Demyelination and Cognitive Decline from Doxorubicin Treatment:
Role of Reactive Oxygen Species

One in 8 women in the US will be diagnosed with breast cancer. The anthracycline drug doxorubicin (DOX) is one of the most effective breast cancer chemotherapies. Unfortunately, DOX is associated with severe side-effects. Anthracycline-treated survivors experience a larger decrease in executive functioning, memory, and processing speed than survivors treated with non-anthracyclines. This chemotherapy-induced cognitive deficit is called "chemobrain," and its **mechanism of action is unknown**.

Myelination is necessary during adulthood to maintain neural plasticity, and pathologies that damage the myelin coating of neurons, or "white matter," are associated with cognitive impairment. Anthracycline-treated survivors show a larger decrease in frontal and temporal white matter tracts compared to non-anthracycline-treated survivors. Our **preliminary data** suggests that therapeutic doses of DOX lead to demyelination and cognitive dysfunction in mice. It is known that DOX, which does not cross the blood brain barrier (BBB), initiates an inflammatory response associated with the production of reactive oxygen species (ROS) in the periphery. The peripheral inflammatory response and ROS production may influence the CNS to promote ROS production in the brain, possibly causing axonal damage. Myelination requires differentiation of oligodendrocyte precursor cells (OPCs) in to myelin-producing oligodendrocytes (OLs). It is unknown if and how DOX influences the oligodendrocytic lineage, but the relatively high sensitivity of both myelin and OPCs to ROS opens the question of a ROS-induced demyelinating mechanism in response to DOX. Demyelination is associated with cognitive decline and is an important mechanism to evaluate to improve our understanding of DOX-induced chemobrain.

The overall aim of this study is to elucidate whether DOX treatment has a detrimental effect on cognitive function, the degree of myelination in the brain, and the oligodendrocytic lineage through the production of ROS. The **translational significance** of this project is to clarify part of the mechanism of DOX-induced cognitive deficit, as observed in breast cancer patients, which may finally lead to the **identification of therapeutic targets**. Based on the above and preliminary data, I **hypothesize** that *therapeutic doses of DOX increase ROS in the brain leading to OPC death, demyelination, and cognitive dysfunction.*

Aim I: To determine if DOX induces cognitive dysfunction, demyelination and OPC/OL death in mice.

By using a mouse model of DOX-induced cognitive dysfunction (DICD), I aim to quantify associated changes in myelination and OPC/OL death. I will test 'therapeutic' doses of DOX, defined as a dosing schedule capable of inhibiting tumor growth, to investigate the mechanism. I will:

- **a)** Evaluate cognitive function in our DICD model.
- **b)** Quantify demyelination and structural changes in myelinated fibers in our DICD model.
- **c)** Quantify OPC/OL and determine cell-specific death in our DICD model.

Aim II: To determine if DOX-induced cognitive dysfunction, demyelination and OPC/OL death is mediated by reactive oxygen species (ROS) in mice.

The role of ROS in DOX-induced cognitive dysfunction, demyelination and OPC/OL death will be evaluated by co-administering a BBB-penetrable ROS scavenger, manganese porphyrin (MnP), with DOX. Results will be compared to DOX-only treatment from Aim I. ROS production will be determined by assessing MnSOD nitration, which is a reliable marker of oxidation in the brain. I intend to:

- **a)** Evaluate ROS in the brain following DOX-only and DOX + MnP co-treatment by immunoprecipitating nitrated MnSOD.
- **b)** Investigate if inhibition of ROS with MnP can prevent DICD using methods outlined in aim I.
- **c)** Investigate if inhibition of ROS with MnP can prevent myelin alterations in our DICD model using methods outlined in aim I.
- **d)** Investigate whether inhibition of ROS with MnP can prevent cell-specific death of OPC /OL in our DICD model using methods outlined in aim I.

Upon completion of aims I and II, we will have a better understanding of the mechanism of DOX-induced cognitive dysfunction and its associated deficits which may open a **translational avenue** of using ROS scavengers to prevent the neurotoxic effects of DOX, including cognitive dysfunction.

Significance

Breast cancer, the second most common cancer among women, has a notable 72% survival rate 5 years following adjuvant treatment in a stage III diagnosis. As cancer survivorship increases, so does the need to confront the long-term effects of chemotherapy which include fatigue, depression and cognitive dysfunction. Chemotherapy-induced cognitive dysfunction, or "chemobrain," was observed in 69% of prospective longitudinal studies on breast cancer patients 1 year following chemotherapy treatment[1]. Cognitive dysfunction is characterized by deficiencies in memory, processing speed and executive function. The anthracycline drug DOX is one of the most effective agents in the treatment of breast cancer, but is associated with more severe cognitive deficits in humans than non-anthracyclines[2]. Despite its prevalence, the mechanism through which doxorubicin-induced cognitive dysfunction (DICD) occurs is largely unknown and **therapeutics aiming to prevent or repair these cognitive deficits are nonexistent**. In the literature, the few animal models of DOX-induced pathologies rely on high DOX doses (sub-lethal to lethal) that do not reflect therapeutic treatment[3]. Moreover, existing studies evaluate structure or cognition exclusively and fail to draw associations between the two. Our **novel** animal model of doxorubicin-induced cognitive dysfunction, which is based on a therapeutic dosing schedule that inhibits tumor growth[4], has produced **preliminary data** that suggests a disruption of white matter density and increase in fiber coherency. This research is **innovative** in that it associates structural changes with changes in cognitive functioning in therapeutic-dose DICD mice using accepted behavioral tests for memory and executive function. Such findings have never been published and will represent a new understanding of cognitive dysfunction resulting from DOX treatment. The **significance** of this translational research relies on these associations: quantifying cognitive deficits that coincide with structural and molecular changes allows us to better understand a possible mechanism of DICD that can then be used to define therapeutic targets. The ability to prevent and/or reverse cognitive dysfunction from DOX treatment has the potential to improve the quality of life for a growing population of breast cancer survivors.

Myelination and Oligodendrocytes

The myelin sheath, a lipid-based layer that coats the axons of some neurons known collectively as "white matter" in the CNS, is necessary for efficient signal transduction between neurons. Myelin damage is associated with a decrease in efficiency of information exchange and cognitive decline[2,5]. Myelination requires the differentiation of oligodendrocyte precursor cells (OPCs), which exist as quiescent cells throughout the CNS, into myelin producing oligodendrocytes (OL). Recently, investigations regarding myelination in the adult brain have elucidated a role for myelination in sustaining neuronal growth and adaptive behavior[6]. Neuronal activity led to an increase in myelination and was associated with improved behavioral function. This increase in myelination was dependent on the differentiation of OPCs into mature OLs[6]. In addition, both the myelin sheath and oligodendrocyte lineage are vulnerable to ROS. Increased ROS in the brain is associated with decreased myelination *in vivo* and *ex vivo*, and myelin incubated with ROS *in vitro* led to lipid peroxidation and decompaction of myelin[7,8,9]. The oligodendrocytic lineage is amongst the most sensitive cell types in the brain to ROS; OLs were killed at ROS concentrations that did not affect astrocytes or microglia. This is in part due to high energy demand, low antioxidant glutathione, and high iron content[10]. Importantly, OPCs were shown to be more vulnerable to ROS than mature OLs[11]. The dependence on OPCs during activity-dependent myelination and remyelination make the effects of DOX-induced ROS production on OPCs important mechanisms to evaluate.

Reactive Oxygen Species

The pharmacological target proposed here are ROS, which are highly reactive species derived from molecular oxygen that can modify lipids, proteins and nucleic acids leading to loss of function and cellular apoptosis. Peripheral ROS production is one of the intended mechanisms of action of DOX that leads to cell death, but ROS are also generated in the CNS of mice following high-dose DOX treatment[12]. Although DOX does not cross the blood-brain-barrier (BBB)[13], it has been shown to increase the production of pro-inflammatory cytokines in the periphery (i.e. TNF-α). Circulating cytokines can cross the BBB to stimulate cytokine production and mitochondrial dysfunction in the CNS, leading to production of ROS in the brain in a feed-forward manner[14,15]. The production of ROS in the brain is strongly associated with neuronal death in other neurological pathologies such as Alzheimer's disease and multiple sclerosis[16,17], both of which are associated with alterations in myelination and cognitive function[18,19,20].

Research Plan

Goal:

In order to evaluate the mechanisms of doxorubicin-induced cognitive dysfunction and demyelination, I will use a therapeutic dosing of DOX **(1)** to determine if DOX induces cognitive dysfunction, demyelination and OPC/OL death in mice. Additionally, I will evaluate the role of ROS in DICD by using a BBB-penetrable ROS scavenger, manganese porphyrin (MnP), co-administered with a therapeutic-dosing of DOX **(2)** to determine if DOX-induced cognitive dysfunction, demyelination and OPC/OL death is mediated by reactive oxygen species in mice.

The **overall hypothesis** to be tested in 2 specific aims is that *therapeutic doses of DOX increase ROS in the brain leading to OPC death, demyelination, and cognitive dysfunction.*

Aim I: To determine if DOX induces cognitive dysfunction, demyelination and OPC/OL death in mice

Rationale: A DOX dosing regimen that inhibited tumor growth and increased survivability of mice with breast carcinomas was identified as 6 mg/kg i.p. injections per week for 4 weeks (24 mg/kg total), with higher doses causing death[4] and lower doses (12 mg/kg total) showing no effect on tumor volume[21]. Cognitive function was not tested in either study. A separate study demonstrated that 5 mg/kg injections per week for 3 weeks (15 mg/kg total) caused a 22.4% reduction in newly dividing cells in the dentate gyrus, but did not analyze cognitive function or antitumor activity[22]. Using these results, our lab defined a dosing schedule of 5 mg/kg i.p. injections of doxorubicin HCl (Pfizer) per week for 4 weeks (20 mg/kg total) for our DICD model to maintain antitumor potential while considering the decrease in neural proliferation, which may or may not be related to cognitive function. 7 days after the final dose, behavioral tests were performed and tissues were harvested. Our preliminary data in Figure 1 suggests that DOX impairs performance in the puzzle box test, which is an accepted measure of executive functioning, and provides support for our therapeutic dosing model of DICD which is the foundation for development of this project. In addition, our preliminary staining of myelin in the cortex suggests that DICD is associated with a decrease in myelination, suggesting at least part of the mechanism of DICD may be myelin-dependent.

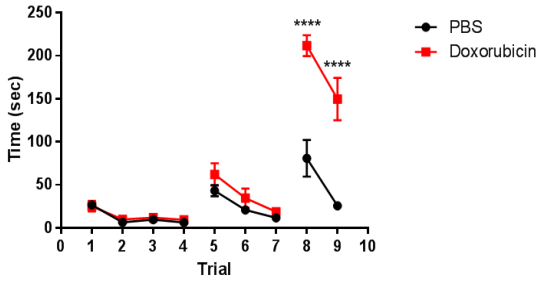

Figure 1: Preliminary data. Female C57 were treated with DOX or PBS (4 weekly i.p. injections of 5mg/kg for a total of 20mg/kg). Puzzle box performance was decreased in DOX mice 7 days after the last injection, as the escape time increased. Puzzle box requires mice to complete an escape task of increasing difficulty.

What is not known and has never been evaluated is whether demyelination is due to a direct action on myelin or an indirect action on myelin via the loss of OPCs and/or OLs. A preferential loss of OLs would suggest a lack of existing myelin upkeep and axonal support[23]. Fortunately, OPCs have the ability to differentiate and replace dying OLs[24]. A loss of OPCs would suggest a lack of activity-dependent myelination, an inability to replace dying OLs, and an inability to remyelinate axons that have been denuded. Recent research that emphasizes the importance of activity-dependent myelination in adult learning, which is dependent on OPCs, suggests a loss of OPCs would be more detrimental to both myelination and cognition[9]. However, the role of OPCs vs. OLs in myelin upkeep and cognitive function is not well understood and requires the investigation of both cell types.

Aim 1a: Evaluate cognitive function in our DICD model

To confirm the validity of our model, I will employ several established behavioral tests that evaluate different aspects of cognitive function: the novel object and place recognition (NOPR) test, the puzzle box test and the Y-maze test which are accepted measures of working memory, executive function and spatial memory, respectively [25,26,27]. Due to the low instance of breast cancer in men, which is likely due to hormonal and physiological differences between sexes, female mice will be used throughout this study to ensure a translational approach. The objective aim of 1a is to test the hypothesis that therapeutic doses of DOX can induce cognitive dysfunction in mice.

Approach: Adult female C57 mice (n=10) will be treated with DOX or PBS according to the dosing schedule in Figure 1. One week after the final dose of DOX or PBS, cognitive function will be evaluated in both cohorts using the NOPR test, the puzzle box test and the Y-maze test. The NOPR test relies on the observation that mice have an innate preference for novelty. When a novel object preference is observed, it indicates intact spatial and episodic memory[26]. Similarly, the Y-maze relies on a mouse's preference to enter a novel arm in a spatial maze. The amount of perfect spontaneous alterations are catalogued, indicating a preference for novel environments and intact spatial memory[27]. The puzzle box test evaluates general cognition and executive function by training mice to complete an escape task of increasing difficulty and measuring the time required to complete the task[25]. It consists of 2 training days and 1 testing day. It is on the testing day that DICD is evident: DOX-treated mice required more than twice as much time to escape the puzzle box than control mice in our preliminary results (Fig. 1).

Aim 1b: Quantify demyelination and structural changes in myelinated fibers in our DICD model

Damage to white matter, made up of myelinated axons, is evident in neurodegenerative diseases that are associated with cognitive decline such as Alzheimer's and multiple sclerosis[16,17]. Previous neuroimaging studies have found associations between white matter damage and adjuvant chemotherapy involving DOX in humans[2], but animal studies focused on DOX-only treatment and white matter damage do not exist. Furthermore, this is the first proposed study to evaluate myelin fiber structure and cognition in DICD, allowing us to draw associations between the two. Myelin fiber structure can be visualized by staining using antibodies directed against myelin proteins, such as myelin basic protein (MBP), as well as techniques that involve gold-based salts such as the Black Gold reagent[28,29]. I will perform both protocols to ensure the validity of our model. The objective of aim 1b is to test the hypothesis that DOX-induced cognitive dysfunction is associated with structural changes in myelinated fibers and demyelination in mice.

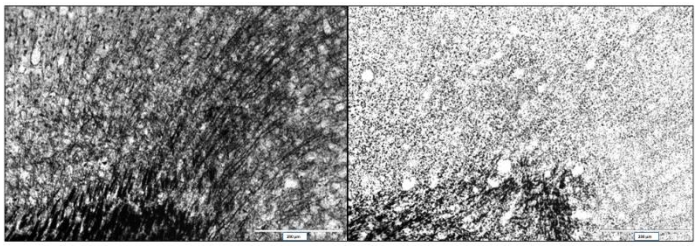

Figure 2: Preliminary data. Black Gold staining at the cingulate cortex shows a decrease in myelination in the DICD mouse model (20x).

Approach: Adult female C57 mice (n=10) will undergo DOX or PBS treatment as described in Figure 1, followed by behavioral tests. Mice will be euthanized after completion of the tests, 12 days after the final injection of DOX or PBS. Mice will be perfused with paraformaldehyde and brains will be collected for myelin analysis on free-floating cryosections stained for MBP or with the Black Gold reagent. Characterization of myelin will occur in the cingulate cortex, corpus callosum and SVZ because these regions are involved in learning, functional connectivity and OPC generation, respectively[30,31,32]. Characterization will be accomplished by evaluating length and percent area, which indicate a general degree of myelination, as well as junctions and fiber coherency, which analyze myelin fiber structure. Junctions, which represent axonal branching, will be counted manually. Coherency is an additional measure of axonal branching and indicates how co-aligned features of an image are from 0 (not aligned) to 1 (all features aligned). A high degree of axonal branching would have a low coherency value since the features of the image are less-aligned. A decrease in axonal branching compared to healthy control mice indicates a pathological abnormality.

Aim 1c: Quantify OPC/OL and determine cell-specific death in our DICD model

Understanding the mechanism of DICD and demyelination requires the evaluation of cell-specific death of both OLs and OPCs. This is because the two cell types play different roles in myelination and axonal function in adults. Recent research has uncovered the importance of OPCs in the adult brain: OPCs are necessary to **1)** myelinate new axonal growth, **2)** remyelinate denuded axons and **3)** replace dying OLs associated with myelinated axons. In an elegant study, stimulation of the premotor cortex of mice promoted OPC differentiation and increased myelination in the activated circuit[9]. Importantly, blockage of OPC differentiation prevented activity-dependent myelination[9]. Therefore, a loss of OPCs would result in a decrease in *de novo* myelination. Furthermore, evidence suggests that OPCs can migrate and differentiate to replace dead OLs[33], and are required to remyelinate denuded axons in toxin-induced models of demyelination[24,34]. Therefore, a loss of OPCs would

also result in an inability to replace dead OLs and remyelinate denuded axons. OPCs are more sensitive to ROS than mature OLs, supporting our proposed mechanism of DICD introduced in aim 2[11].

The role of mature OLs in the adult CNS is less understood. Mature OLs are post-mitotic, non-migratory and are unable to remyelinate lesions or myelinate new axonal growth[24,35,34]. Recent research suggests a role for OLs in axonal support and energy metabolism[23,36]. The few papers in the literature that focus on selective loss of oligodendrocytes report selective demyelination[37]. Therefore, a preferential loss of OLs may lead to cognitive dysfunction firstly via axonal dysfunction, and secondarily via selective demyelination if the OLs are not replaced.

Taken together, DICD and demyelination is probably the result of several interacting mechanisms: **1)** direct damage of myelin by a downstream effect of DOX, presented in aim 2 as ROS, **2)** a decrease in axonal support due to mature OL death leading to selective demyelination and axonal dysfunction, and likely the most important, **3) a decrease in myelination of new axonal growth, a decrease in the brain's ability to remyelinate and a decrease in OL replacement due to the death of OPCs**. This proposal does not aim to differentiate between demyelination due to a lack of repair vs. a lack of myelination of new axonal growth, rather, to expose the hypothesized association between OPC death and demyelination in our DICD model. Since the production of ROS is known to induce apoptosis, as explained in aim 2, I propose to evaluate apoptosis in the oligodendrocytic lineage in our DICD model with TUNEL staining. The objective of aim 1c is to test the hypothesis that DOX-induced cognitive dysfunction is associated with OPC apoptosis in mice.

Approach: Adult female C57 mice (n=10) will undergo DOX or PBS treatment as described in Figure 1, perform behavioral tests and be euthanized after completion, 12 days after the final injection of DOX or PBS. Mice will be perfused with paraformaldehyde and brains will be collected and stained with immunofluorescence for stage-specific oligodendrocytic markers on free-floating cryosections: Olig2/Ki67 and NG2 for OPCs[33,38] and Olig2/CNPase and CC1[38,39] for mature OLs. Stains will be imaged and OPCs and OLs will be counted with a focus on the cingulate cortex, corpus callosum and SVZ, as described in aim 1b. Using this data I can evaluate whether a decrease in OPCs, as stated in the hypothesis, is associated with demyelination and cognitive decline. To evaluate if a change in OPC/OL is due to apoptosis, I will co-stain TUNEL with stage-specific oligodendrocytic markers listed above, and count apoptotic OPC/OLs in the locations described.

Expected Results: In aims 1a and 1b, we have preliminary data that suggests significant cognitive dysfunction, changes in myelin structure and demyelination from therapeutic doses of DOX in our DICD model (Fig. 1 and 2). In aim 1c, established literature suggests a predominant loss of OPCs via apoptosis, as this loss is likely more detrimental to myelination and cognitive function than a loss of OLs and because OPCs are more sensitive to ROS than OLs. However, a concurrent loss of OLs is not unexpected and still provides a stronger understanding of DICD than currently exists.

Potential Problems and Alternative Strategies: It is possible that there may be a decrease in cognitive function and demyelination without a significant change in OPCs/OLs. In this case, I will evaluate whether the proposed downstream effector, ROS, is interacting directly with myelin to induce demyelination without affecting OPCs/OLs. This can be accomplished with a 4HNE stain, which is a marker for lipid peroxidation, co-stained with MBP to visualize ROS with myelin, a lipid-heavy structure[40]. If the staining is negative for 4HNE it is possible that ROS does not play the significant role expected in DICD.

Aim II: To determine if DOX-induced cognitive dysfunction, demyelination and OPC/OL death is mediated by reactive oxygen species (ROS) in mice

Rationale: The production of ROS is one of the intended mechanisms of action of DOX that can lead to apoptosis via caspase-3 activation[41]. Although DOX does not cross BBB, it initiates a pro-inflammatory response that causes production of pro-inflammatory cytokines in the CNS. This is possible through action on endothelial cells at the BBB by peripheral ROS and cytokines, causing them to secrete pro-inflammatory signals such as TNF-α and PGE2 in to the brain. This inflammatory response is accompanied by macrophage infiltration and microglia activation, which stimulates further pro-inflammatory cytokine production in the brain[42]. CNS inflammation is

closely associated with the production of ROS in the brain via increased activity of the mitochondrial respiratory chain and activation of NADPH oxidase[43,44.45,42]. Both sources produce the superoxide anion, O_2^-, from which other ROS derivatives are formed[46]. In a pro-inflammatory environment, the increased production of ROS creates a pro-oxidant network: ROS can modify lipids, proteins and nucleic acids leading to a loss of function, further ROS production and cellular apoptosis[44]. In high dose DOX models, protein oxidation was observed in the brain of mice[47].

The production of ROS in the brain is strongly associated with cell death in other pathologies such as multiple sclerosis, which is associated with alterations in myelination and cognitive function[17]. The brain is especially vulnerable to ROS because of increased energy demands and a low levels of antioxidant activity compared to the periphery[46]. The high polyunsatured fatty acid content in myelin is vulnerable to peroxidation, leading decompaction of myelin and production of aldehydes that may themselves induce apoptosis[9,46]. Oligodendrocytes are highly sensitive to ROS due to their high iron content, high energy consumption during myelination and characteristically low antioxidant defense[10]. Importantly, OPCs were shown to be significantly more vulnerable to ROS than mature OLs[11]. In order to evaluate the role of ROS in the brain following DOX treatment, I will co-administer DOX with the ROS scavenger manganese porphyrin (MnTnBuOE-2-PyP^{5+} or MnP) and compare to DOX treatment alone. MnP is a potent scavenger of O_2^-, can cross the BBB, has been shown to reduce ROS-related damage, increase survival of neurons, and exhibited no toxicity in human breast epithelia *in vitro*[48].

Aim 2a: Evaluate ROS in the brain following DOX treatment and whether DOX + MnP co-treatment will reduce ROS in the brain by immunoprecipitating nitrated MnSOD

To establish a role of ROS in demyelination and DICD, I first need to identify an increase in ROS in response to a therapeutic dosing of DOX in the brain. Following this, I will evaluate whether our proposed ROS scavenger, MnP, decreases ROS in brains of DOX-treated mice. An accepted method of evaluating ROS in the brain is by quantifying nitrated MnSOD, which is responsible for reducing the superoxide radical O_2^- to H_2O_2 and water[44]. Nitration of MnSOD causes inactivation of the enzyme, preventing reduction of O_2^- and increasing further ROS production in the brain. MnSOD nitration is accomplished by the highly reactive peroxynitrite, which is generated by a reaction between O_2^- and the endogenous signaling molecule, NO. Since MnSOD nitration is mediated by an O_2^- derivative, its inactivation is dependent on ROS[49]. The objective of aim 2a is to test the hypothesis whether therapeutic dosing of DOX increases ROS in the brain of mice, and whether co-treatment with MnP lowers the quantity of ROS in the brain of mice.

Approach: Adult female C57 mice (n=10) will be treated in a 2 x 2 factorial design to control for DOX, MnP and the stress of a double injection. I will use the same dosing schedule as in Figure 1, but co-administered with either 3 mg/kg of MnP, as described in previous literature[48], or with PBS. The 4 cohorts will be as follows: DOX + PBS, DOX + MnP, PBS + PBS, and PBS + MnP. Similar to aim 1, mice will receive weekly i.p. injections (5 mg/kg DOX, 3mg/kg MnP, or PBS depending on cohort) for 4 weeks. 12 days after the final dose, to allow a 7 day resting period and 5 days for behavioral tests, mice will be euthanized and brains removed. To evaluate nitrated MnSOD, the total fraction of nitrated proteins is immunoprecipitated and Western blot analysis of MnSOD levels from the immunoprecipitated fractions is completed. This allows us to evaluate nitrated MnSOD specifically. The level of expression of the MnSOD protein band, verified with β-actin, is visualized and quantified using ImageQuant LAS.

Aim 2b: Investigate if inhibition of ROS with MnP can prevent DICD using methods outlined in aim I

To evaluate whether the production of ROS is associated with cognitive dysfunction, and whether co-treatment of DOX with MnP decreases DICD, treated mice will undergo behavioral tests as defined in aim 2a. Indeed, ROS production is associated with cognitive impairment in pathologies such as Alzheimer's disease[16]. The objective of aim 2b is to test the hypothesis that co-treatment of DOX with an ROS scavenger, MnP, prevents or reverses cognitive dysfunction in our DICD model.

Approach: Adult female C57 mice (n=10) will be treated in a 2 x 2 factorial design as described in aim 2a. Behavioral tests, as described in aim 1a, will be performed 7 days after the final dose.

Aim 2c: Investigate if inhibition of ROS with MnP can prevent myelin alterations in our DICD model using methods outlined in aim I

To evaluate whether the production of ROS is associated with structural changes in myelinated fibers and demyelination in mice, and whether co-treatment of DOX with MnP prevents or reverses structural changes in myelinated fibers and/or demyelination, treated mice will be stained for myelin and myelin will characterized as described in aim 1b. The high lipid content, vulnerable to peroxidation, and literature that describe an association between ROS and white matter damage suggests a positive association between ROS and the perturbation of myelination and myelinated fiber structure. The objective of aim 2c is to test the hypothesis that co-treatment of DOX with an ROS scavenger, MnP, prevents or reverses demyelination and/or structural changes in myelinated fibers in our DICD model.

Approach: Adult female C57 mice (n=10) will be treated in a 2 x 2 factorial design as described in aim 2a. Myelin will be stained and characterized, as described in aim 1b, 12 days after the final dose.

Aim 2d: Investigate whether inhibition of ROS with MnP can prevent cell-specific death of OPC /OL in our DICD model using methods outlined in aim I

To evaluate whether the production of ROS is associated with OPC/OL cell death via apoptosis, and whether co-treatment of DOX with MnP prevents or reverses OPC/OL loss, treated mice will be stained for OPC/OL-specific markers to quantify OPCs/OLs and co-stained with TUNEL to quantify apoptosis. Research suggests that OPCs are more sensitive to ROS than OLs, suggesting a preferential perturbation of OPCs by ROS[11]. The ability of ROS production to lead to apoptosis requires investigation of the mechanism of cell death, and suggests that the ROS scavenger, MnP, may reduce apoptosis in our DICD model. The objective of aim 2d is to test the hypothesis that co-treatment of DOX with an ROS scavenger, MnP, prevents or reverses OPC apoptosis in our DICD model.

Approach: Adult female C57 mice (n=10) will be treated in a 2 x 2 factorial design as described in aim 2a. OPC/OL will be stained and quantified, and co-stained with TUNEL as described in aim 1c.

Expected Results: In aim 2a, I expect to observe an increase in MnSOD nitration in the brain. This is consistent with the hypothesis that ROS is associated with DICD and demyelination. It is also expected that MnP treatment decreases MnSOD nitration in response to DOX. In aim 2b, I anticipate improved cognitive function in DOX + MnP mice compared to DOX + PBS mice, suggesting a role for ROS in the development of DICD. In aim 2c, I anticipate less structural changes in myelinated fibers and less demyelination in DOX + MnP mice compared to DOX + PBS mice. A result that found positive correlations between preservation of myelin and reduced cognitive dysfunction in our DICD model would suggest an association between myelination and cognitive function in DICD. Finally, I expect to see a decrease in OPC apoptosis in DOX + MnP treated mice compared to DOX + PBS mice. This result would suggest that ROS are involved in OPC apoptosis, and that apoptosis can be prevented by sequestering ROS.

Potential Problems and Alternative Strategies: One issue that could arise is a potential ineffectiveness of MnP, although this is not expected according to literature[48]. Alternatively, another ROS scavenger that has been shown to reduce ROS in the CNS could be used, like epicatechin[50]. If sequestering ROS does not improve DICD, but does improve the structure of myelinated fibers and demyelination, then it is possible there is an alternative mechanism of DICD that is not related to myelin and is a result itself. This could be related to axon functioning, due to a metabolic imbalance initiated by DOX and the associated inflammatory response. Another potential problem is if sequestering of ROS does not improve DICD, alterations to the structure of myelinated fibers and demyelination, or OPC/OL death. In this case, ROS production may not be crucial in these mechanisms, which is another positive result. Other mechanisms that do not necessitate ROS as mediators are possible, such as a direct effect of cytokines or microglia activation leading to apoptosis.

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