Elucidation of Cognitive Deficits Associated with Doxorubicin Treatment and Ameliorating Effects of Phenyl-2-Amino-Ethyl Selenide (PAESe)

by

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ABSTRACT

A growing body of evidence suggests that acute and chronic cognitive impairments are a common side-effect of chemotherapy. In fact, chemotherapy induced cognitive deficits or "chemobrain" are known to occur in up to 75% of cancer survivors, but the mechanisms of these impairments are not fully understood. Elucidating the mechanisms of memory deficits due to chemobrain is crucial for developmental specific therapies. In the present study, we investigated the effects of a chemotherapeutic agent, doxorubicin (DOX), on memory using a rodent model of chemobrain. Additionally, we explored the therapeutic potential of Phenyl-2-Amino-Ethyl Selenide (PAESe) on altered synaptic plasticity and memory due to chemobrain. In addition, we investigated the direct effect of different concentrations DOX in *in vitro* and *ex vivo* models of chemobrain. Hippocampal neurons were exposed to three different concentrations of DOX (250nM, 500nM, and 1000nM) for 6 hours while acute brain slices were incubated with two concentrations of DOX (250nM and 1000nM) for the same time. Our results indicated that DOX exposure increased Akt, ERK1/2, and p38 phosphorylation, but it did not affect GSK3β phosphorylation. This result suggests that the possible effects of chemobrain are due to p38 dysregulation. Indeed, several studies illustrate the correlation between increased p38 phosphorylation and cognitive decline. We also explored the effects of intravenously injection of DOX (cumulative dose 25 mg/kg), DOX+PAESe (cumulative dose 25 mg/kg and 50 mg/kg), and PAESe (cumulative dose 50 mg/kg) on spatial memory. Nude mice received five doses of assigned drugs for four weeks. The results revealed that DOX treated animals had impaired

memory, reduced LTP, and reduced CREB-1 phosphorylation. These deficits were rescued by PAESe treatment. The principal goal of our research is to develop a new novel therapeutic strategy for the treatment of chemobrain.

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List of Abbreviations

AKT	Protein Kinase B
BBB	The blood–brain barrier
CREB	cAMP response element-binding protein
DOX	Doxorubicin
ERK1/2	Extracellular signal Regulated Kinases 1/2
fEPSP	field excitatory postsynaptic potential
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSK3β	Glycogen synthase kinase 3 beta
ILK	Integrin-linked kinase
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
PAESe	Phenyl-2-Amino-Ethyl Selenide
PI3K	Phosphatidylinositol-3-Kinase
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
BDNF	Brain-derived neurotrophic factor

CHAPTER 1

Introduction

Chemotherapy induced cognitive impairment or "chemobrain" is characterized by cognitive impairments occurring during and/or after cancer therapy. This neuropsychological disorder is induced by chemotherapeutic agents and ultimately results in cognitive impairment in 70% of cancer patients. These cognitive deficits range from a mild inability to perform simple behavioral tasks to serious attention and memory problems 5-10 years after receiving chemotherapy (Joshi et al., 2007; MacLeod et al., 2007; Reid-Arndt, Yee, Perry, & Hsieh, 2009; Silverman et al., 2007,Dubois et al., 2014; Silberfarb, 1983).

The effective treatment of cancer can involve many different medical approaches including surgery, radiation, and/or chemotherapy (Sudhakar, 2009; Urruticoechea et al., 2010). Chemotherapeutic agents and treatment regimens have greatly reduced the risk of cancer recurrence and has significantly increased patient survival rate (Johnson, Bryant, Miles, Hogberg, & Cornes, 2011; Kaneko et al., 2002). However, close to 75% of cancer survivors who received chemotherapy have reported various degrees of cognitive impairments (Cheung, Lim, Ho, & Chan, 2013; Jim et al., 2012,Ahles, Root, & Ryan, 2012). Studies have shown that chemotherapy induced cognitive deficits can impact several different types of memory including verbal memory, working memory and attention (Acevedo, 2012; S. Kesler et al., 2013; S. R. Kesler & Blayney, 2016).

Due to the staggering rate of cancer in the United States, the use of chemotherapy is only predicted to rise. Thus, the burden of chemobrain is also expected to increase in cancer patients and survivors. Therefore, it is important to determine the molecular mechanisms through which

chemotherapy drugs alter cognition. Neuroimaging studies have revealed that a myriad of different brain regions are altered following chemotherapy regimens. For instance, the anthracycline doxorubicin (DOX) injected intravenously altered brain activity in both cortex and hippocampus as well as decreased glucose metabolism in rats (Lim, Joung, Yu, Shim, & Kim, 2016). Data from a human study showed that systemic chemotherapy administration reduced the glucose metabolism in the brain (Horky et al., 2014). In addition, Cyclophosphamide, Methotrexate, and 5-fluorouracil (CMF) treatments have been shown to alter acetylation and deacetylation events in the hippocampus of rats (Briones & Woods, 2011). Additionally, several studies have intensively investigated the impact of co-administration of other compounds or special diets on reducing the symptoms of chemobrain. (J. G. Hou et al., 2013; Kwatra et al., 2016; Lim et al., 2016; Wu et al., 2016).

DOX is a potent broad-spectrum anticancer drug in the anthracycline class and is a well-known and an effective treatment for a wide-range of cancers, including breast, prostate, lung, and leukemia (Cortes-Funes & Coronado, 2007). As with many other drugs, chronic administration of DOX can induce toxicity to other non-targeted tissues such as the heart, kidneys, and brain (Ayla et al., 2011; Christie et al., 2012; Manchon et al., 2016; Volkova & Russell, 2011). DOX induced cardiotoxicity has been well studied and is attributed to multiple different mechanisms such as calcium metabolism, mitochondrial dysfunction, increased production of reactive oxygen species (ROS) and cell apoptosis (Gilliam et al., 2013; Ichikawa et al., 2014). However, the mechanisms underlying DOX induced cognitive deficits have not been fully elucidated.

Protein kinases are important regulators for general body functions such as development, metabolism, synaptic plasticity, and memory (Giese & Mizuno, 2013; G. M. Thomas & Huganir, 2004). Protein kinases also function as second messengers for most receptors that mediate signal transduction (Hofer & Lefkimmiatis, 2007). For instance, protein kinase B (Akt), extracellular signal-regulated kinase 1/2 (ERK1/2), and cAMP response element binding (CREB-1) pathways are broadly expressed in the central nervous system and they are involved in development, normal brain function, and synaptic plasticity (Casadio et al., 1999; Z. Z. Chong, Li, & Maiese, 2005; Ishii, Furusho, Dupree, & Bansal, 2014). In neurons, Akt is induced by growth factors such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), insulin, cytokines and neurotransmitters (Beaulieu, 2012; Yoshii & Constantine-Paton, 2007; B. C. Zhang, Li, & Harbrecht, 2011). Akt is well-known for facilitating neuronal survival (Brunet, Datta, & Greenberg, 2001) as well as playing an essential role in regulating both survival and apoptosis (H. Diez, Garrido, & Wandosell, 2012). Moreover, Akt, ERK1/2, and CREB-1 signaling can mediate cell survival and metabolism that facilitate learning and memory processes (Alonso, Medina, & Pozzo-Miller, 2004; Horwood, Dufour, Laroche, & Davis, 2006; Khan, Zhuang, & He, 2016; Schulz, Siemer, Krug, & Hollt, 1999). Dysregulation of these proteins is also involved in various diseases including neurodegenerative diseases, epilepsy, schizophrenia and other neurological disorders (Y. H. Chong et al., 2006; H. K. Lee, Kumar, Fu, Rosen, & Querfurth, 2009; Nadam et al., 2007; Zheng et al., 2012). Currently, there are no studies examining the impact of DOX on these proteins. Therefore, we evaluated the activity of Akt and ERK1/2, and CREB-1 in the hippocampus of nude mice after peripheral exposure to saline, DOX, DOX+PAESe, or PAESe alone to assess their role in chemobrain. It is not known whether chemobrain is a direct or indirect effect of DOX treatment. Therefore, we performed *in vitro*

(cultured hippocampal neurons) and *ex vivo* (brain slices from ovariectomized rats) studies to investigate the direct effect of DOX on synaptic plasticity and neuronal death by measuring expression and activities of several proteins. In addition, we also performed MTT assay, lipid peroxidation, and mitochondrial complex I activity and function to test the cell viability and oxidative stress. Pharmacokinetic studies indicate that DOX can reach the brain only in small concentrations (Sardi et al., 2013). Thus, we used clinically relevant concentration, 250nM of DOX, which is the reported concentration to reach the rodent brain when administrated intravenously. Additionally, we used 500nM, and 1000nM of DOX for 6 hours to validate our results. The activity of protein kinases Akt, ERK1/2, P38, caspase-3, ILK, and GSK3β were examined as they are well established to play an essential role in regulating memory function.

In summary, DOX may be directly or indirectly contributing to chemotherapy induced cognitive impairment. Understanding the molecular mechanisms by which DOX causes these deficits will shed light onto new therapeutic interventions for the prevention of chemobrain. Our study was designed to establish the *in vitro* impact of DOX on several protein kinases involved in learning and memory as well as determine the effect of DOX on *ex vivo* synaptic plasticity and *in vivo* behavior. Additionally, we tested the impact of PAESe co-administration with DOX to evaluate its potential as a possible protective agent. The results of this study reveals an important mechanism by which DOX can cause memory deficits and how PAESe can ameliorate these deficits in a mouse model of chemobrain.

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CHAPTER 2

Literature review

The hippocampus and memory:

The hippocampus, a bilateral limbic structure in the medial temporal lobe located beneath the cerebral cortex (Wible, 2013). It is composed of sub-regions known as the Cornus Ammonis (CA) and Dentate Gyrus (DG). The CA is further classified as the CA1, CA2, CA3, and CA4. The DG is adjacent to the CA regions and is the main site of adult neurogenesis (Friedman & Goldmanrakic, 1988).

The hippocampus consists of three major pathways: the Schaffer Collaterals, the Mossy Fibers and the Perforant Pathway. The Schaffer Collateral pathway runs between the CA3 and CA1, while the Mossy Fibers run between CA3 and DG. The Perforant Pathway provides inputs to DG from entorhinal cortex (Kumar, 2011) (**Figure 1**). The major excitatory neurotransmitter in the hippocampus is glutamate and it carries out its effects primarily through as the glutamate family of receptors.

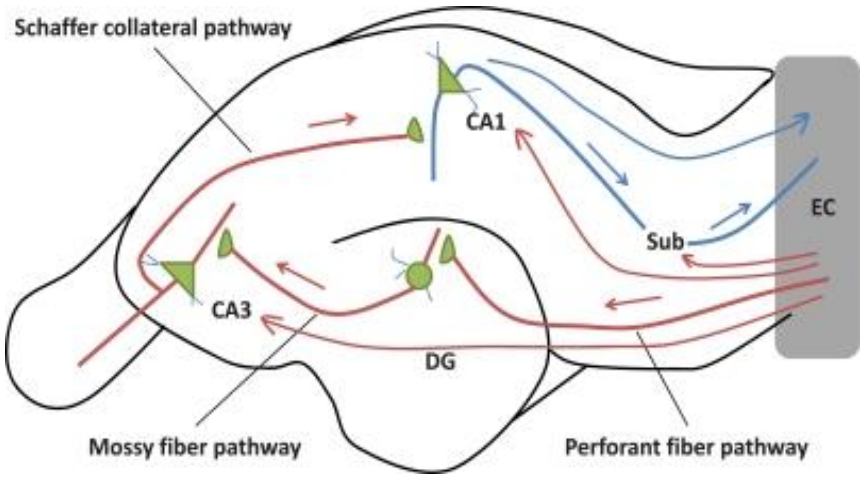

Figure 1: Figure shows the three major pathways in the hippocampus. *Adapted from Zhang, X. M., (2011). Kainic Acid-induced neurotoxicity: targeting glial responses and glia-derived cytokines. Current Neuropharmacology.*

The main function of the hippocampus is to act as a temporary storage site for new, short-term memories. However, it is also involved in the consolidation and storage of long-term memories. Although human and animal studies alike support the hippocampus's role as a structure essential for learning and memory, several of the detailed mechanisms are still under investigation. However, several studies have identified discrete regions of the hippocampus that are associated with specific types of behavioral tasks. For example, emotional and fear memory is linked to the ventral hippocampus due to its close proximity to the amygdala, while verbal and working memory are associated with the dorsal hippocampus. The rest of this chapter will delve deeper into the known mechanisms of hippocampus-based memories.

The Glutamatergic neurotransmission:

Glutamate is the major excitatory neurotransmitter in the central nervous system and it plays a role in both normal brain functions and in the pathogenesis of various neurological disorders. Glutamate receptors and transporters regulate the release of glutamate and maintain extracellular glutamate concentrations to establish the dynamic synaptic signaling processes required for proper cognition. The α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptors and N-methyl-D-aspartate (NMDA) receptors are the main ionotropic glutamate receptors that are well known to regulate learning and memory processes (Lamprecht & LeDoux, 2004). Once the presynaptic neurons are stimulated, Ca^{2+} rushes into the presynaptic terminal, which activates the vesicular release of glutamate into the synaptic cleft. Glutamate then binds to AMPA receptors and elicits an influx of Na^{+} causing depolarization of the postsynaptic neurons. At resting potential (-70 mV), NMDA receptors are blocked by Mg^{2+}, but depolarization of the neuron caused by influx of Na^{+} through AMPA receptors, removes this blockade allowing Ca^{2+} and more Na^{+} into the cell. Ca^{2+} is known to play an important role in downstream signaling by acting on calcium calmodulin-dependent kinases II/IV (CaMKII/CaMKIV) (Fink & Meyer, 2002). This leads to a complex signaling cascade downstream of AMPAR and NMDAR involving other kinases such as protein kinase A (PKA) and protein kinase B (PKB/Akt). Together, Ca^{2+} influx affects expression and trafficking of AMPARs and NMDARs, leading to altered synaptic plasticity mechanisms such as long-term potentiation (LTP) (Luscher & Malenka, 2012). Decreased levels of Ca^{2+} cause a decrease in expression of AMPARs and NMDARs, which suppresses synaptic activity. Therefore, cellular Ca^{2+} balance is necessary for the induction of hippocampal LTP and long-term depression (LTD), which are essential processes for memory formation.

Glutamate in the synaptic cleft can be taken up by excitatory amino acid transporter (EAAT) to be converted to glutamine by glutamine-synthetase. Presynaptic glutamate neurons then reuptake glutamine and convert it into glutamate using the enzyme glutaminase. Glutamate is then stored into vesicles by vesicular glutamate transporter (VGLUT) to await release by the next action potential. The efficient release of glutamate and reuptake of glutamine required for optimal memory function and can be assessed at the circuit level using electrophysiology (**Figure 2**) (Hall & Guyton, 2011; Sanacora, Zarate, Krystal, & Manji, 2008).

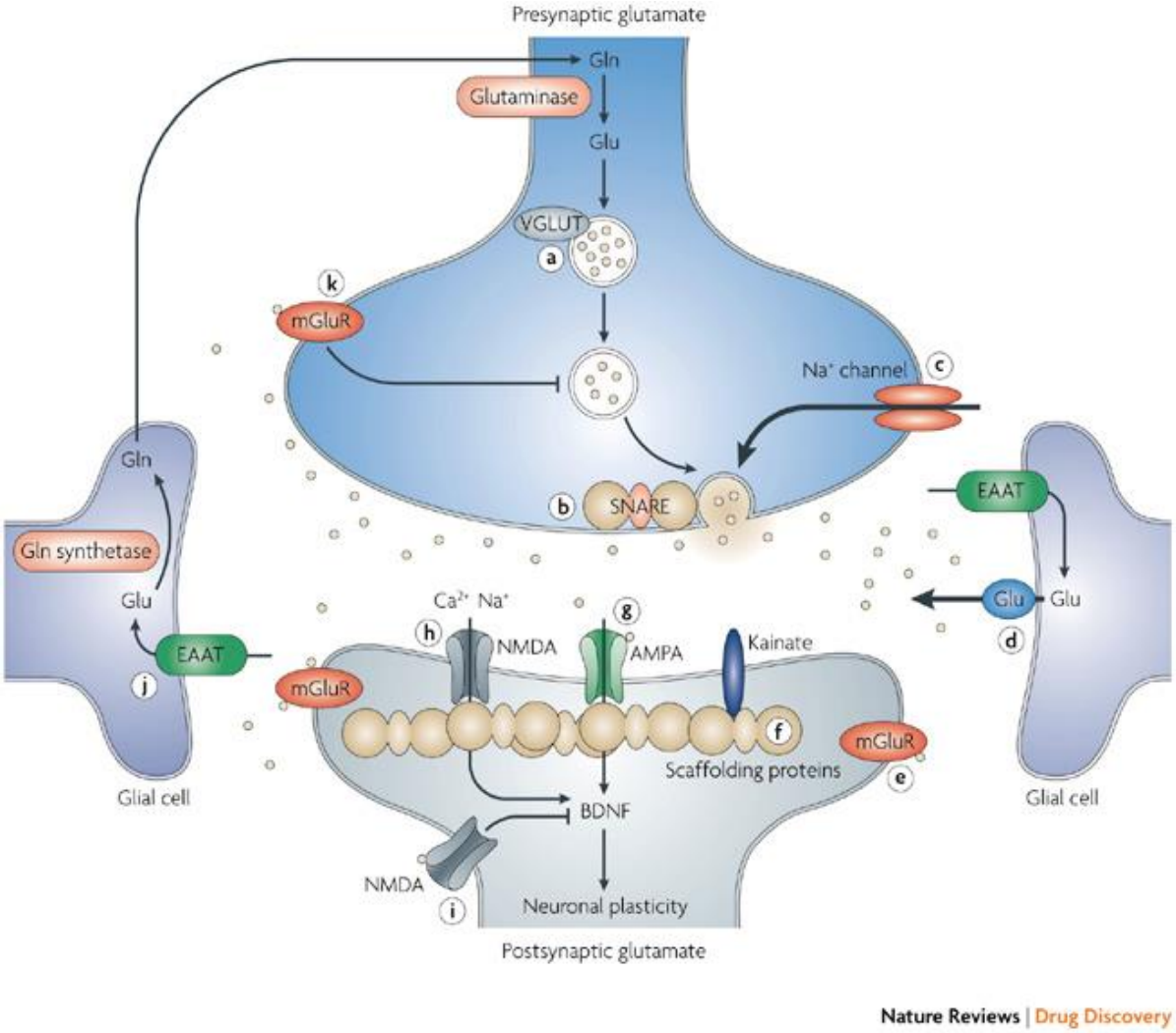

Figure 2: Figure shows the mechanism of glutamate release and regulation of glutamate.

Adapted from Sanacora, G. (2008). Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. Nature Reviews Drug Discovery

Animal models of memory function

The maze is the most commonly used approach to test hippocampal learning and memory. Using spatial cues, animals must learn and remember the location of a novel stimulus and/or a desirable object such as food. Various categories of mazes have been established and successfully used to assess learning and memory in animals. In the following text, I will discuss the most popular types of mazes.

The Y-maze:

The Y-maze test assesses the ability of an animal to recognize places already explored and its propensity to explore a new place by assessing working and spatial memory (Roullet & Crawley, 2011; B. B. Thomas et al., 2007). The Y-maze apparatus is constructed of three arms at a 120-degree angle to each other and placed under ambient lighting so that each arm is illuminated equally (**Figure**). To begin testing, the animal is trained in the maze for 15 minutes during which time the animals explore two arms freely with the third arm occluded by an opaque barrier. The arm in which they were originally placed is recorded as the Entry Arm and the occluded arm is recorded as the Novel Arm. The animals are then removed from the maze and returned to their home cage for a rest period of three hours. After rest, the animals are returned to the maze with the Novel Arm open and allowed to explore all three arms for 10 minutes. . All sessions are video recorded and the second session is scored using a software-tracking program assesses the number and order of arm entries as well as time spent in each arm.

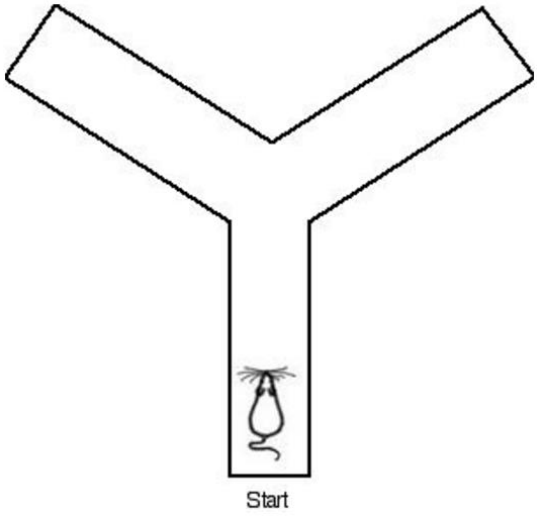

Figure 3: Structural figure for Y-maze. *Adapted from http://www.ratbehavior.org/RatsAndMazes.htm*

The radial arm maze (RAM):

The radial arm maze (RAM) was designed by Olton and Samuelson to test spatial learning and memory in the 1976. The RAM consists of eight arms radiating from a central location. To assess short-term and long-term memory, the ends of four out of eight arms are baited with food for each daily training trial. The arms that contain food remain constant throughout the trials. After sufficient food deprivation, each animal is placed in the center of the maze and allowed to freely explore the maze. Each training session continues until either all four food rewards are found or five minutes have elapsed. As training proceeds on consecutive days, long-term memory is assessed by the animals' ability to remember which arms are baited and by finding all four rewards faster. Additionally, short-term memory is established by animals remembering which arms have already been visited and collecting all of the rewards without

searching the same arm twice. Although food deprivation is necessary for the completion of the RAM, it has the unique ability to assess both short-term memory and long-term memory.

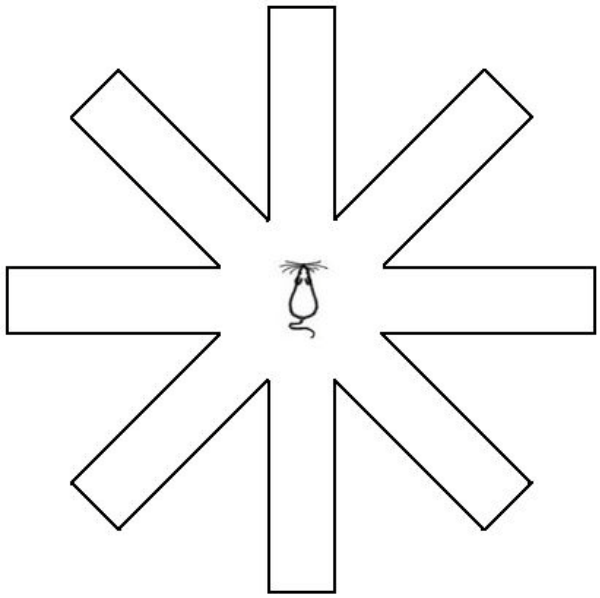

Figure 4: Structural figure of the radial arm maze. *Adapted from http://www.ratbehavior.org/RatsAndMazes.htm*

The Morris Water Maze (MWM):

The Morris Water Maze (MWM) was created by Richard Morris in 1981 to assess spatial memory and learning. The MWM consists of a large round tub filled with room temperature water that is rendered opaque by powdered milk. A platform is placed in the center of one of the four quadrants just below the surface of the water so that it is hidden from view. Each animal is released from one of four cardinal points around the maze and allowed to freely find the platform or is guided to it if five minutes have elapsed. All animals are trained to the platform location for four consecutive days. On the fifth day, a probe trial is conducted in which the animals are placed back in the pool, but the platform is now removed. Training trials are assessed for latency

and path length to reach the platform, while the probe trial is scored for time spent in the target quadrant. The MWM has a distinct advantage over the RAM in that it does not require food deprivation. However, swimming may be stressful for some animals, thus MWM should be used with caution in models for hypertension and anxiety.

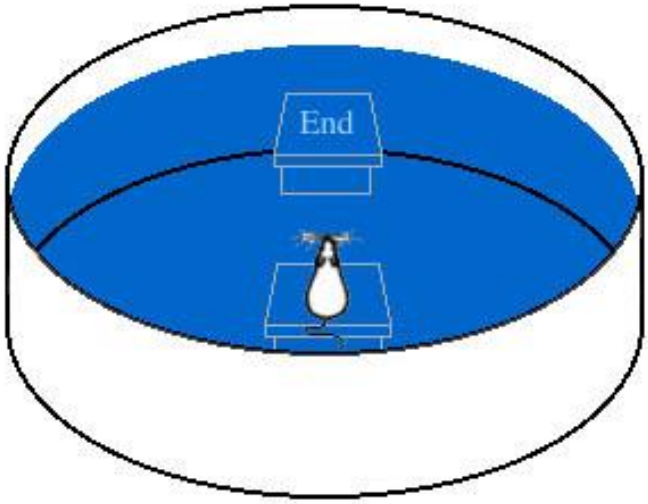

Figure 5: Figure shows the tub of water and the hidden platform, a target object of this test. *Adapted from http://www.ratbehavior.org/RatsAndMazes.htm*

The contextual fear-conditioning task:

Fear and strong emotions are initiated from the amygdala and linked to the hippocampus through ventral structures such as CA3 and DG (Maren, 2003). In this type of assessment, animals are exposed to a novel location (context) and a mild electrical stimulus is delivered to their feet. The animals are then moved back to their home cage for an inter-trial session. In the next trial, animal are returned to the same context and if the properly remember the electrical

shock associated with the context they will freeze in anticipation (S. Bhattacharya et al., 2016; Maren, Phan, & Liberzon, 2013). Contextual fear conditioning is a quick method of assessing ventral hippocampal function that does not require food deprivation. However, as with the MWM, fear conditioning induces stress and may not be suitable for all animal models.

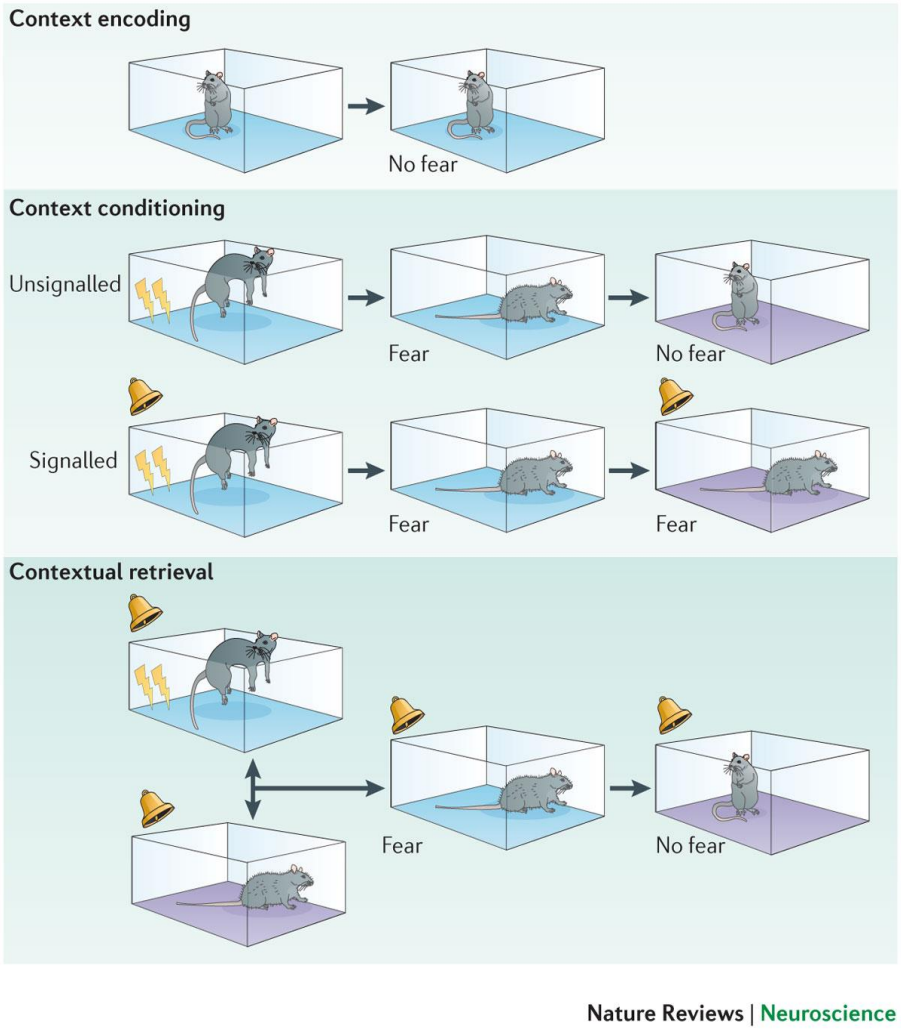

Figure 6: Diagram illustrates the context, conditioning and retrieval tasks in rodents in fear memory processes. *Adapted from Maren, S., Phan, K. L., & Liberzon, I. (2013). The contextual brain: implications for fear conditioning, extinction and psychopathology. Nature Reviews*

The novel object recognition (NOR) and novel location recognition (NLR) tasks:

The novel object recognition task measures object memory that is dependent on the ventral hippocampus due to its close connections to the thalamus. In the novel object recognition experiment, animals are introduced to and arena containing two identical objects and allowed to explore them for 5 minutes. Animals are returned to their home cage for three hours then placed back into the arena, with one of the initial objects replaced with a novel object of similar size.

Time spent exploring the familiar and novel object are recorded. The novel location recognition task is similar to the novel object recognition except instead of replacing one of the familiar objects to a novel object; one of the familiar objects is placed in a new location (Antunes & Biala, 2012; Broadbent, Gaskin, Squire, & Clark, 2010). Both of these tasks take advantage of the natural curiosity of rodents. Animals with intact memory are expected to explore the novel object or novel location more than the familiar one.

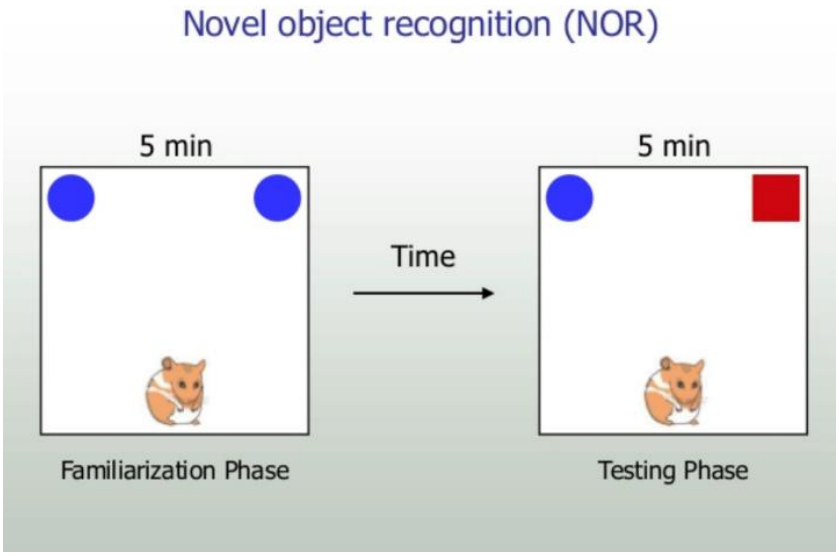

Figure 7: Figure illustrates the procedure of the two phases of NOR. *Adapted from https://www.slideshare.net/plus15campaign/dsrtf-webinar-dr-h-craig-heller-stanford-university*

The electrophysiological studies:

The brain is made up of billions of neurons functioning together in a complex circuitry system. Different regions are connected through these circuits to relay information and initiate integrated responses. Electrophysiological studies are critical for fully understanding the details of these connections and findings from these experiments yield important information on cognition at a cellular and molecular level. Consequently, an understanding of hippocampal electrophysiology response is essential to assess the functional integrity of neural pathways behind the behaviors assessed in the aforementioned behavioral tasks. A deeper understanding the functioning of the brain is attained through *in vitro* (cultured neurons), *ex vivo* (brain slices) and *in vivo* (anesthetized animals) electrophysiological experiments. *Ex vivo* studies, however, provide useful and interesting information because they not only allow for whole animal manipulation before sacrifice, but they also allow for changes to be made to the single slice *post mortem*. For instance, the impact of drugs on the hippocampal Shaffer collaterals may be investigated after administration to live animal while the direct effect of pharmacological agents on a slice, may be assessed by incubating the sliced brain with a certain compound.

Memory and synaptic plasticity:

The idea that synapses change their shape and function to accommodate memory formation and storage has been hypothesized since late of 1800s. This idea is now called synaptic plasticity and it is now known that synaptic transmissions are bidirectional for cell-to-cell communication (Bailey, Kandel, & Harris, 2015; Takeuchi, Duszkiewicz, & Morris, 2014). Bliss and Lømo were the first to describe the long term potentiation (LTP) in 1973. LTP is a widely studied phenomenon in learning and memory measurement and is generally considered as a cellular model of learning and memory encoding. The characteristics and maintenance of LTP

rely on changes in synaptic plasticity to support prolonged cell-cell communication. Increased glutamate release, for example, from presynaptic to the postsynaptic neuron is found to enhance field excitatory postsynaptic potentials (fEPSPs) in the hippocampus during spatial learning tasks and in LTP by strengthening glutamatergic synapses and prolonging action potentials (Richter-Levin, Canevari, & Bliss, 1995).

Cognitive deficits after chemotherapy and potential therapies:

Chemobrain was reported several decades ago, but only a few studies have examined the pathophysiology and mechanisms related to chemotherapy induced cognitive deficits. In the last decade, however, there have been a few studies exploring potential therapies for chemobrain. In 2013, Hou et al., evaluated the effect of the chemotherapeutic agent cyclophosphamide in an animal model and the demonstrated cognitive deficits and reduced neurogenesis in experimental groups. This study also assessed the effect of a compound called "K" when co-administered with cyclophosphamide, improved memory (J. G. Hou et al., 2013). Moreover, Lim et al. found that donepezil co-administered with DOX improved memory function in behavioral test and brain imaging methods indicated that donepezil could be a potential therapy for chemobrain (Lim et al., 2016). However, the weakness of these studies is that they did not demonstrate whether the anti-tumor efficacy of chemotherapy was altered after combining these compounds. Previously, we found that co administration of Phenyl-2-aminoethyl selenide (PAESe), a selenium-containing compound that can function as antioxidant drug, does not affect DOX anti-tumor efficacy (J. Y. Kang et al., 2015). Therefore, the current study intended to expand upon our previous research by evaluating the potential ameliorating effect of PAESe on an animal model of chemobrain.

Studies of human brain structure and function after chemotherapy:

Published human studies report significant cognitive dysfunction after chemotherapy, however most of the mechanistic studies use animal models as a representation of human chemobrain. Only few studies have investigated the alteration in human brain structure after chemotherapy and these studies described that white and grey matters are altered after multiple chemotherapeutic agents were given to the cancer patients (de Ruiter et al., 2012; Inagaki et al., 2007; Lepage et al., 2014). Moreover, study conducted by Horky et al., using sequential FDG-PET/CT imaging shows that glucose metabolism is reduced in both white and grey matters in patients who received chemotherapy (Horky et al., 2014). This study was confirmed by using rat chemobrain models (Lim et al., 2016).

Chemotherapy induced oxidative stress in brain:

Reactive oxygen species (ROS) are molecules that are naturally generated by the body as a byproduct of several biological processes and are vital for the signal transduction and cell function (Kishida & Klann, 2007; Knapp & Klann, 2002). Anti-neoplastic drugs such as anthracyclines, however, can cause excessive generation of ROS, which induce lipid peroxidation and produce electrophilic aldehydes that cause cell cycle arrest (Pizarro et al., 2009). This is crucial in reducing cancer growth, but excessive production of ROS can also affect the normal cell function especially in the brain (Thiels et al., 2000). Oxidative stress alters normal cell function by activating the neuroprotective pathways such as PI3K/AKT and MAPK-ERK1/2 (Cavanaugh, Jaumotte, Lakoski, & Zigmond, 2006; Gines, Paoletti, & Alberch, 2010; J. Hou et al., 2015). Several lines of evidence suggest a correlation between oxidative stress and memory dysfunction in several disease states such as Alzheimer's and Parkinson's disease (Dias, Junn, & Mouradian, 2013; Perry, Cash, & Smith, 2002; Pratico et al., 2002). However, the

relationship among chemotherapeutic agents, oxidative stress, and memory deficits is not well established.

Anthracyclines:

Anthracycline antibiotics, such as doxorubicin, daunorubicin, and epirubicin, are an important class of chemotherapeutic agents that are widely used anticancer drugs for a variety of cancers including leukemia, breast, prostate, ovary and lung cancers (Cortes-Funes & Coronado, 2007). Several clinical trials have reported that chemotherapy protocols, those including anthracyclines, prolong survival time of cancer patients compared to others. However, doxorubicin's efficacy is limited by a cumulative dose-dependent cardiotoxicity, which could cause irreversible heart failure.

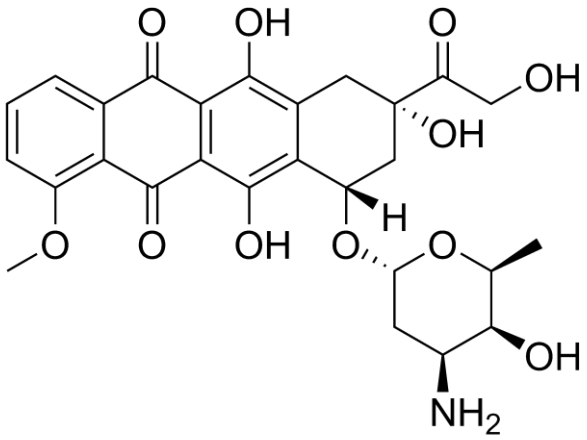

Figure 8: Chemical structure of doxorubicin (DOX), an anthracyclines antibiotic.

Anthracyclines are known to inhibit the activity of topoisomerase II that is required to cleave the DNA (Moro et al., 2004; Yang, Kemp, & Henikoff, 2015). Anthracyclines can also intercalate DNA and RNA, which inhibits the replication of cancer cells, but impairs normal function in healthy cells (M. A. Kang, So, Simons, Spitz, & Ouchi, 2012; S. Y. Kim et al., 2006).

Phenyl-2-aminoethyl selenide (PAESe):

Phenyl-2-aminoethyl selenide (PAESe) is a selenium-containing compound that can function as antioxidant drug. Selenium, the critical component of PAESe, is known to enhance the glutathione peroxidase activity and thus reduce oxidative stress (Takahashi & Cohen, 1986). Previous studies have demonstrated that PAESe has antitumor effect and it reduces the cytotoxicity when it is co-administered with DOX (J. Y. Kang et al., 2015). Several studies have also reported that PAESe reduces the cytotoxicity of DOX and acts as a cardioprotectant both *in vivo* and *in vitro* (J. Y. Kang et al., 2011; J. Y. Kang et al., 2015). In addition, it has been shown that the combination of PAESe with DOX decreases the intracellular reactive free radicals

formed by DOX treatment and the results were dose-dependent: as PAESe increased, ROS decreased.

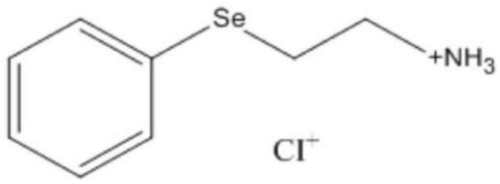

Figure 9: Chemical structure of phenyl-2 aminoethyl selenide (PAESe). *Adapted from Kang, J. Y., et al., (2015). Pharmacokinetics, antitumor and cardioprotective effects of liposome-encapsulated phenylaminoethyl selenide in human prostate cancer rodent models. Pharmaceutical Research*

Dissertation Rationale and Hypothesis:

The hypothesis of this dissertation is that DOX treatment impairs memory in rodents by modifying synaptic transmission and synaptic protein expression in the hippocampus. Additionally, these deficits can be ameliorated by co-administration of PAESe with DOX. The study performed in four groups, saline treated, DOX treated, DOX+PAESe treated, or PAESe treated nude mice. Glutamate receptors play an important role in memory formation; therefore, one aim of this study is to characterize the effects of DOX on glutamatergic function using both *in vitro* (hippocampal H19-7 cells) and *ex vivo* (hippocampal slices) (Chapter 3) methods. Chapter 4 will examine the effects of PAESe when it is co-administered with DOX using behavioral tasks, electrophysiological recordings, and molecular studies to evaluate the memory function. The results obtained provide a substantial support for the idea that a peripheral injection of DOX contributes in memory dysfunction and suggests that PAESe as a potential therapy for DOX induced chemobrain.

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CHAPTER 3

Doxorubicin induces Memory Dysfunction by Modulating ERK1/2, AKT, and P38 Phosphorylation

Abstract:

Doxorubicin (DOX) is an antitumor drug in the anthracycline class that is commonly used in cancer chemotherapy. DOX is effective in the treatment of various types of tumors; however, the optimal clinical effectiveness is limited due to secondary effects including cognitive impairment, which is also recognized as "chemobrain" or "chemofog". Chemobrain refers to a phenomenon in which cancer survivors exhibit significant cognitive impairment following chemotherapy. There are only few reports on the molecular mechanisms involved in anthracycline-induced memory dysfunction. Our own investigation showed deficits in glutamatergic neurotransmission as a result of DOX administration. Drugs modulating ERK1/2 and P38 MAPK signaling pathway can considerably alter memory encoding. Thus the current study investigated the *in vitro* effects of DOX on H19-7 hippocampal cell survival and its effect on ERK1/2 and P38 MAPK signaling pathway associated with memory dysfunction. In addition, *ex vivo* effects of DOX on synaptic plasticity were evaluated using acutely isolated hippocampal slices. DOX caused increased ERK1/2, P38 and AKT phosphorylation in H19-7 cells compared to the controls in a dose dependent manner. Therefore, we conclude that DOX induce hippocampal based memory dysfunction by modulating MAPK pathway.

Introduction:

Many studies have reported an association between chemotherapy and cognitive deficits, which is known as chemotherapy-induced cognitive impairment or "chemobrain" (Janelsins,

Kesler, Ahles, & Morrow, 2014; Janelsins et al., 2011). However, the mechanism of chemobrain is still not clearly understood. Doxorubicin, a chemotherapeutic agent, is used to treat various types of tumors, and is often used as part of a multi-drug chemotherapy regimen (Fisher et al., 1989). Research from both our lab and others has revealed that DOX can cause memory impairment through modulation of glutamatergic and serotonergic systems (R. Y. Liu, Zhang, Coughlin, Cleary, & Byrne, 2014). The present study was conducted to examine the cellular and molecular mechanisms underlying chemobrain caused by DOX.

The proposed mechanisms of DOX as anti-tumor drug are through the inhibition of topoisomerase-II, intercalation into the strands of DNA, and excessive production of reactive oxygen species (ROS) resulting to initiation of apoptosis. Some proposed mechanisms DOX induced chemobrain are chromatin modifications, increase-circulating cytokines, inhibition of protein synthesis, and decreased antioxidant production (Christie et al., 2012; Vichaya et al., 2015). These mechanisms may be further responsible for reduction of long-term potentiation and synaptic plasticity in hippocampal neurons. This project was designed to study the effects of DOX treatment and resulting memory deficits.

The brain and especially the hippocampus require a significant amount of energy for proper function. The anthracycline class of compounds including DOX, is known to cause mitochondrial dysfunction, by either directly inhibiting the respiratory chain through binding cardiolipin in the inner mitochondrial membrane or by negatively interacting with mitochondrial DNA (mtDNA) (Gouspillou et al., 2015). These processes cause defects to the respiratory chain and enhance the formation of ROS. Therefore, in this study, we measured the effect of DOX on lipid peroxidation and mitochondrial complex I activity in the brain slices after 6 hours of incubation with two different concentrations of DOX.

Thus, the main goal of this study was to elucidate the impact of DOX on synaptic plasticity and memory function in both *ex vivo* (hippocampal slices) and *in vitro* (hippocampal H19-7 cells) Specifically, how DOX administration impacts the ERK1/2 and P38 MAPK signaling pathways and other proteins that associated with memory function.

Materials and Methods:

Cell culture:

Embryonic rat hippocampal neuronal cells (H19-7/IGF-IR) were purchased from ATCC (number: CRL-2526, USA). The cells were cultured in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) at 34°C, the optimal temperature for cell growth. When the cells are over 70% confluent DOX was added to the cells for 6 hours before extraction.

Pharmacological treatment:

The use of DOX in H19-7 has not been reported. The concentration that is utilized for the cell culture is based on the DOX pharmacokinetic studies. It is reported that DOX can assess the brain by very small amount. This amount is equal to concentration of 250 nM in rat brain (Sardi et al., 2013). In addition, several cancer studies have used multiple concentrations from 1μM to 10 μM of DOX for treating cancer cells. The concentration of 250 nM of DOX has been used to correspond to actual clinical studies. The 1μM concentration of DOX is used to be a positive control to compare the effect between the 250 nM and 1μM.

MTT reduction assay:

The cell viability was determined by the quantitative colorimetric with MTT assay (3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma). H19-7 cell line was

plated on 24-well culture plates at a density of 2000 cells per well. Cells were cultured in media for 24 hours and then the media were replaced with either control media or media containing DOX for 6 and 24 hours. The medium was removed and mixed with a solution of 1 mg/ml MTT for 4 hours in a 5% CO2 humidified atmosphere at 34℃. The supernatant was removed, and the formazan crystals in the cells were solubilized with DMSO. Absorbance was read at 570 nm on a microplate reader.

Western blot analysis:

DOX treated cells and control cells were lysed with lysis buffer from Thermo Scientific N-PER Neuronal Protein Extraction Reagent. Total protein was estimated by bicinchoninic acid assay (BCA assay) and then stored at −80 °C until use. The sample was mixed thoroughly with 4x Laemmli buffer and was loaded into 10% SDS-page gel. The proteins were transferred to PVDF membranes (Immobilon-p Millipore, Germany), and blocked 5% non-fat dry milk for 2 hours in Tris-Buffered Saline (TBS) containing 0.01% Tween 20. Membranes were washed with TBST and incubated with anti-caspase-3, anti-Akt, anti-phospho-Akt, anti-GSK3β, anti-phospho-GSK3β, anti-p44/42, anti-phospho-p44/42, anti-P38, anti-phospho-P38, anti-ILK, or anti-GAPDH overnight at 4°C. All primary antibodies were purchased from Cell Signaling Technology, Danvers, Massachusetts, and used as a 1:1000 dilution. Then, membranes were probed with secondary anti-rabbit antibody (1:5000) that is conjugated with fluorophore DyLight 550 at room temperature for 1 hour. Then, the membranes were scanned to be visualized by utilizing FluorChem Q System imager with the 606 nm green lasers and the green filter set (Proteinsimple, San Jose, California, USA). These scans were performed using excitation at λ_{535} nm and emission at λ_{606} nm. Finally, the densities of these bands were compared over GAPDH for both DOX treated cells and control.

Lipid peroxidation assay:

Lipid peroxidation was determined by methods previously described (Garcia, Rodriguez-Malaver, & Penaloza, 2005) with some modifications. Brain slices from ovariectomized rats were incubated in oxygenated submerged chambers containing artificial cerebral spinal fluid (ACSF). One chamber contained only ACSF, but the other two contained different concentrations of DOX (250nM and 1000nM). These slices were incubated for 6 hours followed by weighing the tissue and homogenization with PBS and lysis. The samples were sonicated using (Qsonica) for 2-3 minutes. Then samples were centrifuged at 12000 g for 20 minutes at 4 °C. The supernatant was collected and added in new centrifuge tubes. The proteins were quantified before running the lipid peroxidation assay. In brief, 50 µL of the brain tissue homogenate was mixed with 100 µL TCA (10g/50 ml H2O), and 400 µL of TBA (50mg/10ml H2O) and added 500 µL of H2O. the mixture was heated for 15 minutes at 80°C. After cooling, spin the mixture for 5 minutes and the absorbance of the supernatant was measured at 532 nm.

Mitochondrial complex I activity:

The brain slices from ovariectomized rats were incubated in ACSF and ACSF containing DOX for 6 hours. These brain slices were homogenized with Phosphate-buffered saline (PBS) and centrifuged at 14000g and 2-4 °C for 15 min. The supernatant was collected into new, clean 1.5 ml Eppendorf tubes and frozen at -80°C until use. The proteins from each sample were quantified before starting assessment of mitochondrial complex I activity. The samples were diluted with PBS. Then, NADH was added to the samples and total activity was recorded for three minutes. The readings in time 0, 60, 120, and 180 seconds were taken for the assessments. The result of both two concentrations of DOX were normalized and percentage to the controls,

which is 100% (Thrash, Karuppagounder, Uthayathas, Suppiramaniam, & Dhanasekaran, 2010).

Preparations of acute hippocampal slices:

Ovariectomized rats (4 months old) were euthanized with CO_2 and decapitated to remove the brain. The brain was then washed with ice-cold oxygenated cutting solution. Leica VT1200 S Automated Vibratome (Leica Biosystems Inc., Buffalo Grove, IL, United States) was used with an oxygenated cutting solution (NaCl 85, KCl 2.5, MgSO4 4.0, CaCl2 0.5, NaH2PO4 1.25, NaHCO3 25, glucose 25, sucrose 75, kynurenic acid 2.0, ascorbate 0.5) to slice the brain (350 micrometers). The slices were preserved in a holding chamber, submerged in oxygenated aCSF. Some slices were incubated in aCSF with 250 nM or 1µM of DOX for 4-6 hours at 30◦C, before starting the LTP recording.

Extracellular field recordings:

Slices were transferred into a submerge-type recording chamber, under the microscope (Nikon SMZ 745T microscope), and held between two nylon nets. The submersion chamber is continuously perfused with oxygenated ACSF (33°C) with a flow rate of 2-3 ml/minute. A platinum bipolar electrode was placed on the CA3 region of the hippocampus and a glass microelectrode, 1.5 mm outer diameter (World Precision Instruments, Sarasota, Florida), was pulled using a micropipette puller (Narishigie scientific instruments Lab, Tokyo) and filled with ACSF solution approximately 200µl. It was placed on the stratum radiatum in the CA1 region of hippocampus to record field excitatory postsynaptic potentials (fEPSPs) from the Schaffer collateral pathway. Model 4D Digital Stimulus Isolation Amplifier (SIU) was used to stimulate the CA3 region (0.1Hz) for at least 10 minutes once a stable baseline was obtained. The stimulus intensity response was recorded in response to increasing stimulus intensity between 0 µA to 100 µA in steps of 20 µA and measuring the fEPSPs slope and the fiber volley amplitude. For fEPSP

recording, the stimulus intensity was increased until a population spike was observed and stimulus intensity is reduced by 50%. The LTP was induced using theta burst stimulation (TBS). Five TBS was applied with an inter-TBS interval of 20s. Each TBS consists of 10 bursts delivered at 5Hz, each burst containing 4, 0.2 ms, pulses at 100Hz. Field potentials were recorded using LTP software with Axoclamp 2B (Axon Instruments, Foster City, CA) and analyzed using WinLtp software (Anderson & Collingridge, 2001).

Statistics:

All biochemical data from *in vitro* study was analyzed utilizing one-way ANOVA test. All experiments were repeated for n=5 and p value of less than 0.05 were considered significant.

Results:

Effect of DOX on hippocampal neuronal survival:

To determine the sensitivity of hippocampal neurons to the cytotoxic effects of DOX, the cells were seeded into 24 well plates and incubated with various concentrations of DOX as mentioned in the materials and methods. As shown in (Figure-11), increasing concentrations DOX (250nM, 500nM, and 1μM) did not show significant changes after 6 hours of treatment. However, 24 hours of DOX treatment at concentrations of 500nM and 1 μM decreased cell viability by 11±2% and 22±3%, respectively. In contrast, 24 hours of DOX at a low concentration (250nM) had no significant effect (cell survival 98%).

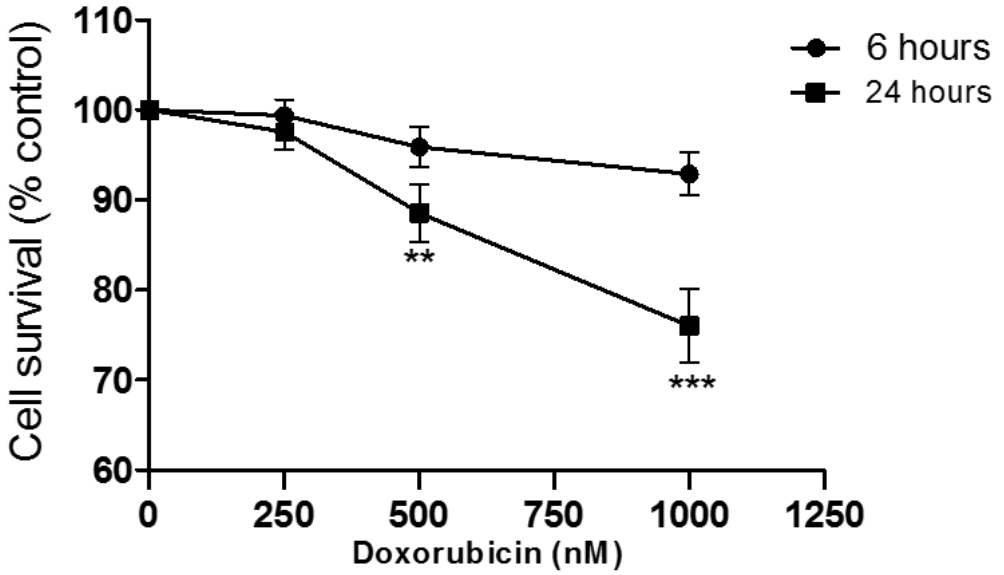
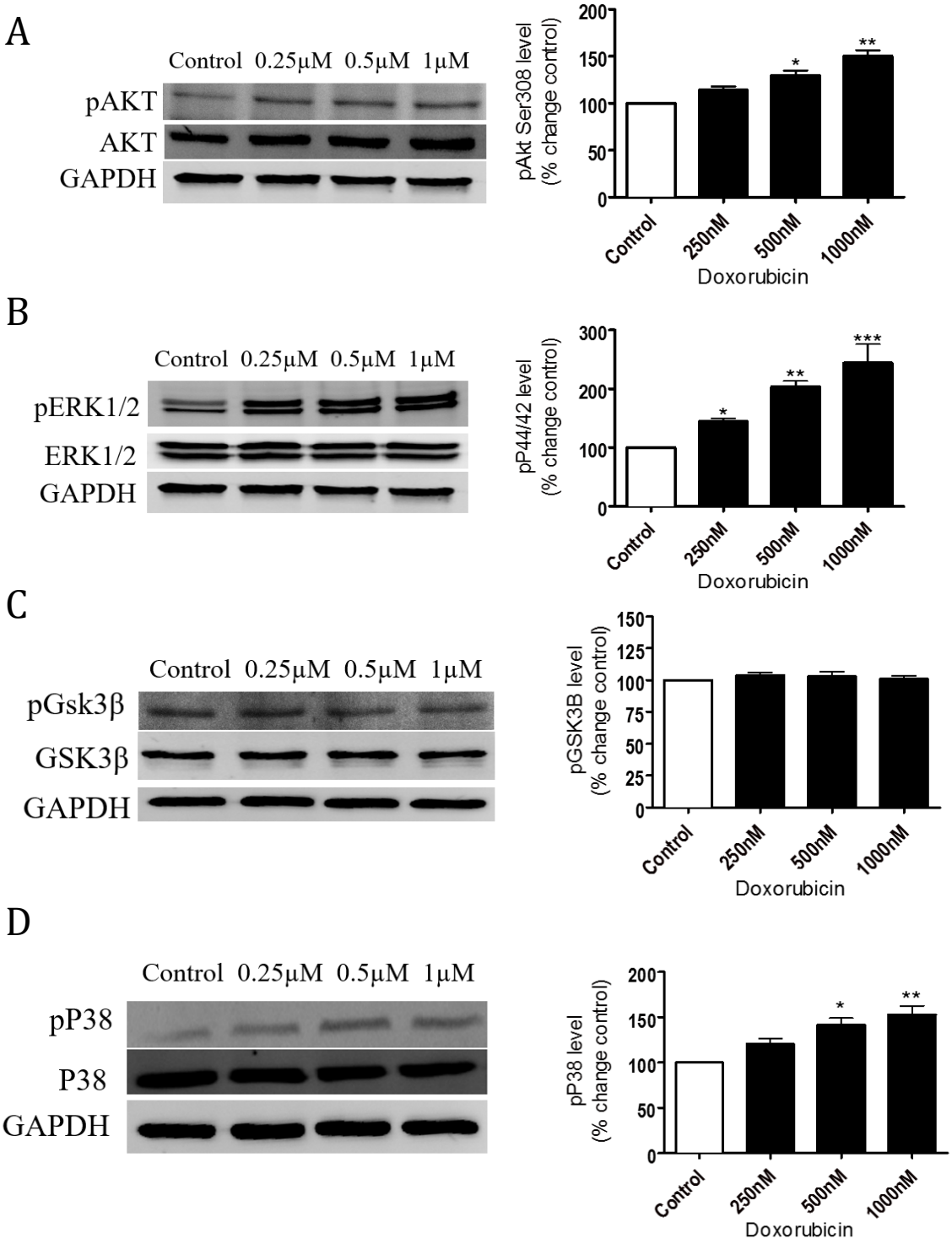


Figure 10: Effect of DOX on cell survival of hippocampal neurons determined by Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. H19-7 cells were plated on 24-well at different concentrations of DOX 250 nM, 500 nM, and 1000 nM and allowed to grow for 6 hours and 24 hours. The cultures at each concentration were subjected to MTT assays to quantify cell survival. At 6 hours, it is observed that there are no significant changes between control and all the three concentrations of DOX. However, at 24-hours, it is noted that MTT values for 250 nM of DOX slightly lower than controls, but is not significantly different. However, in 500 nM and 1000 nM of DOX concentrations, there are significant changes (** $p<0.01$ relative to the control, and *** $p<0.001$ relative to the control; n=5).

DOX alters protein expression and activity in hippocampal neurons:

In this study, hippocampal neuronal cultures were used to study the effects of DOX in the hippocampal protein expression and activity. We began by investigating the protein expression and phosphorylation in the hippocampal neurons following different concentrations of DOX (Figure 12). Western blot analyses revealed that DOX in increasing concentrations (250nM, 500nM, and 1μM) increases the phosphorylation levels of AKT, ERK1/2, and P38 proteins (Figure-12-A-B-D), but the total protein levels of AKT, ERK1/2, and P38 are not significantly altered (Figure-11-A-B-D). Furthermore, we quantified the levels of GSK-3β phosphorylated at the serine-9 residue (p-GSK-3β) to the total expression of GSK-3β, and the results revealed there is no significant change in p-GSK3β compared to total GSK3β (Figure-11-C). Integrin linked kinase (ILK) is an important regulator of synaptic plasticity, therefore, we analyzed the expression of ILK and the results show no difference after treatment with DOX (Figure-11-E). The caspase-3 is well known to initiate the apoptosis processes. Western blot shows that increasing DOX concentrations increased caspase-3 expression, but only significant with 1μM of DOX after 6 hours of treatment (Figure-11-F).


A
Control 0.25μM 0.5μM 1μM
pAKT
AKT
GAPDH
pAkt Ser308 level (% change control)
200
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GAPDH
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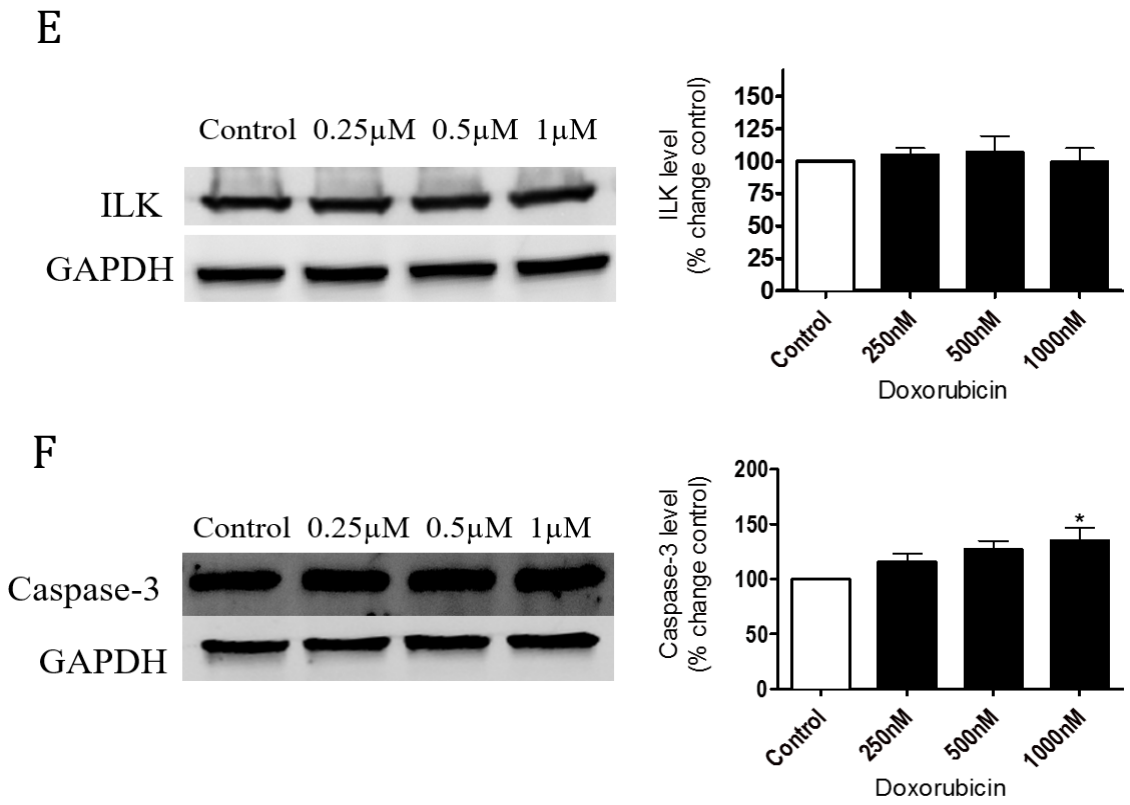

Figure 11: Effect of DOX treatment on hippocampal neuronal protein expressions and phosphorylation (Akt, GSK3B, ILK, ERK1/2, and P38) after 6 hours of treatment. Western blot analysis revealed that protein expression levels were not altered following DOX treatment (A, B, C, D, and E). However, the levels of protein phosphorylation of AKT, ERK1/2, and P38 were significantly increased in respond to DOX treatment (A, B, and D), but not for GSK3β. (E) Illustrates gradual increase in caspase-3 expression and it is statistically significant with 1μM DOX after 6 hours. All protein levels were normalized to GAPDH and stated as a percentage of the control group, which was set as 100%.

DOX reduces LTP in acute hippocampal slices:

In order to assess the direct effect of DOX on LTP, we used acute hippocampal slices obtained from experimentally naïve rats as an animal model corresponding with cell culture. The animals were euthanized and 350 □m hippocampal slices were obtained. The slices were treated with two different concentrations of DOX (250nM and 1μM) for 4 hours before recording. Synaptic plasticity was examined in the control and DOX treated slices by inducing long-term potentiation with theta burst stimulation (TBS). The result showed that LTP was significantly impaired with both 250nM and 1μm of DOX, LTP was 60% in control slices, 40% in 250nM slices, and 20% in 1μM slices ($n=4$; $p<0.001$) (Figure-3-A-B). In addition, Input-output relationship was constructed to evaluate the strength of synaptic transmission between the control slices and DOX treated slices. Doxorubicin treated slices revealed a reduction in fEPSP slope across a range of stimulus intensities (Figure-3-C). However, the presynaptic fiber volley amplitude showed no significant differences between the control slices and DOX treated slices (Figure-3-D), indicating that the alteration is likely in the postsynaptic response and not in the presynaptic axonal depolarization.

Figure-3

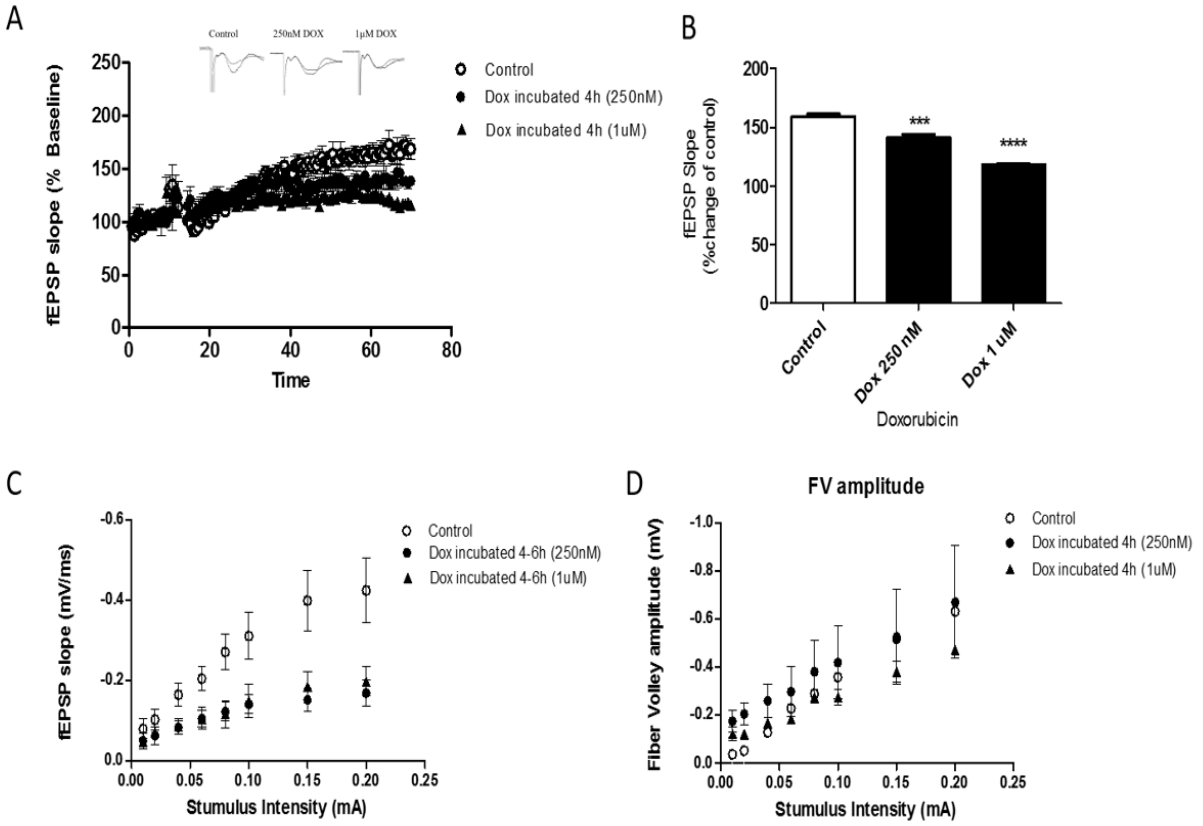

Figure 12: Recording of Long-term potentiation from Schaffer collateral in CA1 area of hippocampus. The data shows the field excitatory postsynaptic potentials (fEPSP) of hippocampi of DOX-incubated slices (250nM and 1000nM) and control slices. (A) Long-term potentiation was induced by theta burst stimulation (TBS) and measured at 1 hour after TBS. (Black circles), representative synaptic responses indicate that LTP was higher and persistent after TBS. (Red circles 250nM, and Blue circles 1000nM) illustrative of synaptic responses after 4 hours of DOX incubation indicating that LTP dose dependently decreased compared to the control slices; control 160%, 250nM of DOX is about 40%, and 1000nM of DOX is about 20%. ($p < 0.05$; n=4). (B) Illustrates the LTP average for the last 10 minutes of recordings for each the three

groups. (C) Illustrates the average slope of DOX-incubated slices (Red circles 250nM, and Blue circles 1000nM). Responses were significantly reduced at all stimulus level compared with control slices. (D) Shows the average of fiber volley amplitude for each stimulus intensity for the two different concentrations of DOX-incubated slices; no significant alteration were noted at all stimulus levels compared to control.

DOX treatment alters lipid peroxidation:

In order to investigate lipid peroxidation with DOX treatment, DOX was incubated with brain slices for six hours and the tissue was homogenized with PBS. The sample was examined for the oxidative biomarkers, thiobarbituric acid reactive substances (TBARS) (Figure-13).

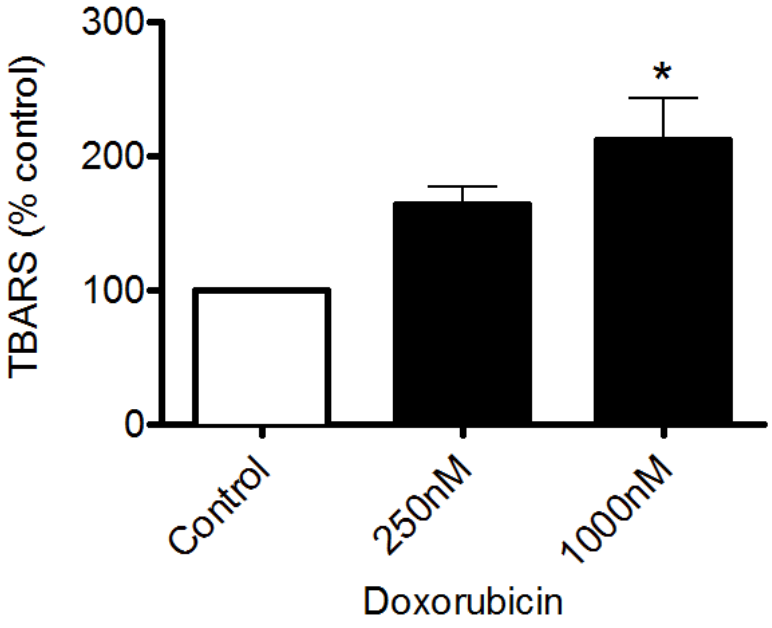

Figure 13: Effects of DOX treatment on Lipid peroxidation (TBARS) levels on brain slices. There is no significant change following 250 nM DOX treatment, however there is a significant difference following 1µM of DOX treatment. ($p<0.05$; $n=4$).

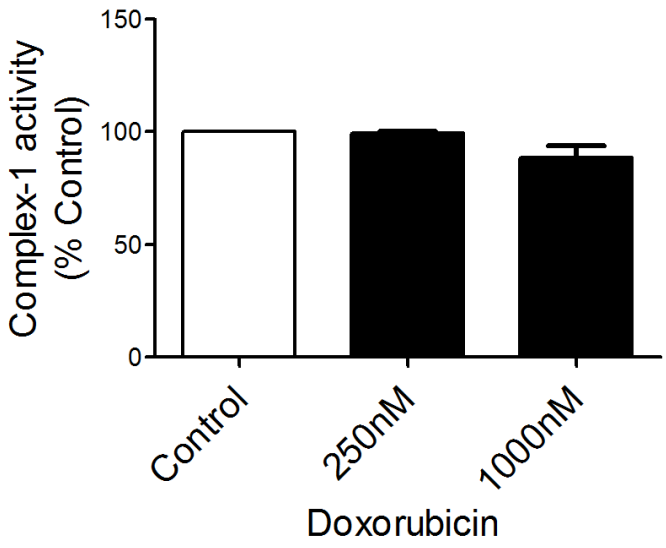

Figure 14: Mitochondrial respiratory chain complex I activity for control and DOX treated (250nM, and 1µM for 4 hours) (n=3 for each group) acute brain slices. There is no significant difference in mitochondrial complex I activity noted but there is a trend of reduced activity in 1µM.

Discussion:

In this study, we investigated the effects of DOX on both *ex vivo* (brain slices from animals) and *in vitro* (cultured hippocampal neurons) models of chemobrain and assessed the alterations in electrophysiological parameters, protein expression and function. The goal of this study was to investigate the cellular and molecular mechanism of chemotherapy induced cognitive dysfunction.

Effects of DOX Treatment on Caspase-3 Expression

Caspase-3 belongs to cysteinyl aspartate specific proteases family. Caspase-3 is known to play a crucial role in regulation cell apoptosis. It also recognized to have non-apoptotic functions. One of the non-apoptotic function is that caspase-3 is it's role in synaptic plasticity mechanisms required for memory formation (D'Amelio et al., 2011). In this study, we have investigated the expression of caspase-3 in hippocampal cells (H19-7 cell line) treated with different concentrations of DOX. The result shows that hippocampal neurons exposed to 250nM, 500nM, and 1μM concentrations of DOX for 6 hours did not have significant alteration in total caspase-3 expression compared to control, although there was a trend of increased expression as the concentration was increased. Caspase-3 is known to regulate synaptic plasticity by modulating AMPA receptor internalization (Han et al., 2013). The observation from our results that caspase-3 had slightly increased expression in neuronal cells due to DOX treatment might have contributed to the deficits in synaptic plasticity leading to memory impairment.

DOX treatment up-regulates Akt activity of H19-7 cells

The phosphoinositide 3-kinase (PI3K)/protein kinase B (PkB/ also known as Akt) pathway is widely expressed in the central nervous system and plays an important role in

development and function of neurons (Del Puerto, Wandosell, & Garrido, 2013; Diez, Garrido, & Wandosell, 2012; Huang & Reichardt, 2001). In the CNS, PI3K is induced by growth factors including brain-derived neurotrophic factor, nerve growth factor, insulin-like growth factor-1, and insulin in addition to extracellular matrix proteins, neurotransmitters, and cytokines (Brunet, Datta, & Greenberg, 2001; Shu et al., 2013). This pathway appears to be important for facilitating neuronal survival in wide range of conditions (Brunet et al., 2001). Activation of Akt by PI3K leads to phosphorylation of numerous downstream signaling proteins, which can control cell survival and apoptosis (Diez et al., 2012). Moreover, Akt activation can mediate cell survival by deactivating the pro-apoptotic mediators and activation of anti-apoptotic proteins (P. Liu, Cheng, Roberts, & Zhao, 2009). Akt is a key regulator for proliferation, survival, metabolism, neuronal differentiation, and apoptosis as well as learning and memory processes (Chiang, Wang, Xie, Yau, & Zhong, 2010; Horwood, Dufour, Laroche, & Davis, 2006). In addition, Akt dysfunction is involved in several diseases including epilepsy and other neurological disorders (Griffin et al., 2005; Lee, Kumar, Fu, Rosen, & Querfurth, 2009; Nadam et al., 2007).

Currently, there are a few reports implicating the role of Akt in the development of cognitive deficits caused by DOX. In the present study, we assessed the dynamic changes of Akt protein expression and phosphorylation in hippocampal neurons after 6 hours of DOX treatment with different concentrations. Akt is important for regulation of synaptic plasticity including LTP and LTD via phosphorylation of various protein kinases such as GSK3β, mTOR, and CREB. Our results indicate that DOX treatment increased Akt phosphorylation, however this may not be due to direct effect of DOX but due to over production of ROS following DOX treatment. This notion is supported by the fact that ROS mediates the activation of Akt and P38 signaling

required for inducing apoptosis (Ahn et al., 2013).

Effects of DOX treatment on GSK-3 beta and ILK regulation

Glycogen synthase kinase-3 (GSK-3β) is an enzyme primarily known to regulate glycogen synthesis in response to insulin (McManus et al., 2005). The alteration of this protein expression or phosphorylation is known to influence memory encoding. GSK-3β is an important downstream protein of the PI3K/Akt pathway, and its activity can be inhibited by Akt-Ser-473 phosphorylation at Ser-9 of GSK-3β. GSK-3β phosphorylation is implicated as a critical regulator for learning and memory (Fuchs et al., 2015; Giese, 2009; Venna, Benashski, Chauhan, & McCullough, 2015). Thus, alteration in GSK-3β phosphorylation at Ser-9 could result in memory impairment. However, our results showed that Akt phosphorylation was upregulated but GSK-3β phosphorylation was not altered at Ser-9 of GSK-3β.

Integrin linked kinase (ILK), an adhesion molecule important for connecting cells, plays a vital role in synaptic regulation (Bhattacharya et al., 2015). ILK regulates the dendrite formation through GSK-3β phosphorylation and activity (Naska et al., 2006). This study investigated the ILK expression pattern following DOX treatment. Our results revealed that expression of ILK was not altered indicating that DOX induces cognitive deficits by alternative mechanisms.

Activation of P38 MAPK by DOX in hippocampal neurons

P38 MAPK is widely expressed in the CNS and is involved in a range of signaling pathways that regulate several biological functions (Lemke et al., 2001). P38 phosphorylation in the brain is found to play an important role in several disease conditions such as Alzheimer disease, hypertension, inflammation, cognitive dysfunction, and synaptic plasticity (Correa & Eales, 2012; Dai et al., 2016; Munoz et al., 2007). In addition, p38 MAPK inhibition has been

shown to ameliorate cognitive deficits caused by hypertension and improved synaptic plasticity by increasing LTP (Dai et al., 2016). In our model of chemobrain, we have found that p38 phosphorylation is increased in hippocampal neurons treated with DOX. This elevated level of p38 phosphorylation could be one of the mechanisms contributing to cognitive impairment following chemotherapy.

Activation of ERK1/2 (P44/42) by DOX in hippocampal neurons

Extracellular signal-regulated kinase-1/2 (ERK1/2) belongs to a subfamily of mitogen-activated protein kinases (MAPKs). Several lines of evidence indicate that ERK1/2 activation is required for learning and memory process. Additionally, inhibition of ERK1/2 results in memory deficits and reduced LTP (Thomas & Huganir, 2004) apoptosis (Lu & Xu, 2006). Moreover, it is also reported that elevated levels of ERK1/2 impairs cognitive function in animal models of autism (Seese, Maske, Lynch, & Gall, 2014). In this study, we found that 6 hours of DOX exposure increased ERK1/2 phosphorylation dose dependently compared to controls. Therefore, it is reasonable to presume that hyperphosphorylation of ERK1/2 following DOX treatment, at least, in part, contributed to hippocampal neuronal death. Future studies are planned to explore the altered synaptic plasticity mechanisms contributing to cognitive impairment resulting from phosphorylation of ERK1/2 following DOX treatment.

Chemotherapy and Mitochondrial function

Mitochondria are essential organelles present in all cells of the body and play an important role in maintaining the functions of the nervous system. Mitochondria contain their own genome (mtDNA), which encodes proteins of the respiratory chain that combines electrons with oxygen to generate energy. This chain, known as the electron transport chain, is composed of four complexes that function to generate ATP. Alteration in mitochondrial function can affect

the tissues that require high-energy demands including the central nervous system and myocardium. Furthermore, there are a several lines of evidence that illustrate the involvement of mitochondrial dysfunction and cognitive deficits (Scaglia, 2010). Several studies have revealed that DOX treatment can induce cell death and mitochondrial dysfunction (Green & Leeuwenburgh, 2002; Sardao, Oliveira, Holy, Oliveira, & Wallace, 2009). Our results suggest that there are no significant changes in the hippocampal slices incubated with 250nM of DOX. However, the functionality of mitochondria was significantly decreased in the slices incubated with 1µM of DOX. We conclude that acute doses of DOX could lead to alteration in mitochondrial function and possibly contribute to cognitive impairment in cancer survivors.

In conclusion, this study has identified a number of key proteins that may be altered following DOX treatment. This investigation also demonstrated that DOX could modulate synaptic function by altering protein phosphorylation, mitochondrial function, and by increasing lipid peroxidation. These findings indicate that DOX treatment could have acute and direct effect on neuronal function.

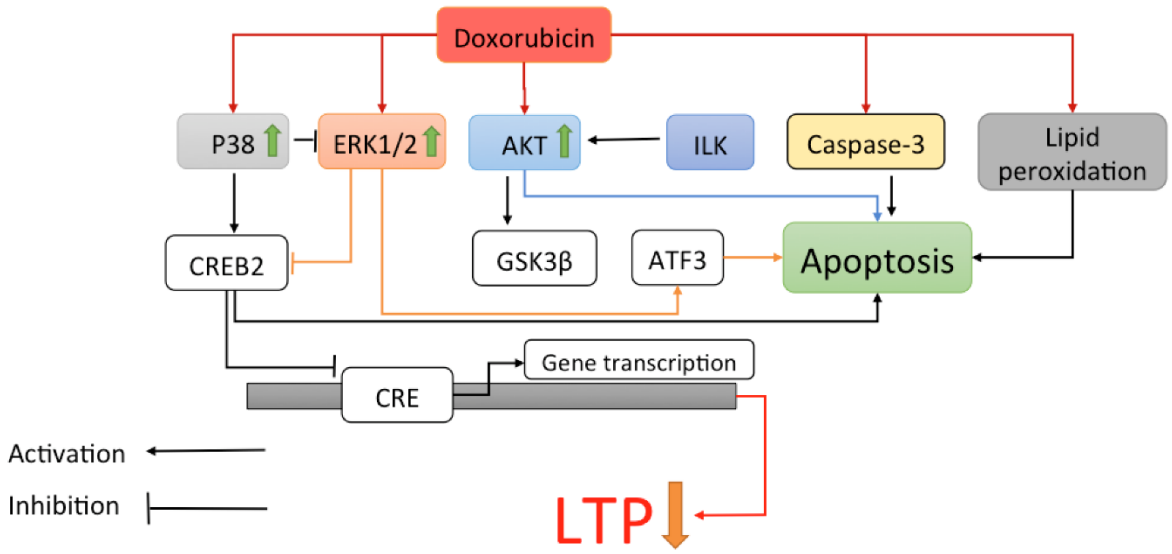

Figure 15: Schematic diagram showing mechanisms by which DOX regulate proteins expression and phosphorylation. DOX activates ERK1/2, AKT, P38 and caspase-3. Akt and ERK1/2 function as neuroprotective proteins; however, activation of P38 and caspase-3 enhance apoptosis through CREB-2 activation and thus inhibiting gene transcription as well as LTP. Also, DOX activates ATF-3 through ERK1/2, which promote apoptosis.

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CHAPTER 4

Phenyl-2-Aminoethyl Selenide Ameliorates Hippocampal Long Term Potentiation and Cognitive Deficits Following Doxorubicin Treatment

Abstract:

Doxorubicin (DOX) is a widely used chemotherapeutic agent whose optimal clinical effectiveness is limited due to the secondary effects such as memory impairment. Chemotherapy induced memory loss is referred as “chemobrain” and is observed in about 75% of patients exposed to chemotherapy and persists in 17-34% of cancer survivors. The mechanisms through which DOX induces cognitive dysfunction are not clear. Our previous work indicates that animals treated with DOX have reduced cellular memory and altered synaptic protein expression. Unfortunately, there are no commercially available therapies to prevent or treat chemobrain. Therefore, the aim of this study is to establish a mouse model of chemobrain using DOX, elucidate the mechanisms of cognitive deficits and to determine the therapeutic potential of the antioxidant drug, Phenyl-2-Aminoethyl Selenide (PAESe). We used male, NOD SCID mice that received five, weekly tail vein injections of either saline, DOX (cumulative dose 25 mg/kg), PAESe (cumulative dose 50 mg/kg), or DOX+PAESe (cumulative dose 25 mg/kg and 50 mg/kg). Spatial memory was evaluated by Y-maze and novel object location tasks, while synaptic plasticity was assessed by measurement of field excitatory post-synaptic potentials from the Schaffer collateral circuit. Additionally, Western blot was performed to assess hippocampal protein expression and phosphorylation. The results revealed that DOX impaired synaptic plasticity and memory by reducing CREB phosphorylation, which is an important regulator of protein synthesis and brain function. Co-administration of PAESe increased CREB

phosphorylation and ameliorated synaptic and memory deficits associated with DOX treatment

Introduction:

Chemotherapy, while an effective treatment for most cancers, has many negative side effects. Cancer patients primarily report fatigue, pain, loss of appetite and nausea following chemotherapy treatment. However, cognitive deficits have also been reported in 75% of patients receiving chemotherapy. These cognitive impacts have become so ubiquitous in chemotherapy treatment that they have earned the name "chemobrain." Several published studies including animals and humans have reported deficits in aspects of short-term cognition such as spatial memory, fear memory, and remembering events after chemotherapy. However, the definitive effects of chemotherapy on learning and memory are controversial. While multiple studies report that there is cognitive impairment associated with memory after chemotherapy treatment, one study found that the memory function is impaired both during and shortly after chemotherapy (Lee et al., 2006). Additionally, another study indicated there is no cognitive effect following chemotherapy (Fremouw, Fessler, Ferguson, & Burguete, 2012). Thus, it is imperative to investigate the effects of chemotherapy on cognition to fill the gap in the current literature. A previous study conducted in our laboratory reported that there is impairment in synaptic plasticity and altered in synaptic protein expression following chemotherapy.

DOX is an anthracyclines that is widely used to treat different type of cancers including breast, prostate, lung, and osteosarcoma (Thorn et al., 2011). While DOX has been proven to be effective against cancer growth, its uses are limited by a number of well-known side effects that include cardiotoxicity, nephrotoxicity, and hepatotoxicity (Briones & Woods, 2011; Damodar, Smitha, Gopinath, Vijayakumar, & Rao, 2014; Ichikawa et al., 2014; Lahoti, Patel, Thekkemadom, Beckett, & Ray, 2012; Liedke et al., 2009). Recently DOX has been shown to

also cause “chemobrain”, however, the exact mechanism is still elusive. A few studies have attempted to investigate the molecular mechanisms associated with chemotherapy induced memory function (Antkiewicz-Michaluk, Krzemieniecki, Romanska, Michaluk, & Krygowska-Wajs, 2016; Briones & Woods, 2011; Salas-Ramirez et al., 2015; Wu et al., 2016). Previous findings from our laboratory indicated that **multiple treatments of DOX** altered basal synaptic transmission and plasticity in the hippocampus.

Phenyl-2-aminoethyl selenide (PAESe) is an antioxidant drug that contains selenium as a functional group (Overcast et al., 2001). The molecular mechanism by which PAESe exerts antioxidant activity is not clear. However, PAESe contains selenium, and selenium is as an important constituent that binds to number of enzymes including glutathione peroxidase and enhances antioxidant activity (Kang et al., 2011). PAESe was initially developed to treat hypertension and cardiovascular disease (Overcast et al., 2001) and recently found to have anti-tumor effect besides reducing oxidative stress and cardioprotective effects (Kang et al., 2011; Kang et al., 2015). In the present study, we investigated the therapeutic potential of PAESe in ameliorating the cognitive deficits caused by DOX treatment.

To the best of our knowledge, currently there are no preventive measures or therapies for chemotherapy induced memory impairments. This study will fill an important gap in the existing literature by elucidating the mechanisms of cognitive deficits caused by DOX and the potential ameliorating effects of Phenylamino-Ethyl Selenide (PAESe) when co-administered with DOX.

Materials and Methods:

Drug Treatment:

Six-week-old athymic NCr nude (nu/nu) mice (Taconic Biosciences, Tarrytown, NY) were housed in pathogen-free condition in 12 h light and dark cycle with access to food and water, *ad libitum*, in accordance to protocols approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). All euthanasia procedures were in accordance with guidelines by the American Veterinary Medicine Association (Cima, 2013). In all experiments, mice were treated *via* intravenous (i.v.) tail-vein injection with 5 mg/kg DOX, 10 mg/kg PAESe, 5mg/kg DOX +10mg/kg PAESe together weekly for 4 weeks (cumulative DOX dose 25 mg/kg), (cumulative PAESe dose 50 mg/kg), or (cumulative DOX+PAESe doses 25 mg/kg and 50 mg/kg). An equivalent volume of vehicle (sterile saline, 0.9% w/v) was administrated to all control animals.

Assessment of spatial memory:

The Y-maze was used as a task for assessing working memory and spatial memory functions in DOX-treated, PAESe-DOX treated, PAESe treated, and vehicle treated mice as described previously (Bannerman et al., 2014). The apparatus for Y-maze test was made of 3 plastic arms at 120 degrees to each other, which was placed under equally distributed light. All testing sessions were recorded by video camera for later analysis. Animals were 12 weeks of age at the initiation of training sessions. The training sessions were 15 minutes long, during which time the animals could explore only 2 arms: the arm where they were placed (Entry arm) and one (Known arm) of the 2 other arms placed at the left and right of the Entry arm. The third arm (Novel arm) was occluded by an opaque divider. Animals were then returned to their home cages for 3 hours. After 3 hours had elapsed, the animals were placed back in the maze, but this time

all arms were open. They were allowed to explore the maze for 10 minutes then returned to the home cage. The second session was scored for number and order of arm entries. An arm entry was defined as more than half of the mouse's body within any of 3 arms. Dwell time spent in each arm was also recorded for all animals. Number of entries and time spent to the novel arm were scored and analyzed.

Novel object location (NOL) test:

The novel object location task is a working memory task that mainly relies on both cortical and hippocampal functioning. To perform this test, a transparent box open (45 cm × 45 cm × 45 cm) and video recording system were used. Each side of this box has a different cue adhered to the wall. During the first day, mice were given one habituation session (10 min) in the box without any objects. The next day, two identical objects were placed adjacent to each other. Mice were placed in the middle of the box and allowed to explore the two objects for 10 minutes. The mice were returned to their home cage for 1 hour and then placed back into the box, but one of the objects was moved 90 degrees from the original position. Mice were allowed to explore the objects for 10 minutes. The time spent exploring the novel location versus the familiar position was calculated and analyzed for all groups.

Preparations of acute hippocampal slices:

Animals were euthanized by CO_2 inhalation then decapitated for brain collection. Leica VT1200 S Automated Vibratome (Leica Biosystems Inc., Buffalo Grove, IL, United States) was filled with an oxygenated cutting solution (NaCl 85, KCl 2.5, MgSO4 4.0, CaCl2 0.5, NaH2PO4 1.25, NaHCO3 25, glucose 25, sucrose 75, kynurenic acid 2.0, ascorbate 0.5) and 350 µm hippocampal slices were obtained. The slices were allowed to rest in a holding chamber,

submerged in oxygenated-ACSF (artificial cerebral spinal fluid) for 2 hours at 30◦C, before starting the LTP recording.

Extracellular field recordings:

Slices were transferred into a submerge-type recording chamber and held between two nylon nets. The chamber was continuously perfused with oxygenated ACSF (33°C) with a flow rate of 2 ml/minute. A platinum bipolar electrode was placed on the CA3 region of the hippocampus and a glass microelectrode (1.5 mm outer diameter, World Precision Instruments, Sarasota, Florida) filled with ACSF was placed on the stratum radiatum in the CA1 region of hippocampus to record field excitatory postsynaptic potentials (fEPSPs) from the Schaffer collateral circuit. The CA3 was stimulated with Model 4D Digital Stimulus Isolation Amplifier (SIU) instrument at 0.33Hz for 10 minutes to monitor basal synaptic transmission. Long-term potentiation (LTP) was induced using 5 theta burst stimulations (TBS) with an inter-TBS interval of 20 s. Each TBS consists of 10 bursts delivered at 5Hz, each burst containing 4, 0.2 ms pulses at 100Hz. Field potentials were recorded using LTP software with Axoclamp 2B (Axon Instruments, Foster City, CA) and analyzed using WinLtp software (Anderson & Collingridge, 2001).

Immunoprecipitation (IP) assay:

An additional group of 12 week old treated mice were euthanized and the hippocampi were dissected and immediately immersed into ice. Hippocampi were homogenized in lysis buffer (Thermo Scientific N-PER Neuronal Protein Extraction Reagent) with protease inhibitors. The homogenates were centrifuged for 15 minutes at 12,000g, at 4°C to remove cellular debris, and supernatants were collected. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Pure-Proteome A/G magnetic beads (Millipore) were washed using

1X IMP buffer and then PSD-95 antibody (cell signaling) was added to coat the beads. The protein samples were added to prepare earlier to the coated beads and left to rotate overnight. PSD-95 was immunoprecipitated from the hippocampal tissue lysate utilizing 1:10 anti-PSD95 using vendor-supplied direct IP protocol. The immunoprecipitated fraction was purified through 3 washing steps with 1X IMP buffer. Then, Laemmli Buffer was added and the reaction heated at 90°C for 5 minutes. The samples were run on a SDS PAGE gel and analyzed after Western blotting.

Western Blot analysis:

The PSD-95 immunoprecipitated samples were probed with PSD-95 rabbit primary antibody (1:1000, Cell Signaling) to detect for presence of PSD-95 is successfully pulled down. Equal amounts of samples were loaded to analyze the interaction of PSD-95 with GluR1 using rabbit primary antibodies (1:1000, Cell Signaling). Also, hippocampi from the four treatment groups were dissected and homogenized with Thermo Scientific N-PER Neuronal Protein Extraction Reagent including proteases inhibitors. The homogenates were centrifuged for 15 minutes at 12,000g, at 4°C and the supernatant was collected. The protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). The sample was mixed with 4x Laemmli buffer in ratio 1:3 and was loaded into 10% SDS-page gel. The proteins were transferred to PVDF membranes (Immobilon-p Millipore, Germany), and blocked by 5% non-fat dry milk for 2 hours in Tris-Buffered Saline (TBS) containing 0.01% Tween 20 (TBST). Membranes were washed with TBST and incubated with anti-phospho-AKT, anti-AKT, anti-ERK1/2, anti-phospho-ERK1/2, anti-ILK, anti-phospho-CREB, anti-CREB, or anti-GAPDH overnight at 4°C. All primary antibodies were purchased from Cell Signaling Technology, Danvers, Massachusetts, and used as a 1:1000 dilution. Then, membranes were probed with

secondary anti-rabbit antibody (1:5000) that is conjugated with fluorophore DyLight 550 at room temperature for 1 hour. Then, the membranes were scanned and visualized by FluorChem Q System imager with the 606 nm green lasers and the green filter set (Proteinsimple, San Jose, California, USA). Finally, the densities of these bands were compared over GAPDH for all different groups of treatment versus control.

Statistics:

All biochemical data from *in vitro* study was analyzed utilizing one-way ANOVA test. All experiments were repeated for n=5 and p value of less than 0.05 were considered significant.

Results:

*Behavioral performance in Y-maze**:***

In this study, there were no differences in the total spent time of the novel arm among the all four groups (Control, DOX, DOX + PAESe, and PAESe) **Figure 20-A**. The four groups did not exhibit preference to choose the novel arm at the beginning of the test session of Y-maze. DOX alone and PAESe alone present a significant reduction in total number of entries into the novel arm ($p<0.005$ and $p<0.05$ respectively). However, there was no significant changes observed between control and DOX + PAESe **Figure 20-B**. Furthermore, the results revealed that in DOX+PAESe group novel arm entries were significantly higher compared to DOX alone indicating the ameliorating effects of PAESe when co-administered with DOX.

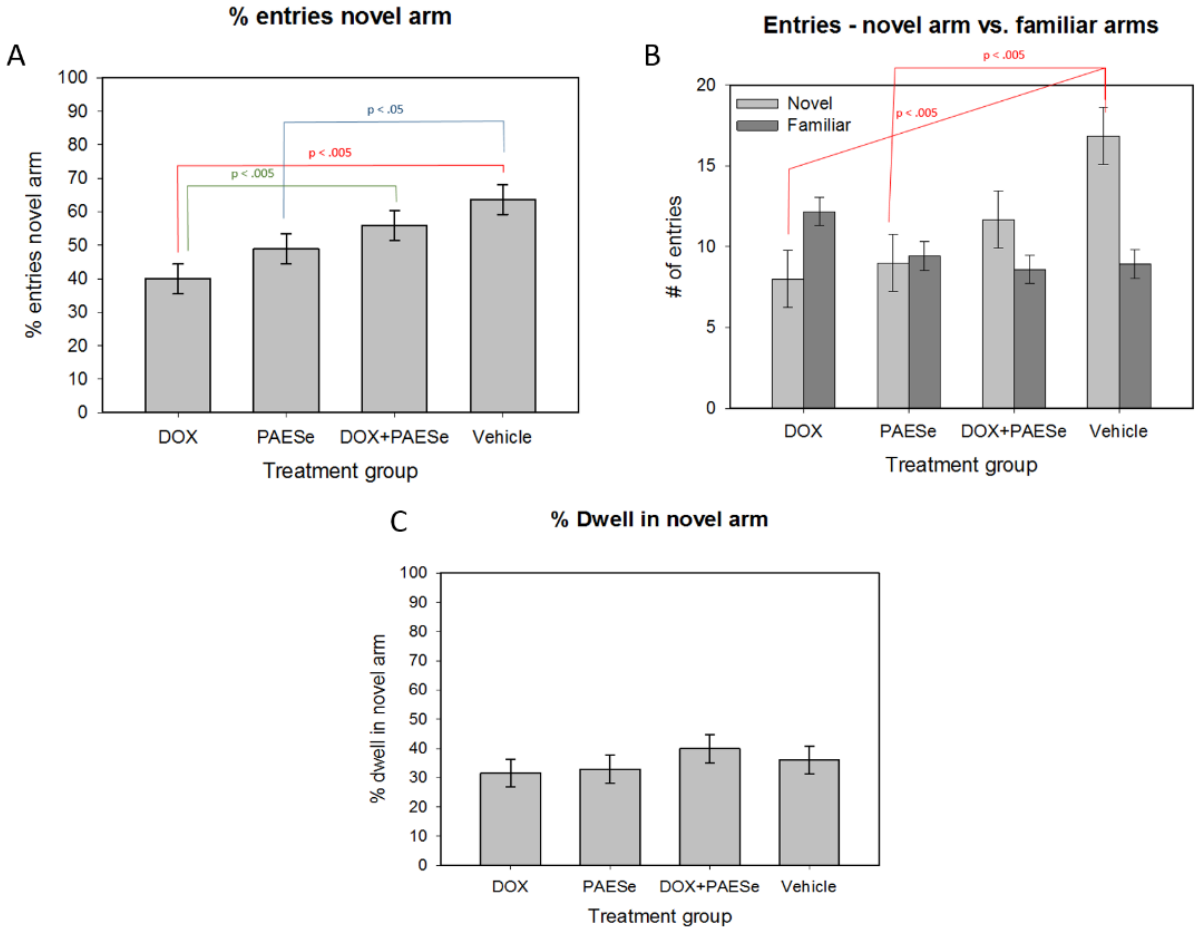


Figure 16: Illustration of Y-maze test performance; DOX-treated mice showed cognitive deficits compared to non-treated control mice. PAESe treatment rescued cognitive deficits n = 8-7/group. *Statistical significant difference ($p < 0.05$) between DOX and DOX + PAESe, DOX and DOX + PAESe, and DOX and non-treated controls.

Effects of Dox treatment on novel object-recognition test:

The result of NOR test indicated that there are no significant changes induced by DOX in object recognition memory. The results illustrate that neither DOX, PAESe, nor combination of these drugs altered the memory function in treated mice.

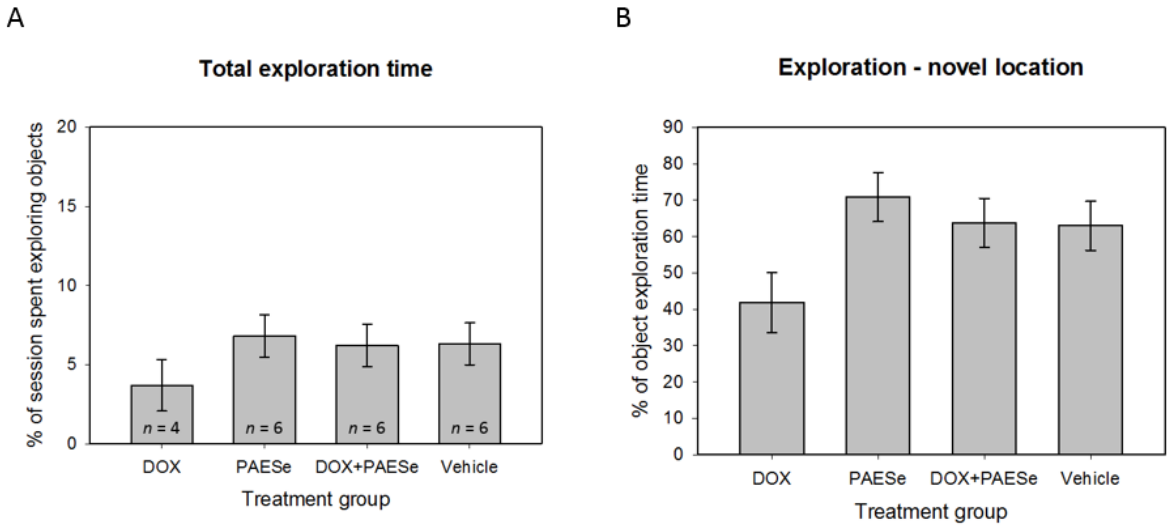


Figure 17: Illustration of NOR test results. The results indicate that there are no significant differences in all treated groups.

PAESe ameliorates impaired synaptic plasticity in the hippocampus caused by DOX:

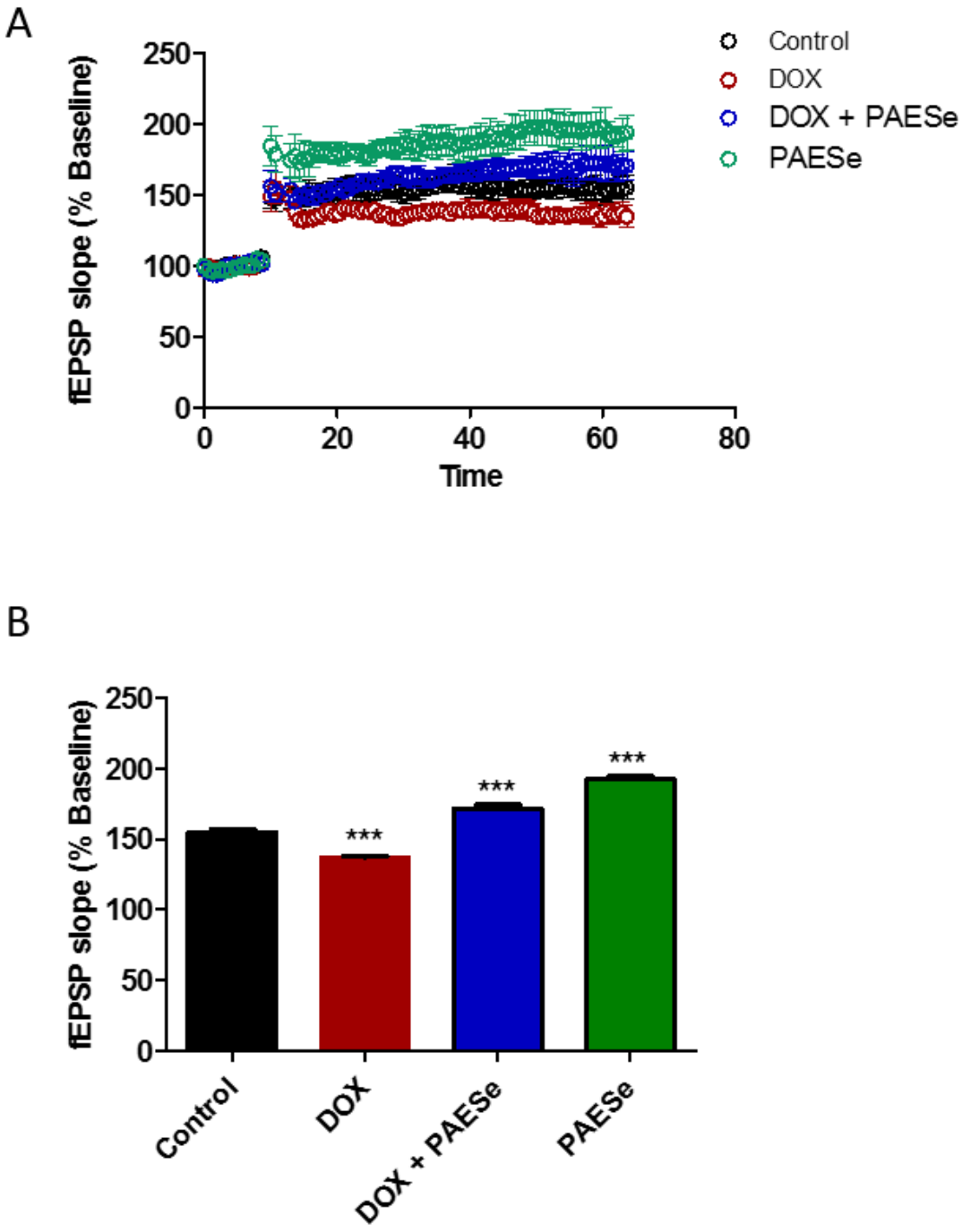


Figure 18: Effect of DOX treatment on long term potentiation (LTP). LTP was induced by high frequency stimulation (5 trains of 100 pulses with 20 seconds within train interval) in the hippocampal slices using Schaffer collateral pathway in CA1 region from control animals, DOX-

treated animals, DOX + PAESe animals, and only PAESe animals. Representative traces illustrate that the baseline 10 minutes before HFS and 55 minutes post-HFS for control, DOX-treated, DOX + PAESe, and only PAESe animals. Normalized fEPSP slopes 55 min post-HFS averaged 154.42% for control and 136.60% for DOX animals. Administration of PAESe along with DOX, rescued LTP. Normalized fEPSP slopes 55 min post-HFS averaged 154.42% for vehicle treated control, 136.60% for DOX treated animals, 171.35 % for the doxorubicin + PAESe treated animals and 192.04% for the only PAESe treated group.

PAESe ameliorates impaired basal synaptic transmission in the hippocampus caused by DOX:

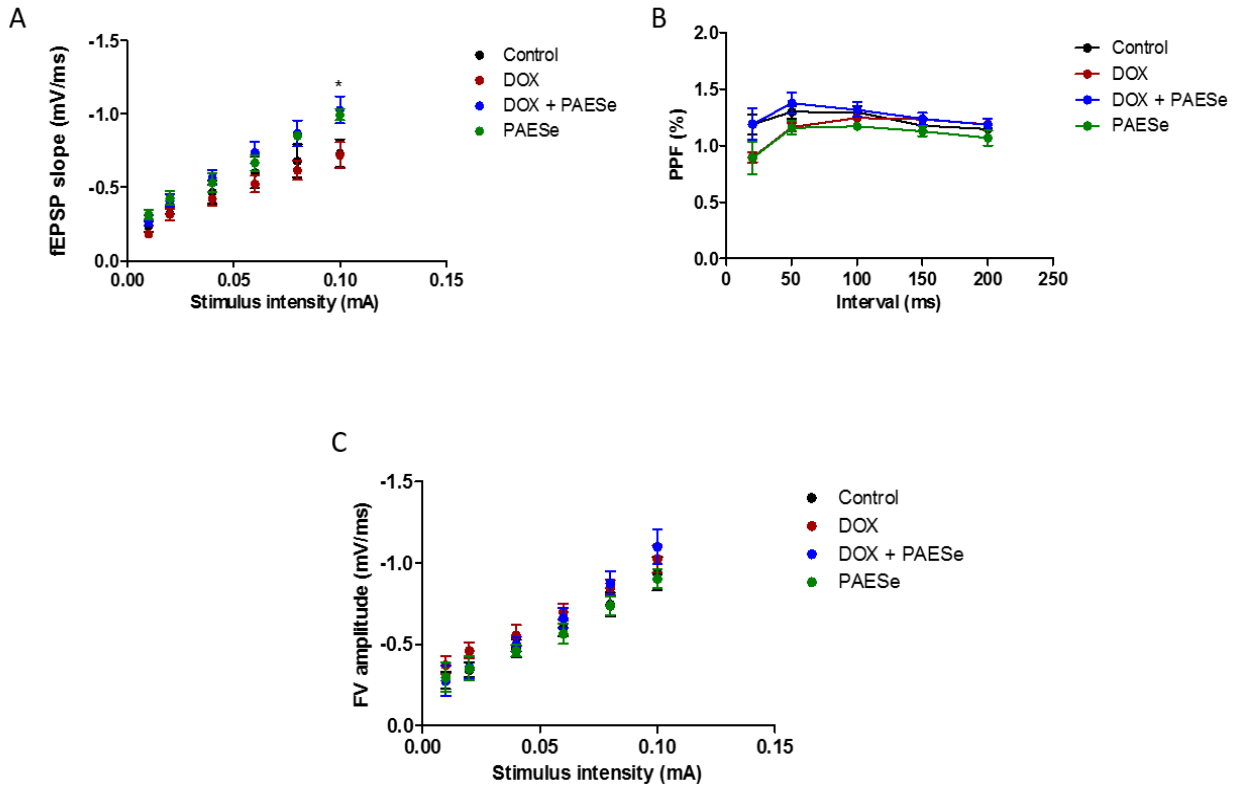
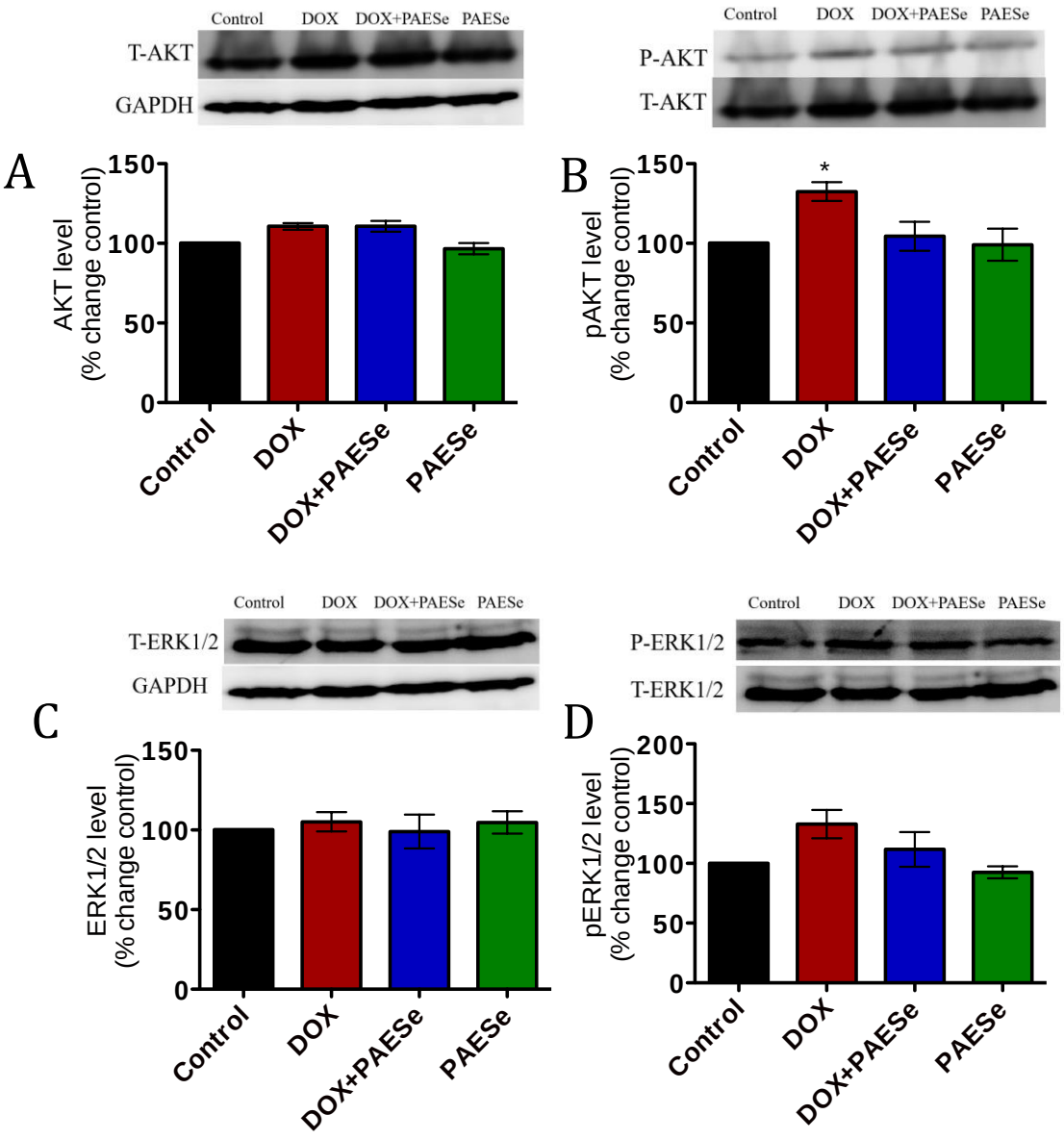


Figure 19: Illustration of basal synaptic transmission in control, DOX-treated animals, DOX + PAESe treated animals, only PAESe-treated animals. (A) The average slope of control and DOX-animals is not significantly different except at 0.08mV and 0.10mV. However, the average slope of DOX-PAESe treated animals and only PAESe-treated animals show no significant differences in all points. In addition, the average slopes of both DOX-PAESe treated animals and only PAESe-treated animals show no significant difference most points except the 0.10mV indicating an increase in the basal synaptic transmission compared to control and DOX alone. (B) Illustrative symbol of paired-pulse facilitation (PPF). PPF ratio (slope2/slope1) in DOX, DOX+PAESe, only PAESe animals measured at different interpulse intervals were not significantly different from controls ($P>0.05$; $n=3$). (C) The average of fiber volley amplitude

was plotted for each stimulus intensity, and there was no difference between DOX, DOX+PAESe, only PAESe animals compared to control animals ($p>0.05$; $n=3$).

Effects of DOX treatment on hippocampal protein expression:

Nude mice treated with DOX have shown increased in Akt and ERK1/2 phosphorylation, but not in the total proteins. On the contrary, DOX treatment caused reduced in CREB phosphorylation. When PAESe ameliorated these deficits when co-administered with DOX.



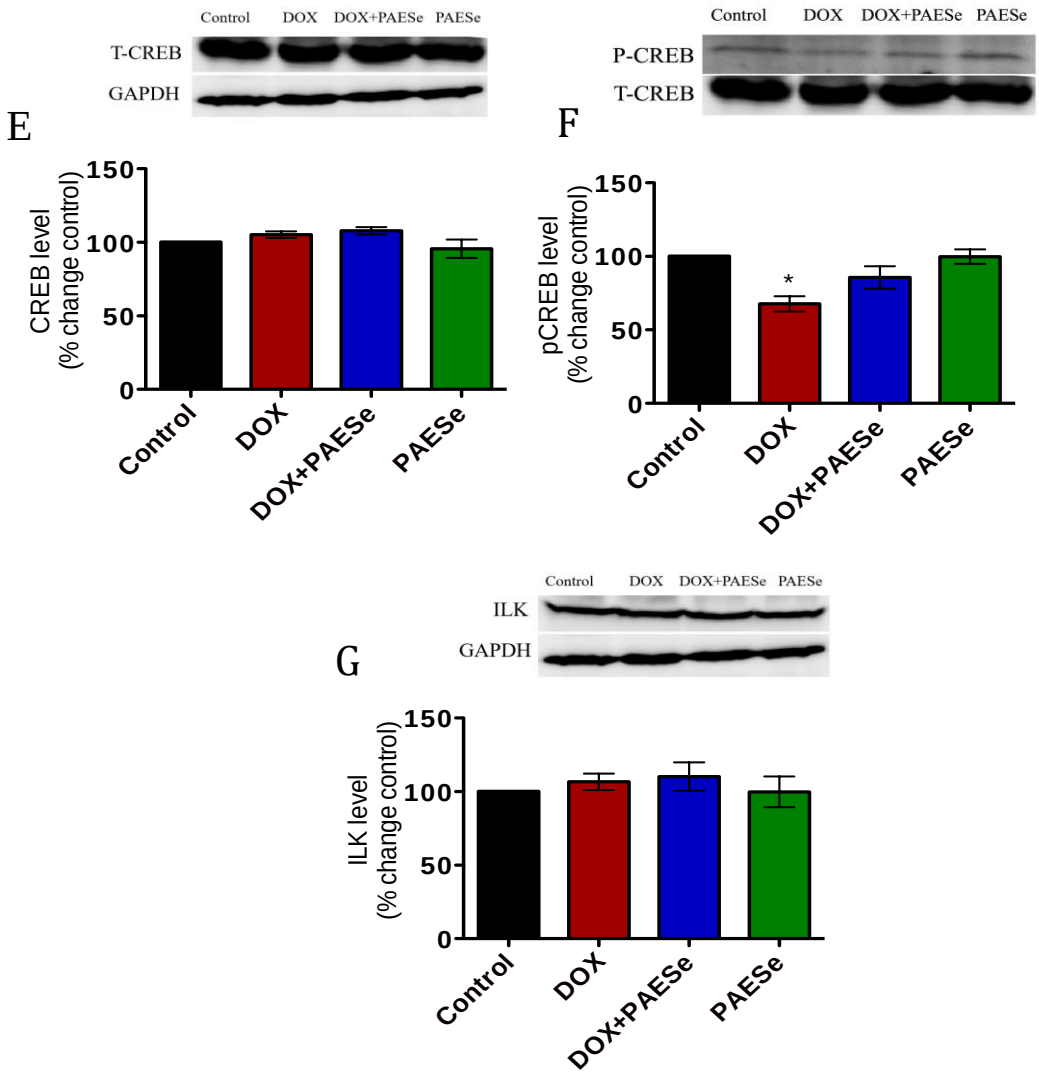


Figure 20: Effects of DOX, DOX+PAESe, and PAESe treatment on hippocampus protein expressions and phosphorylation (Akt, ILK, ERK1/2, and CREB) after 5 doses of treatment. Western blot analysis showed that protein expression levels were not changed following treatment (A, C, E, and G). Conversely, the levels of protein phosphorylation of AKT and ERK1/2 were increased in response to DOX treatment (B, and D), but CREB phosphorylation was decreased in DOX treated animals (F). All protein levels were normalized to GAPDH and presented as a percentage of the control group, which was set as 100%.

Effects of DOX and PAESe treatments on hippocampal surface protein expression:

PSD-95 was polled down and western blot analysis was performed. The results show that synaptic GluA1 was reduced in DOX treated animals, whereas PAESe rescued GluA1 expression and the expression levels were slightly more in the synaptic surface due to PAESe treatment alone.

Discussion:

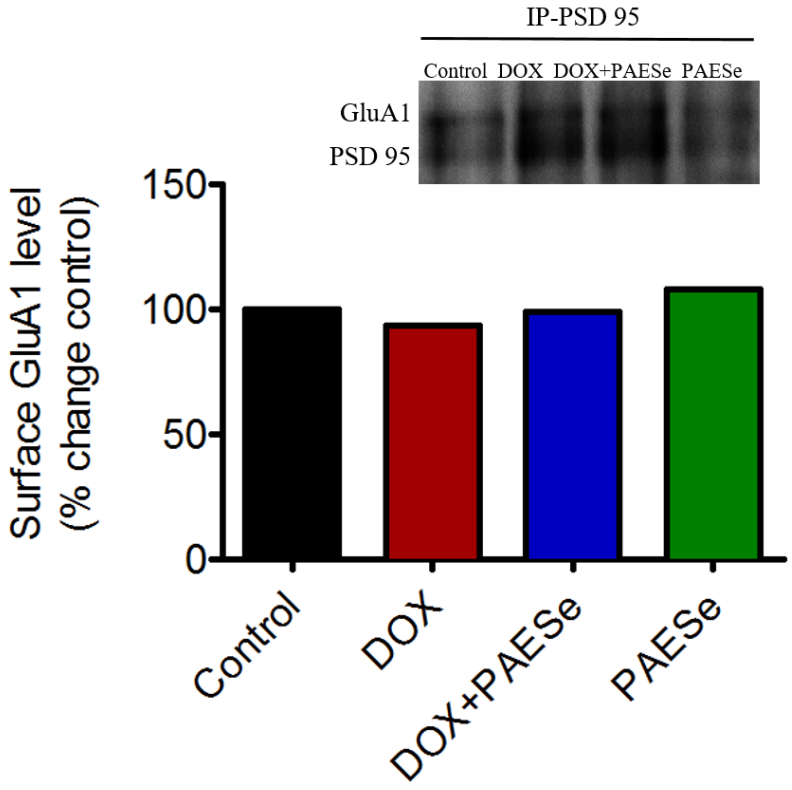


Figure 21: Figure shows the surface expression of GluA1 after DOX treatment in all four groups. GluA1 expression is reduced in DOX treated mice and it is recovered by DOX+PAESe treatment. The GluA1evels were slightly increased in only PAESe treated mice.

In the present study we used an immunocompromised mouse model of chemobrain and hypothesized that the antioxidant PAESe, could protect against the DOX-induced cognitive impairments. We found that DOX-treatment induced deficits in spatial memory in the Y-maze tasks and co-administration of PAESe ameliorated these deficits. We also found that chronic exposure to DOX impairs spatial recognition memory as assessed by NOL task. Both of these tasks are well validated to assess hippocampal function (Broadbent, Gaskin, Squire, & Clark, 2010; Sarnyai et al., 2000; Shipton et al., 2014). We observed significant disruption in hippocampal function in DOX treated animals, which was partially ameliorated by PAESe co-

treatment. It remains to be established whether this co-treatment interferes with the anticancer properties of DOX.

The effects of DOX as chemotherapeutic agent has been well investigated. It is reported to bind to topoisomerase II, arrest the cell cycle causing the death of tumor cells. In addition, it is proposed to increase production of reactive oxygen species that leads to cell death by damaging DNA and inducing lipid peroxidation. However, DOX also cause adverse effects on healthy tissues. DOX treatment is associated with several side effects such as fatigue, inflammation, hypothyroidism, pain, discomfort and cognitive impairment.

Weekly treatments of this chemotherapeutic drug for four weeks caused impaired working and spatial memory in nude mice. A previous study using rats showed that DOX impairs memory in novel place recognition (Christie et al., 2012). Several lines of evidence, including our own, have shown that most chemotherapeutic drugs including DOX cause cognitive dysfunction and that is independent of hormonal manipulations influences. In this study, memory impairment was detected with Y-maze task associated with DOX treatment and PAESe co-administration ameliorated these deficits. Interestingly, PAESe alone caused mild cognitive deficits. This finding suggests that PAESe ameliorates the cognitive impairment following DOX treatment but PAESe treatment alone may have deleterious affect possibly by over activating the glutamatergic neurotransmission. In the novel object recognition tasks, DOX treatment did not impair memory suggesting that not all cognitive functions are affected by DOX. In addition, spatial and working memory is hippocampal-dependent tasks, whereas the novel object recognition is dependent only on ventral hippocampus region. Taken together, this data suggest that DOX treatment can disrupt the memory that relies on intact hippocampal function and PAESe could rescue these deficits.

To investigate the effects of DOX and PAESe on hippocampal synaptic plasticity, we induced LTP in hippocampal slices acquired from the each treatment group. We observed that LTP was impaired in DOX treated slices compared to controls. The impairments in LTP could be due to either alteration of presynaptic release or the postsynaptic responses. Therefore, our study assessed the axonal depolarization by measuring the fiber volley amplitude at various stimulus intensities. The result indicates that fiber volley amplitude is not significantly altered among all groups except in the 0.1 mA. The result revealed that there is no significant difference in the control and DOX-treated mice. However, PAESe, and DOX + PAESe were not significantly different but there were different from the control and DOX-treated mice. Generally, the result indicates that the conversion of the presynaptic stimulus into axonal depolarization was not significantly affected by DOX, DOX+PAESe, and PAESe compared to controls except in 0.1 mA point. Moreover, paired-pulse facilitation measures the pre-synaptic response and there were no significant differences observed in all four groups. This further supports the notion that deficits in LTP due to DOX treatment in mainly due to postsynaptic mechanisms.

In this study, we also examined several proteins associated with learning and memory including AKT, ILK, GSK3 beta, P38, ERK1/2 after DOX, DOX+PAESe, and PAESe treatment in the total hippocampal lysates. The results revealed that total expression did not show any significant differences among control and the treatment groups. However, the protein phosphorylation is altered after the DOX, DOX+PAESe, and PAESe compared to control group. In addition, the results also show that synaptic surface proteins such as GluA1 are decreased after DOX treatment compared to controls.

AMPA and NMDA receptors are made up of different subunits. Presentation of more GluA1 subunit in AMPA receptors in the synaptic membrane will increase Ca^{++} permeability, which could enhance synaptic efficacy (Derkach, Oh, Guire, & Soderling, 2007; Oh et al., 2012; Vandenberghe, Robberecht, & Brorson, 2000). It has been reported that increase in GluA1 in AMPA receptors composition in a GluA2 knockout mice increases Ca^{++} influx into the neuron leading to epileptic seizures resulting in cell death (Feldmeyer et al., 1999). Moreover, increased expression of GluA2 in the synapse will decrease Ca^{++} influx and thus reduce excitotoxicity (Feldmeyer et al., 1999; Wright & Vissel, 2012). Therefore, an alteration in the surface expression of these subunits in the surface plays an important role in the synaptic regulation. Surface expression of GluA1 was assessed by Western blot analysis of PSD-95 pull down assay. Our results show that surface synaptic expression of GluA1 was decreased by DOX treatment. The results indicate that the anchoring of GluA1 into the synaptic surface was reduced in the DOX treated mice compared control and DOX+PAESe, whereas there is an increased of GluA1 expression in only PAESe treated animals.

AKT and ERK1/2 signaling are required for normal neuronal development, function, and synaptic plasticity (Alonso, Medina, & Pozzo-Miller, 2004; Easton et al., 2005; Horwood, Dufour, Laroche, & Davis, 2006; Jansen et al., 2015; Thomas & Huganir, 2004). Phosphorylation is an important signal for synaptic plasticity and memory encoding (Horwood et al., 2006; Thomas & Huganir, 2004). AKT signaling is important for regulating other protein phosphorylation and function, whereas inhibition of AKT phosphorylation inhibits neurogenesis and synaptic plasticity in the dentate gyrus of the adult rodents (Bruel-Jungerman et al., 2009). AKT can be activated by different pathways including PI3K and ILK phosphorylating Ser473 (Farrar, Houser, & Clarke, 2005; Kimura et al., 2010). AKT and ERK1/2 phosphorylation can

mediate neuroprotection during oxidative stress responses and (Kim & Wong, 2009; Song et al., 2008) DOX is well characterized to cause oxidative stress that may lead to apoptosis. Few published studies have reported DOX treatment reduced neurogenesis in the hippocampus (Christie et al., 2012; Inagaki et al., 2007; Kitamura et al., 2015). Thus, we measured the hippocampal AKT and ERK1/2 in our mice model and found that AKT and ERK1/2 phosphorylation were significantly increased in DOX animals compared to all other groups, indicating increased activity. Our finding has shown that AKT and ERK1/2 pathways were activated and in agreement with recently published work by Salas-Ramirez et al., 2016. In addition, ILK expression was also examined and the result revealed there were no differences in the any of the treatment groups compared to control. This data is in agreement with our previous study using hippocampal neurons mentioned in chapter 3, suggesting that there are no significant effects of DOX on ILK expression *in vivo* or *in vitro* models of chemobrain.

The cAMP-response element binding protein (CREB) is transcription factor that plays an essential role in gene regulation (Lonze & Ginty, 2002). CREB is downstream for several pathways including insulin, PI3K/AKT, and MAPK and increase or decrease in CREB activation will alter the gene expression and thus proteins expression in animals and humans. Studies have shown that phosphorylated CREB is required for hippocampal synaptic plasticity and long-term memory formation (Caraci et al., 2015; Porte, Buhot, & Mons, 2008). There are different forms of CREB such as CREB-1 and CREB-2 and these forms play different roles in gene transcription. In the hippocampus, it is shown that increased in CREB-1 phosphorylation increases gene transcription; however, activation CREB-2 suppresses the gene transcription. Therefore, CREB1 phosphorylation could increase protein expression and promote memory formation. On the contrary, CREB-2 phosphorylation suppresses the gene transcription and thus

reduced protein synthesis leading to memory impairment. In this study, we have investigated the phosphorylation pattern of CREB-1 particularly in Ser^{133}. The results indicate that DOX treated mice showed decreased in CREB-1 phosphorylation compared to all other groups. Interestingly, we have found that decrease in CREB-1 phosphorylation is rescued by PAESe treatment indicating that PAESe could ameliorate memory deficits cause by DOX through increased CREB-1 phosphorylation.

In conclusion, this study utilized behavioral, electrophysiological, and biochemical assays to elucidate the mechanisms by which DOX causes memory impairment. DOX treated mice showed elevated levels of AKT and ERK1/2 phosphorylation in the hippocampus indicating that DOX can cause oxidative stress leading to memory impairment. In addition, DOX treated mice exhibited reduction in CREB-1 phosphorylation levels. Reduced CREB-1 phosphorylation has been shown to impair memory encoding. This study also showed that impaired LTP expression leading to memory deficits by DOX treatment is due to decreased synaptic GluA1 expression. Additionally, this study also demonstrated the ameliorating effects of PAESe on DOX induced synaptic deficits and memory impairment indicating a therapeutic role for PAESe in chemotherapy induced memory loss.

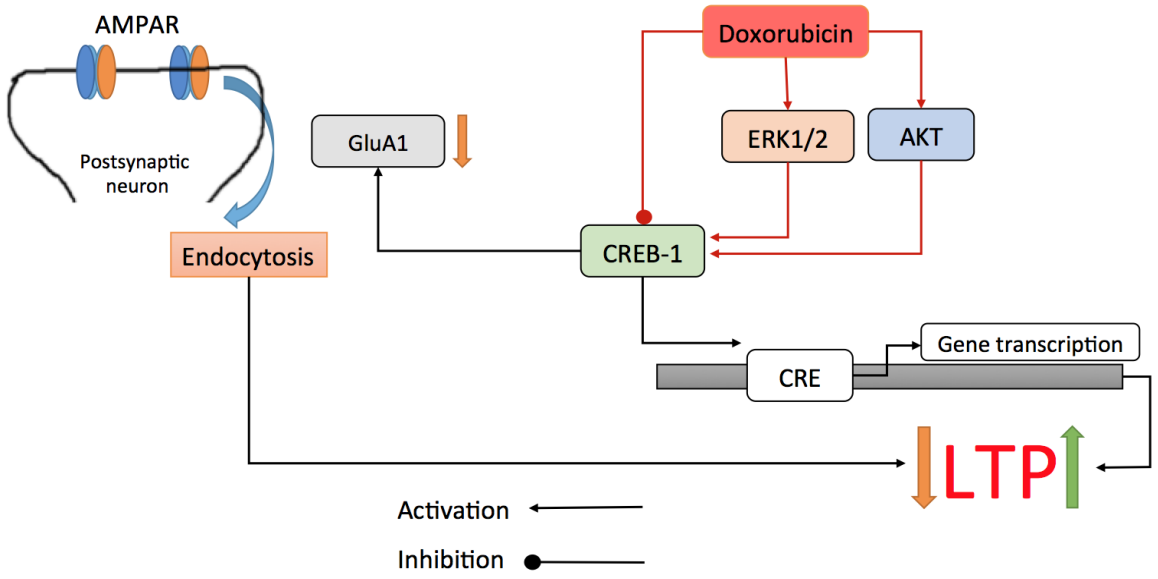


Figure 22: Schematic illustration of how DOX regulates proteins expression and phosphorylation. DOX activates both ERK1/2 and AKT and these proteins activate CREB-1. Also, DOX treatment inhibits CREB-1 phosphorylation in Ser-133 residue, which plays a key role in gene regulation and thus protein transcription. CREB-1 phosphorylation regulates glutamate-AMPA-GluA1 expression. This study has revealed synaptic GluA1 expression was reduced and LTP impaired.

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CHAPTER 5

CONCLUSIONS AND PROSPECTIVE

Clinical studies and scientific reports have demonstrated that chemotherapy treated cancer patients exhibit both acute and chronic cognitive deficits. Cognitive impairments have also been reported in several animal models of chemotherapy. However, the exact pathophysiology of these impairments remains unknown. Our recent study indicated that DOX impairs hippocampal long-term potentiation (LTP) through altered glutamatergic synaptic transmission.

We found that intravenous administration of DOX caused cognitive deficits in memory and hippocampal synaptic plasticity. Additionally, we found that co-administration of PAESe with DOX ameliorated synaptic plasticity and memory. The animals treated with both PAESe and DOX had improved long-term potentiation and Y-maze performance. However, the animals treated with only PAESe showed impaired spatial memory, highlighting the importance of further investigation into effects of PAESe on memory encoding. It is possible that, intravenous injection of DOX could be affecting peripheral organs such as the pancreas, thyroid, kidneys and liver. Insulin, thyroid hormones, and hemoglobin all have well-established roles in regulating memory function. Therefore, future studies will be directed towards the assessment of the impact of DOX and PAESe on these organs to elucidate the alternative mechanisms of DOX induced chemobrain and the ameliorating effects of PAESe.

In conclusion, our research indicates that DOX treatment induces cognitive deficits by altering expression and function of synaptic proteins. Additionally, we show that co-administration of PAESe with DOX can rescue LTP and ameliorate cognitive dysfunction induced by DOX. Thus, PAESe should be considered as a potential therapy for chemotherapy-induced cognitive deficits.