):dju057. [PubMed: 24681605]
12. Hetman M, Gozdz A. Role of extracellular signal regulated kinases 1 and 2 in neuronal survival. *Eur. J. Biochem*. 2004; 271:2050–2055. [PubMed: 15153093]
13. Steelman LS, et al. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging*. 2011; 3(3): 192–222. [PubMed: 21422497]
14. Béique JC, Andrade R. PSD-95 regulates synaptic transmission and plasticity in rat cerebral cortex. *J Physiol*. 2003; 546:859–867. [PubMed: 12563010]
15. Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods*. 1985; 14:149–167. [PubMed: 2864480]
16. Bowman RE, Maclusky NJ, Diaz SE, Zrull MC, Luine VN. Aged rats: sex differences and responses to chronic stress. *Brain Res*. 2006; 1126:156–166. [PubMed: 16919611]
17. Ennaceur A, Aggleton JP. The effects of neurotoxic lesions of the perirhinal cortex combined to fornix transection on object recognition memory in the rat. *Behav Brain Res*. 1997; 88:181–193. [PubMed: 9404627]
18. Mumby DG. Perspectives on object-recognition memory following hippocampal damage: lessons from studies in rats. *Behav Brain Res*. 2001; 127:159–181. [PubMed: 11718890]
19. Winters BD, Saksida LM, Bussey TJ. Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval. *Neurosci Biobehav Rev*. 2008; 32:1055–1070. [PubMed: 18499253]
20. Beck KD, Luine VN. Sex differences in behavioral and neurochemical profiles after chronic stress: role of housing conditions. *Physiol Behav*. 2002; 75:661–673. [PubMed: 12020731]

21. Li C, Brake WG, Romeo RD, Dunlop JC, Gordon M, Buzescu R, Magarinos AM, Allen PB, Greengard P, Luine V, McEwen BS. Estrogen alters hippocampal dendritic spine shape and enhances synaptic protein immunoreactivity and spatial memory in female mice. *Proc Natl Acad Sci (USA)*. 2004; 101:2185–2190. [PubMed: 14766964]
22. Wesierska M, Dockery C, Fenton AA. Beyond memory, navigation, and inhibition: behavioral evidence for hippocampus-dependent cognitive coordination in the rat. *J Neurosci*. 2005; 25:2413–2419. [PubMed: 15745968]
23. Willford JA, Segar TM, Hansen-Trench LS, Barron S. The effects of neonatal cocaine exposure on a play-rewarded spatial discrimination task in juvenile rats. *Pharmacol Biochem Behav*. 1999; 62:137–143. [PubMed: 9972856]
24. Meunier J, Maurice T. Beneficial effects of the sigma1 receptor agonists igmesine and dehydroepiandrosterone against learning impairments in rats prenatally exposed to cocaine. *Neurotoxicol Teratol*. 2004; 26:783–797. [PubMed: 15451042]
25. Walker JM. The bichinchoninic acid (BCA) assay for protein quantitation. *Methods Mol Biol*. 1994; 32:5–8. [PubMed: 7951748]
26. Harlow, E.; Lane, DP. *Antibodies: A Laboratory Manual*. New York, NY: Cold Spring Harbor Laboratory Press; 1988.
27. Yeager CE, Olsen EA. Treatment of chemotherapy-induced alopecia. *Dermatologic Therapy*. 2011; 24:432–442. [PubMed: 21910801]
28. Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem*. 1999; 274:22569–22580. [PubMed: 10428835]
29. Lopes MA, Meisel A, Carvalho FD, Bastos Mde L. Neuronal nitric oxide synthase is a key factor in doxorubicin-induced toxicity to rat-isolated cortical neurons. *Neurotox Res*. 2011; 19(1):14–22. [PubMed: 19924493]
30. Yoshii A, Constantine-Paton M. Postsynaptic localization of PSD-95 is regulated by all three pathways downstream of TrkB signaling. *Front Synaptic Neurosci*. 2014; 6:6. [PubMed: 24744726]
31. Konat GW, Kraszpulski M, James I, Zhang HT, Abraham J. Cognitive dysfunction induced by chronic administration of common cancer chemotherapeutics in rats. *Metab Brain Dis*. 2008; 23(3):325–333. [PubMed: 18690526]
32. Briones TL, Woods J, Wadowska M. Involvement of insulin-like growth factor-1 in chemotherapy-related cognitive impairment. *Behav Brain Res*. 2015; 279:112–122. [PubMed: 24632471]
33. Charoenphandhu N, Nuntapornsak A, Wongdee K, Krishnamra N, Charoenphandhu J. Upregulated mRNA levels of SERT NET, MAOB, and BDNF in various brain regions of ovariectomized rats exposed to chronic aversive stimuli. *Mol Cell Biochem*. 2013; 375(1–2):49–58. [PubMed: 23208077]
34. Winocour G, Vardy J, Binns MA, Kerr L, Tannock I. The effects of the anti-cancer drugs, methotrexate and 5-fluorouracil on cognitive function in mice. *Pharmacol Biochem Behavior*. 2006; 85:66–75.
35. Christie LA, Acharya MM, Parihar VK, Nguyen A, Martirosian V, Limoli CL. Impaired cognitive function and hippocampal neurogenesis following cancer chemotherapy. *Clin Cancer Res*. 2012; 18:1954–1965. [PubMed: 22338017]
36. Seigers R, Loos M, Van Tellingen O, Boogerd W, Smit AB, Schagen SB. Cognitive impact of cytotoxic agents in mice. *Psychopharmacology (Berl)*. 2015; 232:17–37. [PubMed: 24894481]
37. Jim H, Rodin G, Ahles TA. Long-term effects of chemo on the cognitive function of cancer patients. *Oncology (Williston Park)*. 2012; 26:1012–1014. 1042. [PubMed: 23330339]
38. Lee GD, Longo DL, Wang Y, Rifkind JM, Abdul-Raman L, Mamczarz JA, Duffy KB, Spangler EL, Taub DD, Mattson MP, Ingram DK. Transient improvement in cognitive function and synaptic plasticity in rats following cancer chemotherapy. *Clin Cancer Res*. 2006; 12:198–205. [PubMed: 16397043]
39. Fremouw T, Fessler CL, Ferguson RJ, Burguete Y. Preserved learning and memory in mice following chemotherapy: 5-Fluorouracil and doxorubicin single agent treatment, doxorubicin-

- cyclophosphamide combination treatment. *Behav Brain Res.* 2012; 226:154–162. [PubMed: 21930159]
40. Wang CI, Yu JT, Miao D, Wu ZC, Tan MS, Tan L. Targeting the mTOR signaling network for Alzheimer's disease therapy. *Mol Neurobiol.* 2014; 49(1):120–135. [PubMed: 23853042]
41. Wu JJ, Liu J, Chen EB, Wang JJ, Cao L, Narayan N, Fergusson MM, Rovira II, Allen M, Springer DA, Lago CU, Zhang S, DuBois W, Ward T, deCabo R, Gavrilova O, Mock B, Finkel T. *Cell Reports.* 2013; 4:913–920.
42. Xu S, Cai Y, Wei Y. mTOR Signaling from Cellular Senescence to Organismal Aging. *Aging and Disease.* 2014; 5(4):263–273. [PubMed: 25110610]
43. Neff F, et al. Rapamycin extends murine lifespan but has limited effects on aging. *J Clin Invest.* 2013; 123(8):3272–3291. [PubMed: 23863708]
44. Liu RY, Zhang Y, Coughlin BL, Cleary LJ, Byrne JH. Doxorubicin attenuates serotonin-induced long-term synaptic facilitation by phosphorylation of p38 mitogen-activated protein kinase. *J Neurosci.* 2014; 34(40):13289–13300. [PubMed: 25274809]
45. Yoshii A, Constantine-Paton M. BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. *Nat Neurosci.* 2007; 10(6):702–711. [PubMed: 17515902]
46. Jantas D, Pytel M, Mozrzymas JW, Leskiewicz M, Regulska M, Antkiewicz-Michaluk L, Lason W. The attenuating effect of memantine on staurosporine-, salsolinol- and doxorubicin-induced apoptosis in human neuroblastoma SH-SY5Y cells. *Neurochem Int.* 2008; 52(4–5):864–877. [PubMed: 17996985]
47. Jantas D, Lason W. Protective effect of memantine against Doxorubicin toxicity in primary neuronal cell cultures: influence a development stage. *Neurotox Res.* 2009; 15(1):24–37. [PubMed: 19384585]

Highlights

- Chemotherapy impairs working and spatial memory but not anxiety.
- Chemotherapy transiently impairs locomotor activity.
- Chemotherapy induces the Akt and ERK 1/2 signaling pathways.

Figure 1. Ovariectomized females weighed less than intact females upon arrival, however, chemotherapy reduced body weight in both intact and OVX female subjects
On average, female subjects lost 20–40 grams after exposed to chemotherapeutic agents. Two weeks after chemotherapy treatment ended, their body weights stabilized in the chemo-treated females.

Figure 2. Chemotherapy decreases the number of open arm visits, but does not affect the amount of time spent in the open or closed arms in the elevated plus maze

(A) Both the intact and OVX control groups visited the closed arms significantly more than chemo-treated intact and OVX groups (* $p < 0.01$). (B) There was no significant difference between treated and control groups in the total amount of time the animals spent in closed arms. There was no difference between OVX and intact animals.

Figure 3. Chemotherapy decreases locomotor activity in an open field

(A) The chemo-treated groups had a significantly lower level of activity (outer sector plus inner sector visits), compared to the control groups (* $p < 0.001$), independent of hormonal status. (B) Chemo-treated animals visited the outer sectors of the open field significantly less than the control females (* $p < 0.009$) throughout the six minute locomotor test. In addition, chemotreated females had significantly less inner sector visits (* $p < 0.03$) in the second half (3–6 minute interval) of the six minute locomotor test.

Figure 4. Chemotherapy impairs working memory

The chemo-treated females had impaired working memory, as seen by a decreased percent of spontaneous alternations in a Y-maze (* $p < 0.006$).

Figure 5. Chemotherapy does not impact visual memory, however, it does impair spatial memory

(A) There were no significant differences between any of the treatment groups in the visual memory, object recognition task. (B) Chemo-treated females has significantly impaired spatial memory as compared to control groups (* $p < 0.001$), independent of hormonal status.

Figure 6. Hormonal status effects BDNF levels but not chemotherapy

Hippocampal lysates isolated as described in Materials and Methods, were subjected to enzyme linked immunosorbent assays (ELISA) and read at 450 nm. BDNF levels were normalized to total amount of protein in each sample and quantified using BDNF standards. Data are presented as mean value + S.E.M; intact saline n = 12; intact chemo n = 14; OVX saline n = 11; chemo OVX n= 12.

Figure 7. Chemotherapy treatment activates the Akt and Erk/MAPK signaling pathways and upregulates the synaptic protein PSD-95 in the hippocampus of ovariectomized (OVX) rats
Hippocampal lysates were prepared from the intact-saline (N=10), intact-chemotherapy (N=12), OVX -saline (N=10) or OVX-chemotherapy (N=12) injected groups as described under “Methods”. Protein lysates (15 mg, PSD-95 and Erk; 25mg, Akt) were analyzed by SDS-PAGE and immunoblotted with phospho-Akt (S473), phospho-Erk (T204/Y202), and PSD-95 primary antibodies. The blots were scanned and band intensities were normalized to β -actin or their respective total Akt or total Erk protein levels. (A) Representative blots for Erk activation and PSD-95. (C,E) Quantification of Erk (phosphorylated/total) and PSD-95 from intact and OVX animals. (B) Representative blots for Akt activation. (D)

Quantification of Akt (phosphorylated/total) from intact and OVX animals. Data are presented as mean value + S.E.M; * $p < 0.05$ vs. saline.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript