Biological Research for Nursing
1-10

DOI: 10.1177/1099800416644780
brn.sagepub.com
SAGE

Effects of Intraventricular Methotrexate on Neuronal Injury and Gene Expression in a Rat Model: Findings From an Exploratory Study

Ida M. (Ki) Moore, PhD, RN, FAAN[1], Carrie J. Merkle, PhD, RN, FAAN[1], Howard Byrne, Adam Ross, PSM[1], Ashley M. Hawkins, BSN, RN[2], Sara S. Ameli, BSN, RN[1], and David W. Montgomery, PhD[1,2]

Abstract

Central nervous system (CNS)-directed treatment for acute lymphoblastic leukemia, used to prevent disease recurrence in the brain, is essential for survival. Systemic and intrathecal methotrexate, commonly used for CNS-directed treatment, have been associated with cognitive problems during and after treatment. The cortex, hippocampus, and caudate putamen, important brain regions for learning and memory, may be involved in methotrexate-induced brain injury. Objectives of this study were to (1) quantify neuronal degeneration in selected regions of the cortex, hippocampus, and caudate putamen and (2) measure changes in the expression of genes with known roles in oxidant defense, apoptosis/inflammation, and protection from injury. Male Sprague Dawley rats were administered 2 or 4 mg/kg of methotrexate diluted in artificial cerebrospinal fluid (aCSF) or aCSF only into the left cerebral lateral ventricle. Gene expression changes were measured using customized reverse transcription (RT)^2 polymerase chain reaction arrays. The greatest percentage of degenerating neurons in methotrexate-treated animals was in the medial region of the cortex; percentage of degenerating neurons in the dentate gyrus and cornu ammonis 3 regions of the hippocampus was also greater in rats treated with methotrexate compared to perfusion and vehicle controls. There was a greater percentage of degenerating neurons in the inferior cortex of control versus methotrexate-treated animals. Eight genes involved in protection from injury, oxidant defense, and apoptosis/inflammation were significantly downregulated in different brain regions of methotrexate-treated rats. To our knowledge, this is the first study to investigate methotrexate-induced injury in selected brain regions and gene expression changes using a rat model of intraventricular drug administration.

Keywords

methotrexate, brain injury, rat model, neuronal degeneration, gene expression, laser capture microdissection

Cancer affects a significant number of children, with 1 in 330 children diagnosed with cancer before 20 years of age and 1 in 530 young adults between the ages of 20 and 39 being a childhood cancer survivor (Ward, DeSantis, Robbins, Kohler, & Jemal, 2014). The most prevalent cancer among children and adolescents of less than 15 years of age is acute lymphoblastic leukemia (ALL). For this type of tumor, the long-term survival rate approaches 90% (Hunger et al., 2012; Winick, Carroll, & Hunger, 2004). Central nervous system (CNS)-directed treatment is essential for long-term, disease-free survival because the brain is a sanctuary site for leukemia cells (Pui, 2003, 2006; Pui & Evans, 2006) and the primary site of initial relapse among children with ALL who achieve a bone-marrow remission. CNS-directed treatment commonly comprises repeated cycles of intrathecal and intermediate- to high-dose intravenous methotrexate. However, many children experience cognitive and academic problems, including deficits in memory, attention, processing speed, visual–spatial and fine motor skills, and math abilities, during and after ALL therapy (Ashford et al., 2010; Buizer, de Sonneville, & Veerman, 2009; Buizer, de Sonneville, van den Heuvel-Eibrink, & Veerman, 2005; Buizer, De Sonneville, van den Heuvel-Eibrink, Njiokiktjien, & Veerman, 2005; Carey et al., 2007; Caron et al., 2009; Hockenberry et al., 2007, 2015; Kaemingk, Carey, Moore, Herzer, & Hutter, 2004; Krull et al., 2008; Krull, Hockenberry, Miketova, Carey, & Moore, 2013; Montour-Proulx

[1] College of Nursing, The University of Arizona, Tucson AZ, USA
[2] Southern Arizona VA Healthcare System, Tucson AZ, USA

Corresponding Author:
Ida (Ki) M. Moore, PhD, RN, FAAN, College of Nursing, The University of Arizona, 1305N Martin Ave, PO Box 210203, Tucson, AZ 85721, USA.
Email: imoore@email.arizona.edu

et al., 2005; Moore, Hockenberry, & Krull, 2013; Moore, Lupo, et al., 2015; Waber et al., 1995; Waber & Tarbell, 1997).

Despite compelling evidence for the significant long-term impact of CNS-directed treatment on cognitive and academic outcomes, little is known about the mechanisms of injury to specific brain regions. The cortex, hippocampus, and caudate putamen are important brain regions for learning and memory and may be involved in methotrexate-induced brain injury (DeCoteau & Kesner, 2000; Eichenbaum, 2001, 2004; Fortin, Agster, & Eichenbaum, 2002; Grahn, Parkinson, & Owen, 2008; Graybiel, 2005; Kesner & Gilbert, 2006; Miyashita, 1993; Rieckmann, Fischer, & Backman, 2010; Schendan, Searl, Melrose, & Stern, 2003). Knowledge about mechanisms of methotrexate-induced CNS injury is needed to develop interventions that will prevent or minimize cellular injury, resulting in significant cognitive and academic problems for the growing number of long-term survivors of childhood ALL. To date, knowledge of methotrexate-induced CNS injury is based primarily on in vitro cell culture studies, preclinical rodent models of childhood cancer treatment, and a limited number of clinical or postmortem studies of children after CNS-directed treatment (Bisen-Hersh, Hineline, & Walker, 2011; Merkle et al., 2000; Moore et al., 2006; Nagel et al., 2004; Reddick, Glass, Helton, Langston, Li, et al., 2005; Reddick, Glass, Helton, Langston, Xiong, et al., 2005; Reddick et al., 2006). Potential mechanisms of injury to neural systems include disruption of myelination (Moleski, 2000), reduced neural-cell proliferation (Janelsins et al., 2010), increased oxidative stress (Hockenberry et al., 2013; Jahovic, Cevik, Sehirli, Yegen, & Sener, 2003; Miketova et al., 2005; I. M. Moore, Gundy, et al., 2015), apoptosis (Merkle et al., 2000), neuroinflammation, vascular injury, and deficits in DNA repair enzymes (Ahles & Saykin, 2007; Seigers & Faradell, 2011).

Rodent studies have shown that treatment with methotrexate is associated with long-lasting subtle and pervasive impairments in short- and long-term memory neural systems involved in acquisition, retention, or recall of new information (Seigers & Faradell, 2011; Yanovski et al., 1989). Seigers and colleagues (2009) found a significant reduction in hippocampal cell proliferation and a decrease in the thickness of the lateral corpus callosum in adult male Wistar rats 7 days after treatment with methotrexate. The effects on cell proliferation were long-lasting (up to 3 weeks), dose dependent, and associated with impairment in spatial memory (Seigers et al., 2008). Neural-cell proliferation in the dentate gyrus region of the hippocampus was reduced in mice following treatment with chemotherapy that crossed the blood–brain barrier (Janelsins et al., 2010), and density of synapses in the cornu ammonis 3 (CA3) region of the hippocampus was decreased in rat pups following intraperitoneal methotrexate administration (Igarashi, 1989).

In a clinical postmortem study of children with medulloblastoma who were treated with cranial radiation and chemotherapy, Nagel et al. (2004) reported that right and left hippocampal volumes declined significantly during the first 2 years after diagnosis and/or treatment but then gradually returned toward a positive growth curve. Findings suggest that the treatment disrupted, but did not destroy, the ability of neural stem cells or precursor cells to effectively produce hippocampal neurons. Females had a steeper slope of decline in both right and left hippocampal volumes than did males, suggesting sex differences in the susceptibility to the negative effects of radiation and chemotherapy on the hippocampus. Since children received both cranial radiation and chemotherapy, findings cannot be attributed to chemotherapy alone.

In earlier studies, we reported that methotrexate increased both apoptosis in cultured vascular endothelial cells (Merkle et al., 2000) and markers of oxidative stress in cerebrospinal fluid (CSF) samples obtained from children with ALL (Hockenberry et al., 2013; Miketova et al., 2005; I. M. Moore, Gundy, Pasvogel, et al., 2014). It is possible that gene expression modifications underlie chemotherapy-induced brain injury, yet little is known about gene expression changes that may be important for brain injury, protection from injury, or cognitive problems. In the present pilot study, we used a preclinical rodent model of methotrexate-induced neurologic injury to meet the following objectives: (1) quantify neuronal degeneration in selected regions of the cortex, hippocampus, and the caudate putamen and (2) measure changes in the expression of genes with known roles in oxidative stress/oxidant defense, apoptosis/inflammation, and protection from injury before and after treatment with methotrexate.

Material and Method

Animals

We purchased male Sprague Dawley rats at 3 weeks of age from Harlan (Indianapolis, IN) and housed them in the American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at the Southern Arizona VA Health Care System (SAVAHCS, Tucson, AZ). The animals were quarantined for 1 week before use, fed ad-lib, and maintained on a 12/12 hr light/dark schedule. The Institutional Animal Care and Use Committee (IACUC) committees of the University of Arizona and Southern Arizona VA Health Care System (SAVAHCS) approved the animal protocols.

Design

We used an experimental design to investigate neuronal degeneration and gene expression changes in an animal model of CNS-directed treatment with methotrexate. Table 1 summarizes the experimental groups. Animals were treated with either 2 mg/kg or 4 mg/kg of methotrexate or artificial CSF (aCSF; vehicle control) administered by a catheter surgically inserted into the left cerebral lateral ventricle and delivered by an osmotic pump. We left the osmotic pump inserted for an additional day in each of these groups in order to ensure that all treatments were delivered. An additional group did not receive the surgical procedures, but we perfusion fixed the brains to control for potential fixation artifacts (perfusion control).

Surgical Procedures and Brain Fixation

We provided surgical analgesia with 6 mg/ml acetaminophen in water bottles 24 hr prior to and for 48 hr after surgery.

Table 1. Summary of Experimental and Control Groups.

Group[a]	Summary of Procedures[b]
Perfusion Control	No Surgical Procedure
Artificial CSF control	3 Days of drug delivery followed by 1 day with osmotic pump only
Methotrexate 2 mg/kg	3 Days of drug delivery followed by 1 day with osmotic pump only
Methotrexate 4 mg/kg	3 Days of drug delivery followed by 1 day with osmotic pump only
Artificial CSF control	4 Days of drug delivery followed by 1 day with osmotic pump only
Methotrexate 4 mg/kg	4 Days of drug delivery followed by 1 day with osmotic pump only

Note. CSF = cerebrospinal fluid.
[a]For each group, $n = 4$. [b]All brains perfusion fixed.

Animals were anesthetized with isofluorane/oxygen (Hallowell EMC Anesthesia Work Station, Pittsfield, MA). Alzet osmotic pumps (Model 1003D; DURECT Corp., Cupertino, CA) and brain infusion cannulae (Brain Infusion kit 2; DURECT Corp.) were filled with methotrexate solution in aCSF. The cannula was inserted into the left ventricle at 2 mm left of sagittal suture and 1 mm posterior to coronal suture to a depth of 3.5 mm and cemented to the skull (Locktite cement). The osmotic pumps were implanted in a subcutaneous pocket (midscapular region) and surgical wounds closed. The animals were allowed to recover, returned to their cage, and monitored throughout the day.

Depending on treatment group (Table 1), we harvested rat brains at either 4 or 5 days after initiation of treatment. Rats were anesthetized as above and heparinized (100 U heparin intraperitoneally/100 g body weight). Cannula placement was confirmed by injecting 10 µl of Evan's blue dye into the cannula. Presence of dye in all ventricles indicated correct cannula placement. The animals were exsanguinated by severing the vena cava. An angiocatheter was inserted into the left ventricle of the heart, and the brain was retroperfused at room temperature with 100 ml heparinized phosphate-buffered saline (PBS) and then with 100 ml of PBS containing 4% formalin. Perfusion pressure was maintained at 80–90 mm Hg with a sphygmomanometer. After removal, brains were postfixed with 4% formalin for an additional 16–24 hr at room temperature. To prepare the brain for paraffin embedding and histology, we made 2-mm-thick coronal slices of the entire brain using a rat brain slicer matrix and stored the slices in PBS at 4°C.

Histology and Microscopy

Sections stained with hematoxylin and eosin were examined on an inverted microscope (Leica Microsystems type 090-135.002) set for bright-field observation. Images were captured following protocols to assure observation and comparisons from standardized areas (i.e., superior, medial, and inferior areas of the cortex) in the sections of the brain using a 40× objective and a digital camera (SPOT™; Diagnostic Instruments, Sterling Heights, MI). Digital files of the captured images were saved using a standardized labeling system for identification of rat, right versus left side of brain, slice and section numbers, and brain area.

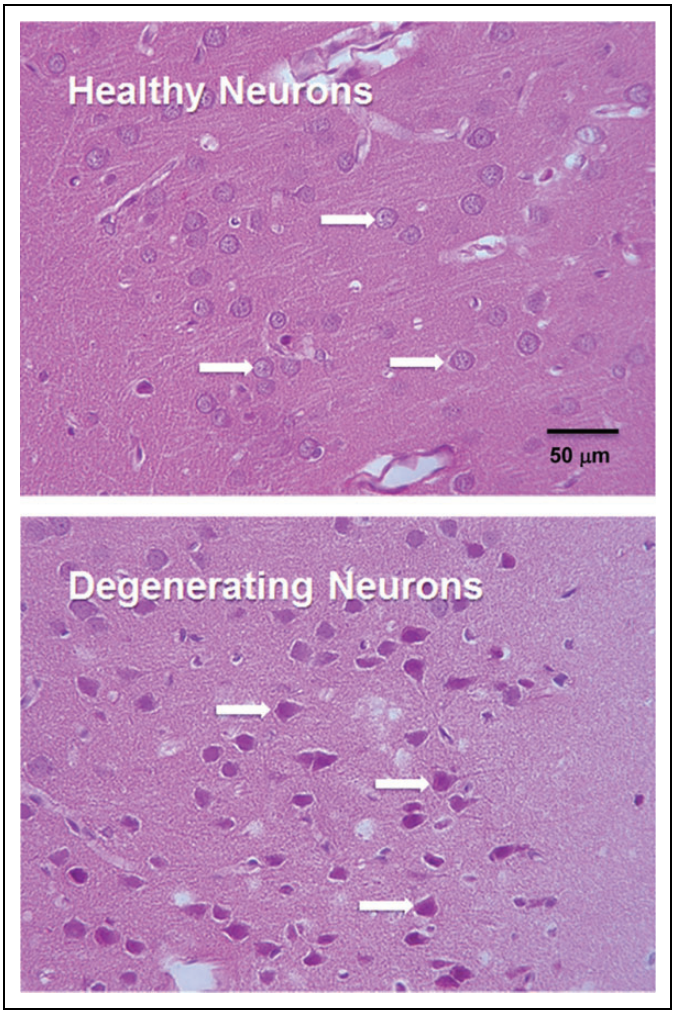

Figure 1. Healthy and degenerating neurons in dentate gyrus region of the hippocampus. Hematoxylin and eosin staining of brain samples from the dentate gyrus region of the hippocampus taken from a control (healthy neurons) and methotrextate-treated (degenerating neurons) animal. Criteria for assessing neuronal injury were decrease in cell size with intense staining of cell contents, eosinophilic neurons due to condensation of mitochondria, and pyknotic nucleus.

Neurons were categorized in the images as healthy versus degenerating (Figure 1) using ImageJ and an optional analysis and cell counter tool (National Institutes of Health) in specified areas defined as a region of interest (ROI) set at 200.08 × 200.08 µm (corresponding to 820 × 820 pixels). Images of the analyzed ROIs with healthy versus degenerating neurons were also saved and numbers of neurons in each category recorded. Using the saved analyzed images of the ROIs, we determined interrater reliability by randomly selecting 10% of the images, having a second rater reanalyze the ROIs, and determining correlation coefficients.

Laser Capture Microdissection (LCM)

Paraffin embedding and tissue sectioning were performed at the Tissue Acquisition and Cellular/Molecular Analysis Shared Resource, a University of Arizona Cancer Center core facility. For gene expression studies, 5-µ-thick serial sections of formalin-fixed, paraffin-embedded (FFPE) brain slices were cut, skipping 50 µ between sections, and mounted on polyethylene naphthalate membrane–coated glass slides (Molecular Devices, Sunnyvale, CA). Microtome blades were cleaned between each tissue slice to prevent cross contamination of tissue sections with RNA. LCM was performed with an Arcturus XT microdissection instrument (Molecular Devices) using a Nikon TE2000 microscope. Tissues were excised from the dentate gyrus region of the hippocampus, caudate putamen, and cortex regions and collected on CapSure™ Macro LCM caps (ThermoFisher Scientific, Waltham, MA). Locations of areas to be cut were based on anatomical landmarks, and removal (capture) of desired areas was confirmed by microscopy and documented by digital imaging. The CA3 region of the hippocampus was not included due to the absence of well-defined anatomical markers.

Microarray Analysis of Gene Expression

Tissue harvested by LCM was deparaffinized and total RNA extracted using the Qiagen (Germantown, MD) RNeasy FFPE RNA isolation kit. RNA concentration and purity (260:280 ratio) were determined with a NanoDrop (ThermoFisher Scientific, Waltham, MA) 2000 (Thermo Scientific) spectrophotometer, and samples were stored at −20°C until they were used. First-strand synthesis and polymerase chain reaction (PCR) preamplification were performed using the Qiagen RT^2 PreAmp complementary DNA Synthesis kit following the kit instructions. We performed eight preamplification PCR cycles in an ABI model 2700 thermal cycler. Custom RT^2 PCR arrays were purchased from Qiagen that contained primers for 11 genes essential for oxidative stress/oxidant defense, apoptosis/inflammation, and injury protection plus three housekeeping gene controls. Table 2 provides a summary of the functional gene groups, associated gene functions, and housekeeping genes. Real-time, quantitative PCR was performed using an ABI model 7500 instrument for 40 PCR cycles, following Qiagen kit instructions. Thermal melting curves were determined for each well in the array to ensure that a single amplicon was produced and to rule out primer–dimer formation. Data were calculated using the $\Delta\Delta C_T$ method as recommended by Qiagen. We performed data analyses using online tools provided by the SABiosciences GeneGlobe Data Analysis Center.

Results

Neuronal Degeneration

Figure 2 presents a summary of the percentage of degenerating neurons in the dentate gyrus and CA3 regions of the hippocampus. In the dentate gyrus region of the hippocampus, the percentage of degenerating neurons was greatest in rats treated with 4 mg/kg of methotrexate over 3 days and was significantly greater in those rats than in the perfusion or aCSF controls and other methotrexate treatment groups. The percentage of degenerating neurons in the CA3 region of the hippocampus was significantly greater in two methotrexate groups compared to the perfusion and aCSF controls. As shown in Figure 3, the percentage of degenerating neurons in the medial region of the cortex was greater in methotrexate-treated animals compared to the aCSF control animals. However, there was no significant difference between experimental and control groups in the superior cortex. An unexpected finding was the greater percentage of degenerating neurons in the inferior cortex of control animals compared to those treated with methotrexate. The number of degenerating neurons was far less in the caudate putamen than in the other regions, and there were no differences among experimental ($M = 3.0$; $SD = 0.30$), perfusion ($M = 2.0$; $SD = 0.30$), and aCSF ($M = 2.0$; $SD = 0.20$) control groups in that region.

Gene Expression Changes

Among the four brain regions, 10 genes were significantly or almost significantly downregulated in the methotrexate group compared to controls (Table 3). In the caudate putamen, five genes had significant fold changes. Fibroblast growth factor-1 (*Fgf1*), which is involved in protection from injury, was the gene with the greatest fold change. Also significantly downregulated in methotrexate-treated animals compared to controls were two genes involved in oxidant defense (glutamine cysteine ligase [*Gclc*] and heme oxygenase-1 [*Hmox1*]) and two genes involved in apoptosis/inflammation (Vcl-2 associated X protein [*Bax*] and glial fibrillary acidic protein [*Gfap*]). In the medial cortex, three genes had significant fold differences between methotrexate-treated and control animals: Manganese superoxide dismutase (*Sod2*, has a key role in oxidant defense), Bcl-2 associated agonist of cell death (*Bad*, involved in regulation of apoptosis), and *Fgf1*. Only one gene, *Sod2*, was significantly downregulated in methotrexate-treated animals in the dentate gyrus.

Discussion

To our knowledge, the present study is the first to investigate methotrexate-induced injury in selected brain regions and associated gene expression changes using a rat model of intraventricular drug administration. The greatest percentage of degenerating neurons in methotrexate-treated rats was in the medial region of the cortex. *Bad*, *Sod2*, and *Fgf1* were all significantly downregulated in this brain region in methotrexate-treated animals. *Sod2* was also significantly downregulated in methotrexate-treated rats in the dentate gyrus, a region of the hippocampus in which there was also a large percentage of degenerating neurons. Collectively these findings suggest that genes involved in regulating oxidative

Table 2. Summary of Functional Gene Groups and Associated Functions.

Gene Groups and Specific Genes	Abbreviation	Gene Functions
Oxidative stress/oxidant defense		
Glutamine cysteine ligase	*Gclc*	Rate-limiting enzyme in glutathione synthesis (Franco, Schoneveld, Pappa, & Panayiotidis, 2007)
Glutathione reductase	*Gsr*	Key cellular antioxidant enzyme (Franco et al., 2007); upregulated by oxidative stress in the hippocampus (Torres, 2012)
Manganese superoxide dismutase	*Sod2*	Enzyme that reduces damage by superoxide (H. F. Huang, Guo, Cao, Shi, & Xia, 2012) and upregulated by oxidative stress in hippocampus (Furuta et al., 1995; Rodriguez-Martinez, Martinez, Espinosa-Garcia, Maldonado, & Rivas-Arancibia, 2013)
Heme oxygenase-1	*Hmox1*	Enzyme strongly upregulated by oxidative stress in brain (Guerra et al., 2013)
Apoptosis/inflammation		
Bcl-2-associated X protein	*Bax*	Proapoptotic member of Bcl-2 family (Engel, Plesnila, Prehn, & Henshall, 2011); key role in regulating intrinsic apoptotic signaling (Cheng, Gulbins, & Siemen, 2011)
Bcl-2-associated agonist of cell death	*Bad*	Positively regulates cell apoptosis (Dave et al., 2011; Siemionow, Klimczak, Brzezicki, Siemionow, & McLain, 2009)
Glial fibrillary acidic protein	*Gfap*	Marker of CNS inflammation (gliosis; Giovannoni, 2006; Siemionow et al., 2009)
Protection from injury		
Tumor necrosis factor	*Tnf*	Regulates survival of injured hippocampal neurons (Bernardino et al., 2005; Viviani, Corsini, Galli, & Marinovich, 1998; Yang, Lindholm, Konishi, Li, & Shen, 2002)
Glial-derived neurotrophic factor	*Gdnf*	Promotes survival and differentiation of dopaminergic neurons (Deng et al., 2013; Tan et al., 2014; Wood, McDermott, & Sullivan, 2005), prevents apoptosis of motor neurons (Brunet et al., 2007; Zhao et al., 2004), and improves cognitive deficits in old rats (Pertusa et al., 2008)
Thrombospondin-1	*Thsd1*	Astrocyte-derived signal promotes CNS synaptogenesis (Christopherson et al., 2005; Raju et al., 2014; Xu, Xiao, & Xia, 2010)
Fibroblast growth factor-1	*Ffg1*	Involved in tissue repair and neuroprotection (B. Huang, Krafft, et al., 2012; Tsai, Shen, Kuo, Cheng, & Chak, 2008)
Housekeeping genes (control genes)		
Peptidylprolyl isomerase (cyclophilin A)	*Ppia*	Cyclosporin-binding protein; accelerates protein folding (Bonefeld, Elfving, & Wegener, 2008; Harris, Reeves, & Phillips, 2009; Langnaese, John, Schweizer, Ebmeyer, & Keilhoff, 2008; Marini et al., 2006; Pernot, Dorandeu, Beaup, & Peinnequin, 2010; Santos & Duarte, 2008)
TATA-Box binding protein	*Tbp*	Central to initiation of RNA transcription; part of the preinitiation complex (Cook, Vink, Donkin, & van den Heuvel, 2009; Pernot et al., 2010; Santos & Duarte, 2008)
Phosphoglycerate kinase 1	*Pgk1*	Plays a phosphate-transfer role in glycolysis and gluconeogenesis; ATP to ADP; ADP to ATP (Langnaese et al., 2008; Santos & Duarte, 2008)
Succinate dehydrogenase subunit A	*Sdha*	Mitochondrial inner membrane protein; participates in the citric acid cycle and the respiratory chain (Cook et al., 2009; Langnaese et al., 2008)

Note. ADP = adenosine diphosphate; ATP = adenosine triphosphate; CNS = central nervous system.

stress and apoptosis could be involved in neuronal injury and cognitive dysfunction associated with methotrexate treatment.

The caudate putamen had the fewest number of degenerating neurons in the methotrexate groups; however, five genes involved in protection from injury (*Fgf1*), oxidative stress (*Gclc* and *Hmox1*), and apoptosis/inflammation (*Bax* and *Gfap)* were significantly downregulated with methotrexate treatment in this brain region. One possible explanation for this finding is that the time course for neuronal injury and degeneration in the caudate putamen in response to treatment is different from that in the cortex and hippocampus. Future studies are needed to more closely investigate regional differences in methotrexate-associated brain injury.

Seigers and colleagues (2008) reported that rats treated with a single dose of intravenous methotrexate (37.5–300 mg/kg injected into the tail vein) had a dose-dependent and long-lasting decrease in hippocampal cell proliferation. Compared to controls, rats treated with 250 mg/kg of methotrexate also had impaired spatial memory and novel object recognition. These investigators did not examine cell death/differentiation in the caudate putamen. Fardell, Vardy, Logge, and Johnston (2010) found that rats treated with a single high dose of intraperitoneal methotrexate (250 mg/kg) performed significantly worse than vehicle controls on tasks that required short- and long-term memory and rule memory, and that these memory impairments lasted for at least 8 months after treatment. In CSF samples obtained from children with ALL during CNS-directed therapy in prior studies, we found increased activity of caspase enzymes that initiate (caspase 8 and 9) and induce (caspase 3/7) apoptosis and increased levels of oxidized phospholipids (I. Moore, Gundy, Montgomery, et al., 2014; I. M. Moore, Gundy, Pasvogel, et al., 2015). These findings lend additional support

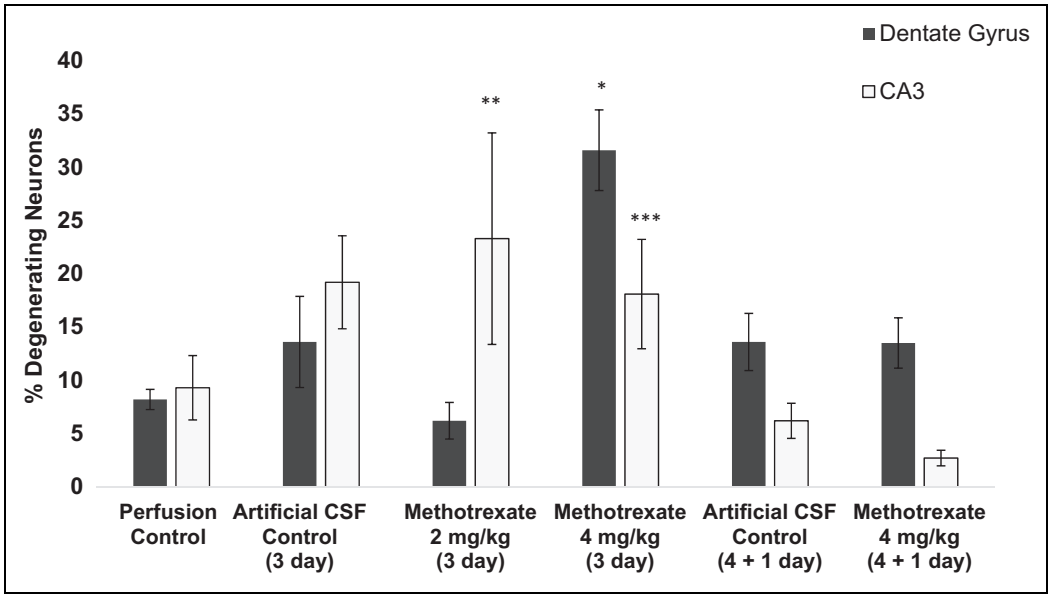

Figure 2. Degenerating neurons in the hippocampus by treatment group. The percentage of degenerating neurons for the dentate gyrus and the cornu ammonis 3 regions are presented by treatment group. Error bars represent standard error. *Significantly more degenerating neurons than methotrexate 2 mg/kg (3 days), artificial cerebral spinal fluid control (3 days), methotrexate 4 mg/kg (4 + 1 days), and perfusion control (all $ps \leq .001$). **Significantly more degenerating neurons than methotrexate 4 mg/kg (4 + 1 days; $p = .012$). ***Significantly more degenerating neurons than methotrexate 4 mg/kg (4 + 1 days; $p = .048$).

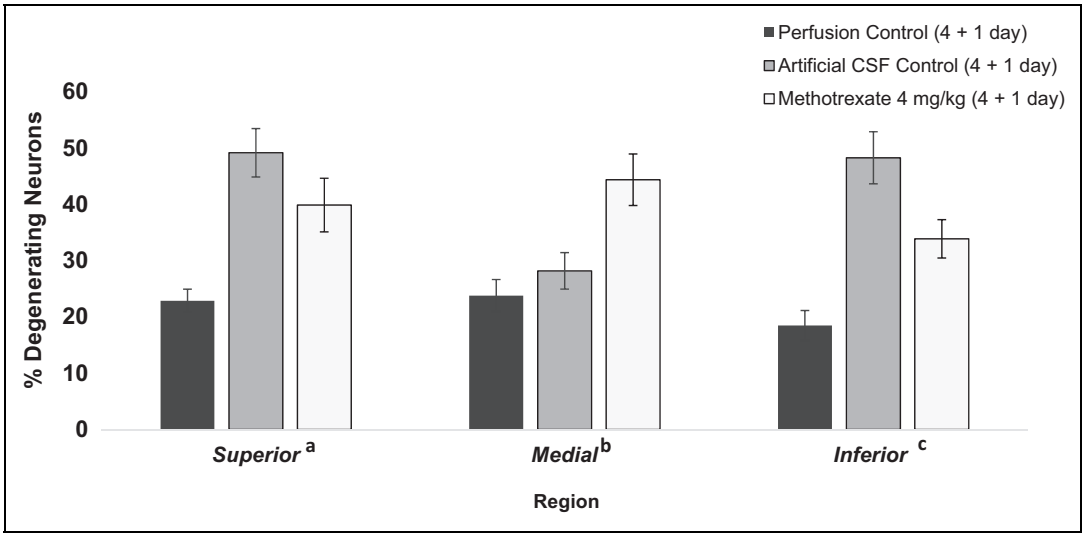

Figure 3. Degenerating neurons in the cortex by region. The percentage of degenerating neurons by treatment group for the superior, medial, and inferior regions of the cortex is presented. Error bars represent standard errors. [a]Methotrexate 4 mg/kg (4 + 1 days) significantly more degenerating neurons than artificial cerebral spinal fluid (aCSF; $p = .05$) and perfusion control ($p = .001$); aCSF significantly more degenerating neurons than perfusion control ($p < .001$). [b]Methotrexate 4 mg/kg (4 + 1 days) significantly more degenerating neurons than aCSF ($p = .003$) and perfusion control ($p = .001$). [c]Methotrexate 4 mg/kg (4 + 1 days) significantly fewer degenerating neurons than aCSF ($p = .007$) and perfusion control ($p = .001$); aCSF significantly more degenerating neurons than perfusion control ($p < .001$).

for the potential role of oxidative stress and apoptosis in methotrexate-induced CNS injury.

In our gene expression studies, we found that, among the brain regions studied, the expression of 10 genes were significantly, or near significantly, downregulated with methotrexate treatment. It is possible that the downregulation may have been due to nongene-specific methotrexate inhibition of RNA synthesis, as investigators have previously reported in studies with cultured lymphocytes, in which methotrexate inhibited cytokine messenger RNA synthesis (Goldminz et al., 2015). However, this possibility seems quite unlikely in the present study because the majority of the 11 target

Table 3. Gene Expression Fold Changes in Methotrexate-Treated Rats Relative to Control Rats by Brain Region.

Gene Symbol	Fold Change	Significance
Caudate putamen		
Fgf1	−22.997	.006
Gclc	−6.364	.004
Hmox1	−4.418	.027
Bax	−3.458	.028
Gfap	−9.063	.010
Dentate gyrus		
Sod2	−1.859	.012
Medial cortex		
Bad	−5.568	.028
Sod2	−2.426	.033
Fgf1	−6.142	.083
Inferior cortex		
Gsr	−2.642	.089

Note. *Fgf1* = fibroblast growth factor-1; *Gclc* = glutamine cysteine ligase; *Hmox1* = heme oxygenase-1; *Bax* = Bcl-2-associated X protein; *Gfap* = glial fibrillary acidic protein; *Sod2* = manganese superoxide dismutase; *Bad* = Bcl-2-associated agonist of cell death; *Gsr* = glutathione reductase.

genes that we evaluated in each of four brain regions were not affected by methotrexate treatment. Rather, the finding suggests that methotrexate treatment selectively downregulated the genes.

Limitations

Because this was a pilot study of neuronal degeneration and gene expression changes in an animal model of CNS-directed treatment with methotrexate, our sample size was only four rats per group, which limited the statistical power to detect differences. However, even with our relatively small sample size, we did find treatment group–dependent increases in neuronal degeneration in all regions except the caudate putamen. Another limitation is that the doses we selected for these studies were based on prior studies, in which single doses of 2 and 4 mg/kg were administered intraperitoneally (Schunior, 1994; van der Kogel, 1985). However, children with ALL receive repeated doses of intrathecal and intravenous methotrexate; it is possible that the magnitude of neuronal degeneration and gene expression fold changes could be different following repeated methotrexate treatments than what we observed with single doses. Finally, the time course of our experimental design did not allow for measures of learning and memory. Future studies are needed to determine potential associations among neuronal degeneration, gene expression changes, learning, and memory.

Acknowledgments

We thank Mr. Jim Averill, research specialist, for his technical assistance; the Department of Veterans Affairs Biorepository Brain Bank for providing laser capture microdissection equipment; and the Tissue Acquisition and Cellular/Molecular Analysis Shared Resource, University of Arizona Cancer Center, for performing histology. We thank Dr. Alice Pasvogel for assistance with statistical analyses and Ms. Kari Marano for editorial assistance and preparation of graphs.

Author Contribution

Ida M. (Ki) Moore contributed to conception, design, analysis, and interpretation; drafted and critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. Carrie J. Merkle contributed to conception, design, analysis, and interpretation; drafted and critically revised manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. Howard Byrne contributed to acquisition, critically revised manuscript, gave final approval, and agreed to be accountable for all aspects of work ensuring integrity and accuracy. Adam Ross contributed to acquisition and analysis, critically revised manuscript, gave final approval, agreed to be accountable for all aspects of work ensuring integrity and accuracy. Ashley M. Hawkins contributed to acquisition and analysis, critically revised manuscript, gave final approval, agreed to be accountable for all aspects of work ensuring integrity and accuracy. Sara S. Ameli contributed to acquisition and analysis, critically revised manuscript, gave final approval, and agreed to be accountable for all aspects of work ensuring integrity and accuracy. Davis W. Montgomery contributed to conception, design, acquisition, analysis, and interpretation; drafted and critically revised manuscript; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by a Discovery Grant from Alex's Lemonade Stand Foundation.

References

Ahles, T. A., & Saykin, A. J. (2007). Candidate mechanisms for chemotherapy-induced cognitive changes. *Nature Reviews Cancer*, *7*, 192–201.

Ashford, J., Schoffstall, C., Reddick, W. E., Leone, C., Laningham, F. H., Glass, J. O., … Conklin, H. M. (2010). Attention and working memory abilities in children treated for acute lymphoblastic leukemia. *Cancer*, *116*, 4638–4645.

Bernardino, L., Xapelli, S., Silva, A. P., Jakobsen, B., Poulsen, F. R., Oliveira, C. R., … Zimmer, J. (2005). Modulator effects of interleukin-1beta and tumor necrosis factor-alpha on AMPA-induced excitotoxicity in mouse organotypic hippocampal slice cultures. *Journal of Neuroscience*, *25*, 6734–6744.

Bisen-Hersh, E. B., Hineline, P. N., & Walker, E. A. (2011). Disruption of learning processes by chemotherapeutic agents in childhood survivors of acute lymphoblastic leukemia and preclinical models. *Journal of Cancer*, *2*, 292–301.

Bonefeld, B. E., Elfving, B., & Wegener, G. (2008). Reference genes for normalization: A study of rat brain tissue. *Synapse*, *62*, 302–309.

Brunet, N., Tarabal, O., Portero-Otin, M., Oppenheim, R. W., Esquerda, J. E., & Caldero, J. (2007). Survival and death of mature avian motoneurons in organotypic slice culture: Trophic requirements for survival and different types of degeneration. *Journal of Comparative Neurology*, *501*, 669–690.

Buizer, A. I., De Sonneville, L. M., van den Heuvel-Eibrink, M. M., Njiokiktjien, C., & Veerman, A. J. (2005). Visuomotor control in survivors of childhood acute lymphoblastic leukemia treated with chemotherapy only. *Journal of the International Neuropsychology Society*, *11*, 554–565.

Buizer, A. I., de Sonneville, L. M., van den Heuvel-Eibrink, M. M., & Veerman, A. J. (2005). Chemotherapy and attentional dysfunction in survivors of childhood acute lymphoblastic leukemia: Effect of treatment intensity. *Pediatric Blood & Cancer*, *45*, 281–290.

Buizer, A. I., de Sonneville, L. M., & Veerman, A. J. (2009). Effects of chemotherapy on neurocognitive function in children with acute lymphoblastic leukemia: A critical review of the literature. *Pediatric Blood & Cancer*, *52*, 447–454.

Carey, M. E., Hockenberry, M. J., Moore, I. M., Hutter, J. J., Krull, K. R., Pasvogel, A., & Kaemingk, K. L. (2007). Brief report: Effect of intravenous methotrexate dose and infusion rate on neuropsychological function one year after diagnosis of acute lymphoblastic leukemia. *Journal of Pediatric Psychology*, *32*, 189–193.

Caron, J. E., Krull, K. R., Hockenberry, M., Jain, N., Kaemingk, K., & Moore, I. M. (2009). Oxidative stress and executive function in children receiving chemotherapy for acute lymphoblastic leukemia. *Pediatric Blood & Cancer*, *53*, 551–556.

Cheng, Y., Gulbins, E., & Siemen, D. (2011). Activation of the permeability transition pore by Bax via inhibition of the mitochondrial BK channel. *Cellular Physiology & Biochemistry*, *27*, 191–200.

Christopherson, K. S., Ullian, E. M., Stokes, C. C., Mullowney, C. E., Hell, J. W., Agah, A., . . . Barres, B. A. (2005). Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell*, *120*, 421–433.

Cook, N. L., Vink, R., Donkin, J. J., & van den Heuvel, C. (2009). Validation of reference genes for normalization of real-time quantitative RT-PCR data in traumatic brain injury. *Journal of Neuroscience Research*, *87*, 34–41.

Dave, K. R., Bhattacharya, S. K., Saul, I., DeFazio, R. A., Dezfulian, C., Lin, H. W., . . . Perez-Pinzon, M. A. (2011). Activation of protein kinase C delta following cerebral ischemia leads to release of cytochrome C from the mitochondria via *Bad* pathway. *PLoS ONE*, *6*, e22057.

DeCoteau, W. E., & Kesner, R. P. (2000). A double dissociation between the rat hippocampus and medial caudoputamen in processing two forms of knowledge. *Behavioral Neuroscience*, *114*, 1096–1108.

Deng, X., Liang, Y., Lu, H., Yang, Z., Liu, R., Wang, J., . . . Feng, Z. (2013). Co-transplantation of GDNF-overexpressing neural stem cells and fetal dopaminergic neurons mitigates motor symptoms in a rat model of Parkinson's disease. *PLoS One*, *8*, e80880.

Eichenbaum, H. (2001). The hippocampus and declarative memory: Cognitive mechanisms and neural codes. *Behavioral Brain Research*, *127*, 199–207.

Eichenbaum, H. (2004). Hippocampus: Cognitive processes and neural representations that underlie declarative memory. *Neuron*, *44*, 109–120.

Engel, T., Plesnila, N., Prehn, J. H., & Henshall, D. C. (2011). In vivo contributions of BH3-only proteins to neuronal death following seizures, ischemia, and traumatic brain injury. *Journal of Cerebral Blood Flow & Metabolism*, *31*, 1196–1210.

Fardell, J. E., Vardy, J., Logge, W., & Johnston, I. (2010). Single high dose treatment with methotrexate causes long-lasting cognitive dysfunction in laboratory rodents. *Pharmacology Biochemistry & Behavior*, *97*, 333–339.

Fortin, N. J., Agster, K. L., & Eichenbaum, H. B. (2002). Critical role of the hippocampus in memory for sequences of events. *Nature Neuroscience*, *5*, 458–462.

Franco, R., Schoneveld, O. J., Pappa, A., & Panayiotidis, M. I. (2007). The central role of glutathione in the pathophysiology of human diseases. *Archives of Physiology & Biochemistry*, *113*, 234–258.

Furuta, A., Price, D. L., Pardo, C. A., Troncoso, J. C., Xu, Z. S., Taniguchi, N., & Martin, L. J. (1995). Localization of superoxide dismutases in Alzheimer's disease and Down's syndrome neocortex and hippocampus. *American Journal of Pathology*, *146*, 357–367.

Giovannoni, G. (2006). Multiple sclerosis cerebrospinal fluid biomarkers. *Disease Markers*, *22*, 187–196.

Goldminz, A. M., Suarez-Farinas, M., Wang, A. C., Dumont, N., Krueger, J. G., & Gottlieb, A. B. (2015). CCL20 and IL22 messenger RNA expression after adalimumab vs methotrexate treatment of psoriasis: A randomized clinical trial. *Journal of the American Medical Association Dermatology*, *151*, 837–846.

Grahn, J. A., Parkinson, J. A., & Owen, A. M. (2008). The cognitive functions of the caudate nucleus. *Progress in Neurobiology*, *86*, 141–155.

Graybiel, A. M. (2005). The basal ganglia: Learning new tricks and loving it. *Current Opinion in Neurobiology*, *15*, 638–644.

Guerra, R., Vera-Aguilar, E., Uribe-Ramirez, M., Gookin, G., Camacho, J., Osornio-Vargas, A. R., . . . De Vizcaya-Ruiz, A. (2013). Exposure to inhaled particulate matter activates early markers of oxidative stress, inflammation and unfolded protein response in rat striatum. *Toxicology Letters*, *222*, 146–154.

Harris, J. L., Reeves, T. M., & Phillips, L. L. (2009). Injury modality, survival interval, and sample region are critical determinants of qRT-PCR reference gene selection during long-term recovery from brain trauma. *Journal of Neurotrauma*, *26*, 1669–1681.

Hockenberry, M., Krull, K., Moore, K., Gregurich, M. A., Casey, M. E., & Kaemingk, K. (2007). Longitudinal evaluation of fine motor skills in children with leukemia. *Journal of Pediatric Hematology/Oncology*, *29*, 535–539.

Hockenberry, M. J., Krull, K. R., Insel, K. C., Harris, L. L., Gundy, P. M., Adkins, K. B., . . . Moore, I. M. (2015). Oxidative stress, motor abilities, and behavioral adjustment in children treated for acute lymphoblastic leukemia. *Oncology Nursing Forum*, *42*, 542–549.

Hockenberry, M. J., Taylor, O. A., Gundy, P. M., Ross, A. K., Pasvogel, A., Montgomery, D., . . . Moore, I. (2013). F2-Isoprostanes: A measure of oxidative stress in children receiving treatment for leukemia. *Biological Research for Nursing*, *16*, 303–309.

Huang, B., Krafft, P. R., Ma, Q., Rolland, W. B., Caner, B., Lekic, T., . . . Zhang, J. H. (2012). Fibroblast growth factors preserve blood-brain barrier integrity through RhoA inhibition after

intracerebral hemorrhage in mice. *Neurobiology of Disease*, *46*, 204–214.

Huang, H. F., Guo, F., Cao, Y. Z., Shi, W., & Xia, Q. (2012). Neuroprotection by manganese superoxide dismutase (MnSOD) mimics: Antioxidant effect and oxidative stress regulation in acute experimental stroke. *CNS Neuroscience & Therapeutics*, *18*, 811–818.

Hunger, S. P., Lu, X., Devidas, M., Camitta, B. M., Gaynon, P. S., Winick, N. J., ... Carroll, W. L. (2012). Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: A report from the children's oncology group. *Journal of Clinical Oncology*, *30*, 1663–1669.

Igarashi, H. (1989). The effect of methotrexate on the development of synapses in the neonatal rat hippocampus. *Neuropediatrics*, *20*, 196–198.

Jahovic, N., Cevik, H., Sehirli, A. O., Yegen, B. C., & Sener, G. (2003). Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. *Journal of Pineal Research*, *34*, 282–287.

Janelsins, M. C., Roscoe, J. A., Berg, M. J., Thompson, B. D., Gallagher, M. J., Morrow, G. R., ... Gross, R. A. (2010). IGF-1 partially restores chemotherapy-induced reductions in neural cell proliferation in adult C57BL/6 mice. *Cancer Investigation*, *28*, 544–553.

Kaemingk, K. L., Carey, M. E., Moore, I. M., Herzer, M., & Hutter, J. J. (2004). Math weaknesses in survivors of acute lymphoblastic leukemia compared to healthy children. *Child Neuropsychology*, *10*, 14–23.

Kesner, R. P., & Gilbert, P. E. (2006). The role of the medial caudate nucleus, but not the hippocampus, in a matching-to sample task for a motor response. *European Journal of Neuroscience*, *23*, 1888–1894.

Krull, K. R., Hockenberry, M. J., Miketova, P., Carey, M., & Moore, I. M. (2013). Chemotherapy-related changes in central nervous system phospholipids and neurocognitive function in childhood acute lymphoblastic leukemia. *Leukemia & Lymphoma*, *54*, 535–540.

Krull, K. R., Okcu, M. F., Potter, B., Jain, N., Dreyer, Z., Kamdar, K., & Brouwers, P. (2008). Screening for neurocognitive impairment in pediatric cancer long-term survivors. *Journal of Clinical Oncology*, *26*, 4138–4143.

Langnaese, K., John, R., Schweizer, H., Ebmeyer, U., & Keilhoff, G. (2008). Selection of reference genes for quantitative real-time PCR in a rat asphyxial cardiac arrest model. *BMC Molecular Biology*, *9*, 53. doi:10.1186/1471-2199-9-53

Marini, F., Pozzato, C., Andreetta, V., Jansson, B., Arban, R., Domenici, E., & Carboni, L. (2006). Single exposure to social defeat increases corticotropin-releasing factor and glucocorticoid receptor mRNA expression in rat hippocampus. *Brain Research*, *1067*, 25–35.

Merkle, C. J., Moore, I. M., Penton, B. S., Torres, B. J., Cueny, R. K., Schaeffer, R. C. Jr., & Montgomery, D. W. (2000). Methotrexate causes apoptosis in postmitotic endothelial cells. *Biological Research for Nursing*, *2*, 5–14.

Miketova, P., Kaemingk, K., Hockenberry, M., Pasvogel, A., Hutter, J., Krull, K., & Moore, I. M. (2005). Oxidative changes in cerebral spinal fluid phosphatidylcholine during treatment for acute lymphoblastic leukemia. *Biological Research for Nursing*, *6*, 187–195.

Miyashita, Y. (1993). Inferior temporal cortex: Where visual perception meets memory. *Annual Review of Neuroscience*, *16*, 245–263.

Moleski, M. (2000). Neuropyschological, neuroanatomical, and neurophysiological consequences of CNS chemotherapy for acute lymphoblastic leukemia. *Archives of Clinical Neuropsychology*, *15*, 603–660.

Montour-Proulx, I., Kuehn, S. M., Keene, D. L., Barrowman, N. J., Hsu, E., Matzinger, M. A., ... Halton, J. M. (2005). Cognitive changes in children treated for acute lymphoblastic leukemia with chemotherapy only according to the Pediatric Oncology Group 9605 protocol. *Journal of Child Neurology*, *20*, 129–133.

Moore, I., Gundy, P., Montgomery, D., Pasvogel, A., Taylor, O. A., & Hockenberry, M. (2014, September). *CNS-directed chemotherapy for childhood leukemia increases oxidative stress and apoptosis biomarkers of neurologic injury*. Paper presented at the State of the Science Congress on Nursing Research, Washington, DC.

Moore, I. M., Gundy, P., Pasvogel, A., Montgomery, D. W., Taylor, O. A., Koerner, K. M., ... Hockenberry, M. (2015). Increase in oxidative stress as measured by cerebrospinal fluid lipid peroxidation during treatment for childhood acute lymphoblastic leukemia. *Journal of Pediatric Hematology/Oncology*, *37*, e89–e93.

Moore, I. M., Hockenberry, M. J., & Krull, K. R. (2013). Cancer-related cognitive changes in children, adolescents and adult survivors of childhood cancers. *Seminars in Oncology Nursing*, *29*, 248–259.

Moore, I. M., Merkle, C. J., Miketova, P., Salyer, R. K., Torres, B. J., Schaeffer, R. C. Jr., & Montgomery, D. W. (2006). Cytosine arabinoside induces programmed endothelial cell death through the caspase-3 pathway. *Biological Research for Nursing*, *7*, 289–296.

Nagel, B. J., Palmer, S. L., Reddick, W. E., Glass, J. O., Helton, K. J., Wu, S., ... Mulhern, R. K. (2004). Abnormal hippocampal development in children with medulloblastoma treated with risk-adapted irradiation. *American Journal of Neuroradiology*, *25*, 1575–1582.

Pernot, F., Dorandeu, F., Beaup, C., & Peinnequin, A. (2010). Selection of reference genes for real-time quantitative reverse transcription-polymerase chain reaction in hippocampal structure in a murine model of temporal lobe epilepsy with focal seizures. *Journal of Neuroscience Research*, *88*, 1000–1008.

Pertusa, M., Garcia-Matas, S., Mammeri, H., Adell, A., Rodrigo, T., Mallet, J., ... Sanfeliu, C. (2008). Expression of GDNF transgene in astrocytes improves cognitive deficits in aged rats. *Neurobiology of Aging*, *29*, 1366–1379.

Pui, C. H. (2003). Toward optimal central nervous system-directed treatment in childhood acute lymphoblastic leukemia. *Journal of Clinical Oncology*, *21*, 179–181.

Pui, C. H. (2006). Central nervous system disease in acute lymphoblastic leukemia: Prophylaxis and treatment. *Hematology American Society of Hematology Education Program*, *2006*, 142–146.

Pui, C. H., & Evans, W. E. (2006). Treatment of acute lymphoblastic leukemia. *New England Journal of Medicine*, *354*, 166–178.

Raju, R., Palapetta, S. M., Sandhya, V. K., Sahu, A., Alipoor, A., Balakrishnan, L., ... Gowda, H. (2014). A network map of FGF-1/FGFR signaling system. *Journal of Signal Transduction*, *2014*, article id: 962962.

Reddick, W. E., Glass, J. O., Helton, K. J., Langston, J. W., Li, C. S., & Pui, C. H. (2005). A quantitative MR imaging assessment of

leukoencephalopathy in children treated for acute lymphoblastic leukemia without irradiation. *American Journal of Neuroradiology*, *26*, 2371–2377.

Reddick, W. E., Glass, J. O., Helton, K. J., Langston, J. W., Xiong, X., Wu, S., & Pui, C. H. (2005). Prevalence of leukoencephalopathy in children treated for acute lymphoblastic leukemia with high-dose methotrexate. *American Journal of Neuroradiology*, *26*, 1263–1269.

Reddick, W. E., Shan, Z. Y., Glass, J. O., Helton, S., Xiong, X., Wu, S., . . . Mulhern, R. K. (2006). Smaller white-matter volumes are associated with larger deficits in attention and learning among long-term survivors of acute lymphoblastic leukemia. *Cancer*, *106*, 941–949.

Rieckmann, A., Fischer, H., & Backman, L. (2010). Activation in striatum and medial temporal lobe during sequence learning in younger and older adults: Relations to performance. *Neuroimage*, *50*, 1303–1312.

Rodriguez-Martinez, E., Martinez, F., Espinosa-Garcia, M. T., Maldonado, P., & Rivas-Arancibia, S. (2013). Mitochondrial dysfunction in the hippocampus of rats caused by chronic oxidative stress. *Neuroscience*, *252*, 384–395.

Santos, A. R. A., & Duarte, C. B. (2008). Validation of internal control genes for expression studies: Effects of the neurotrophin BDNF on hippocampal neurons. *Journal of Neuroscience Research*, *86*, 3684–3692.

Schendan, H. E., Searl, M. M., Melrose, R. J., & Stern, C. E. (2003). An FMRI study of the role of the medial temporal lobe in implicit and explicit sequence learning. *Neuron*, *37*, 1013–1025.

Schunior, A. (1994). Radiation effects on growth are altered in rats by prednisolone and methotrexate. *Pediatric Research*, *35*, 416–423.

Seigers, R., & Faradell, J. E. (2011). Neurobiological basis of chemotherapy-induced cognitive impairment: A review of rodent research. *Neuroscience & Biobehavioral Reviews*, *35*, 729–741.

Seigers, R., Schagen, S. B., Beerling, W., Boogerd, W., van Tellingen, O., van Dam, F. S., . . . Buwalda, B. (2008). Long-lasting suppression of hippocampal cell proliferation and impaired cognitive performance by methotrexate in the rat. *Behavioral Brain Research*, *186*, 168–175.

Seigers, R., Schagen, S. B., Coppens, C. M., van der Most, P. J., van Dam, F. S., Koolhaas, J. M., & Buwalda, B. (2009). Methotrexate decreases hippocampal cell proliferation and induces memory deficits in rats. *Behavioral Brain Research*, *201*, 279–284.

Siemionow, K., Klimczak, A., Brzezicki, G., Siemionow, M., & McLain, R. F. (2009). The effects of inflammation on glial fibrillary acidic protein expression in satellite cells of the dorsal root ganglion. *Spine*, *34*, 1631–1637.

Tan, X., Zhang, L., Zhu, H., Qin, J., Tian, M., Dong, C., . . . Lin, G. (2014). Brn4 and TH synergistically promote the differentiation of neural stem cells into dopaminergic neurons. *Neuroscience Letters*, *571*, 23–28.

Torres, L. (2012). Environmental tobacco smoke induces oxidative stress in distinct brain regions of infant mice. *Journal of Toxicology & Environmental Health Part A*, *75*, 971–980.

Tsai, M. C., Shen, L. F., Kuo, H. S., Cheng, H., & Chak, K. F. (2008). Involvement of acidic fibroblast growth factor in spinal cord injury repair processes revealed by a proteomics approach. *Molecular & Cellular Proteomics*, *7*, 1668–1687.

van der Kogel, A. J. (1985). Effects of intrathecal methotrexate and cytosine arabinoside on the radiation tolerance of the rat spinal cord. *Radiotherapy and Oncology*, *4*, 239–251.

Viviani, B., Corsini, E., Galli, C. L., & Marinovich, M. (1998). Glia increase degeneration of hippocampal neurons through release of tumor necrosis factor-alpha. *Toxicology & Applied Pharmacology*, *150*, 271–276.

Waber, D. P., & Tarbell, N. J. (1997). Toxicity of CNS prophylaxis for childhood leukemia. *Oncology*, *11*, 259–264.

Waber, D. P., Tarbell, N. J., Fairclough, D., Atmore, K., Castro, R., Isquith, P., . . . Schiller, M. (1995). Cognitive sequelae of treatment in childhood acute lymphoblastic leukemia: Cranial radiation requires an accomplice. *Journal of Clinical Oncology*, *13*, 2490–2496.

Ward, E., DeSantis, C., Robbins, A., Kohler, B., & Jemal, A. (2014). Childhood and adolescent cancer statistics, 2014. *CA: A Cancer Journal for Clinicians*, *64*, 83–103.

Winick, N. J., Carroll, W. L., & Hunger, S. P. (2004). Childhood leukemia—New advances and challenges. *New England Journal of Medicine*, *351*, 601–603.

Wood, T. K., McDermott, K. W., & Sullivan, A. M. (2005). Differential effects of growth/differentiation factor 5 and glial cell line-derived neurotrophic factor on dopaminergic neurons and astroglia in cultures of embryonic rat midbrain. *Journal of Neuroscience Research*, *80*, 759–766.

Xu, J., Xiao, N., & Xia, J. (2010). Thrombospondin 1 accelerates synaptogenesis in hippocampal neurons through neuroligin 1. *Nature Neuroscience*, *13*, 22–24.

Yang, L., Lindholm, K., Konishi, Y., Li, R., & Shen, Y. (2002). Target depletion of distinct tumor necrosis factor receptor subtypes reveals hippocampal neuron death and survival through different signal transduction pathways. *Journal of Neuroscience*, *22*, 3025–3032.

Yanovski, J. A., Packer, R. J., Levine, J. D., Davidson, T. L., Micalizzi, M., & D'Angio, G. (1989). An animal model to detect the neuropsychological toxicity of anticancer agents. *Medical and Pediatric Oncology*, *17*, 216–221.

Zhao, Z., Alam, S., Oppenheim, R. W., Prevette, D. M., Evenson, A., & Parsadanian, A. (2004). Overexpression of glial cell line-derived neurotrophic factor in the CNS rescues motoneurons from programmed cell death and promotes their long-term survival following axotomy. *Experimental Neurology*, *190*, 356–372.