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# Hdac6 Inhibition Reverses Long-Term Doxorubicin-Induced Cognitive Dysfunction By Restoring Microglia Homeostasis

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# HDAC6 INHIBITION REVERSES LONG-TERM DOXORUBICIN-INDUCED COGNITIVE DYSFUNCTION BY RESTORING MICROGLIA HOMEOSTASIS

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By

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*To my family*

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**Abstract**. One in 8 women in the US will be diagnosed with breast cancer. Currently, doxorubicin is one of the most effective chemotherapies for breast cancer. Unfortunately, up to 60% of survivors report long-term chemotherapy-induced cognitive dysfunction (CICD) characterized by deficits in working memory, processing speed, and executive functioning. Currently, no interventions for CICD have been approved by the US Food and Drug Administration. I show here that a 14-day treatment with a blood-brain barrier permeable histone deacetylase 6 (HDAC6) inhibitor successfully reverses long-term CICD following a therapeutic doxorubicin dosing schedule in female mice, as assessed by the puzzle box test and novel object/place recognition test. Long-term CICD was associated with a decreased expression of the postsynaptic protein PSD95, but no decrease in the presynaptic protein synaptophysin, in the hippocampus. I did not detect a significant decrease in mitochondrial function or morphology in brain synaptosomes, myelination in the cingulate cortex using Black Gold II staining, or changes in astrocyte reactivity as assessed by anti-GFAP immunofluorescence staining. Using advanced imaging techniques and single-nucleus RNA sequencing, I demonstrate that doxorubicin-induced changes are associated with decreased microglial ramification and alterations in the microglia transcriptome that suggest a neurodegenerative microglia phenotype closely resembling stage 1 disease-associated microglia

(DAMs). HDAC6 inhibition completely reversed these doxorubicin-induced alterations, indicating a restoration of microglial homeostasis. These results suggest that a stage 1 DAM-like microglia phenotype and decreased postsynaptic integrity contribute to long-term CICD. Moreover, HDAC6 inhibition shows promise as an efficacious pharmaceutical intervention to alleviate CICD and improve quality of life of breast cancer survivors.

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## List of Abbreviations

**BBB:** blood-brain barrier

**CA1:** cornu ammonis 1

**CA3:** cornu ammonis 3

**CICD:** chemotherapy-induced cognitive dysfunction

**CNS:** central nervous system

**DAM:** disease-associated microglia

**DG:** dentate gyrus

**DOX:** doxorubicin

**GFAP:** glial fibrillary acidic protein

**HDAC6:** histone deacetylase 6

**IBA1:** ionized calcium binding adaptor molecule 1

**IL-10:** interleukin 10

**IP:** intraperitoneal

**MMTV-PyMT**: mouse mammary tumor virus-polyoma middle tumor-antigen

**NOPRT:** novel object/place recognition test

**OCR:** oxygen consumption rate

**PBS:** phosphate buffered saline

**PBT:** puzzle box test

**PSD95:** postsynaptic density protein 95

**ROI:** region of interest

**ROS:** reactive oxygen species

**TREM2:** triggering receptor expressed on myeloid cells 2

## Chapter 1: Introduction

Chemotherapy-induced cognitive dysfunction (CICD) is a debilitating impairment that severely effects the quality of life of up to 60% of breast cancer survivors [1]. Importantly, CICD has been shown to persist long after the cessation of chemotherapy. Studies on breast cancer survivors have identified deficits in learning and memory, attention, and processing speed 5 years after cancer treatment [2]. Currently, no therapeutic standard for treating CICD exists. While there is evidence that dysregulated neuroinflammation and oxidative stress in the brain as a result of microglia activity may contribute to CICD, the specific phenotype of microglia that sustain CICD long after the cessation of treatment is unknown.

I have developed a doxorubicin-induced model of CICD in female mice to identify mechanisms underlying long-term CICD and to evaluate a candidate pharmaceutical intervention, histone deacetylase 6 (HDAC6) inhibition, to reverse CICD after completion of chemotherapy treatment. In this introduction, I will first discuss breast cancer, chemotherapy treatment, and provide an overview of CICD. Secondly, I will provide an overview of doxorubicin including its mechanism of action, peripheral and central toxicities, and current literature regarding CICD as a result of doxorubicin treatment in animal models. Thirdly, I will discuss synaptic integrity including the functional synaptic unit, the two synaptic proteins evaluated in this dissertation (synaptophysin and postsynaptic density protein 95 (PSD95)), and synaptic alterations in neurodegenerative disorders. Fourthly, I will provide an overview of the microglia cell type including its physiological function, morphology, pathological phenotypes, and role in CICD. Lastly, I will discuss physiological roles of HDAC6, its relationship to neurodegeneration, and the effects of HDAC6 inhibition.

## 1.1 Breast Cancer and Chemotherapy

In this section, I will provide a brief overview of breast cancer and general chemotherapy treatments including peripheral and central side effects. Then, I will describe CICD including associated structural and functional changes in patients, candidate mechanisms, and candidate interventions.

### *1.1.1 Overview of breast cancer and chemotherapy*

Breast cancer is the most female common malignancy in both the developed and developing world. In 2020, it was estimated that 2.3 million women were newly diagnosed with breast cancer, and 685,000 women died with breast cancer listed as the cause of death [3]. Unfortunately, the global incidence of breast cancer is on the rise, with the annual incidence rate of breast cancer up 31% from 1980 to 2010 [4]. In the United States, 1 in 8 women will develop breast cancer in her life. Thankfully, the success of breast cancer treatments has increased the survival rate of breast cancer patients by 38% since 1989 [5]. Indeed, early-stage, non-metastatic breast cancer is curable in ~70-80% of patients. Breast cancer management may involve surgery, radiation therapy, and systemic therapies such as endocrine therapy, bone stabilizing agents, poly (ADP-ribose) polymerase inhibitors and chemotherapy. In 2015, 64.2% of women who were diagnosed with node positive breast cancer received multi-agent chemotherapy regimens including anthracyclines (doxorubicin), taxanes (paclitaxel), 5-fluorouracil, cyclophosphamide, and carboplatin [5]. Prior to 2005, anthracycline-based chemotherapy regimens were most common, with 70-80% of all breast cancer patients receiving anthracyclines, such as doxorubicin, during their treatment schedule [6]. After 2005, the use of taxane-based chemotherapy regimens sharply increased due to increasing concerns regarding anthracycline-induced toxicities including cardiotoxicity [6]. Nevertheless, anthracyclines still play an important role for patients with high-

risk diseases such as triple-negative breast cancer, HER2-positive breast cancer, or breast cancer that involves multiple lymph nodes. In these high-risk patients, data suggests that adjuvant anthracycline- and taxane-based chemotherapy is the most effect treatment regimen [7].

### *1.1.2 Side effects of chemotherapy*

In general, chemotherapy treatment is associated with side effects that affect the quality of life of cancer patients and survivors. While side effects from chemotherapy are treatment-specific, a 2017 study that evaluated side effects from individuals with breast, colorectal, or lung cancer who are undergoing chemotherapy identified that 86% of participants reported at least one side effect throughout the period of study including pain, rash, and dyspnea [8]. While the study focused on physiological side effects, psychological symptoms are also very common in cancer patients and survivors. For example, up to 25% of cancer patients experience depression, while 45% of cancer patients show anxiety during cancer treatment [9]. While the psychological response to a cancer diagnosis is a significant cause of distress, studies suggest that chemotherapy treatment itself may be associated with increased cancer patient anxiety [9]. In addition, chemotherapy-induced neurotoxicities such as neuropathy, fatigue, and cognitive dysfunction are common side effects that can persist for weeks, months and years even after patients have been declared cancer-free [10]. Importantly, severe symptom expression is associated with an increased risk of mortality [11]. While chemotherapy treatments each have unique mechanisms of action, common underlying mechanisms that may initiate and sustain chemotherapy-induced behavioral toxicities have been suggested [10]. For example, cancer-related fatigue has been associated with increased peripheral inflammation that may lead to neuroinflammation [12]. In addition, peripheral neuropathy, which is peripheral nerve damage that is characterized by numbness, tingling, temperature sensitivity, and pain, has been suggested to be partly mediated by

neuroinflammation. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), and IL-6 released by macrophages and Schwann cells at the dorsal root ganglia promote the development of peripheral neuropathy and induce neuroinflammation [13, 14]. In addition to increased neuroinflammation, increased reactive oxygen species (ROS) and altered neuronal mitochondrial function have been suggested to underlie both peripheral neuropathy and fatigue following cancer treatment [15, 16]. Finally, increased neuroinflammation, increased ROS, and altered mitochondrial function have been recognized as being associated with cognitive dysfunction following cancer treatment [17]. Although fatigue, neuropathy, and cognitive dysfunction are all associated with chemotherapy treatment, only fatigue and neuropathy are recognized by the American Society of Clinical Oncology's Survivorship Guidelines to assess and manage cancer treatment side effects [17]. Due to the increase in chemotherapy technologies, and subsequent increase in long-term cancer survivors, there is a great need to understand, manage, prevent, and reverse cognitive dysfunction that may occur following chemotherapy treatment.

### *1.1.3 Chemotherapy-induced cognitive dysfunction*

Chemotherapy-induced cognitive dysfunction (CICD) is characterized by deficits in working memory, processing speed, executive functioning, attention, language, psychomotor function, verbal learning and ability, and visual-spatial skills [1, 17]. Longitudinal studies with neuropsychological evaluation of breast cancer survivors who received standard dose chemotherapy including doxorubicin, cyclophosphamide, and 5-fluorouracil, identified that cognitive dysfunction was associated with chemotherapy treatment [1, 2, 18, 19]. Cognitive dysfunction was evaluated within the domains of attention, processing speed, learning and memory, executive function, and quality of life. One study showed that 1.6 months after the

completion of chemotherapy, 65% of survivors presented with cognitive dysfunction [1]. Of these, 38% showed decline in only 1 cognitive domain, 58% showed decline in 2 cognitive domains, and 4% showed decline in 3 cognitive domains. Additionally, the same survivors completed a late post-treatment evaluation 7.7 months after the completion of chemotherapy. In the late evaluation, 61% of patients demonstrated cognitive decline relative to their performance 1.6 months after the completion of chemotherapy. This suggests that CICD has the potential to be sustained, and even worsen, in the long-term. Indeed, research has found that CICD has been reported to persist up to 5 years after cessation of treatment in long-term breast cancer survivors [2]. As a result, CICD is a is a debilitating impairment that causes interpersonal, emotional, and financial burdens that significantly lower the quality of life of cancer survivors. Unfortunately, no intervention has been approved by the Food and Drug Administration to prevent or reverse CICD. Therefore, more research is required to evaluate the mechanisms that underlie CICD to allow for the development of interventions that target this debilitating side effect of chemotherapy.

### *1.1.4 Structural and functional changes in CICD from patient studies*

Although the mechanisms underlying the initiation and sustaining of CICD are generally unknown, several structural and functional outcomes of systemic chemotherapy treatment that influence the central nervous system (CNS) have been identified. Using brain imaging techniques, structural and functional changes in the brain following chemotherapy include a reduction in the volume of specific brain structures that are required for cognitive functioning such as the prefrontal cortex and hippocampus [20]. In addition, changes in the integrity of white matter, which contains myelinated axons required for efficient transmission of information through the CNS, have also been observed after chemotherapy treatment [20]. Using functional magnetic resonance imaging (fMRI), reduced activation in the prefrontal cortex during a working memory-

based task was observed in breast cancer survivors up to 12 months after treatment. Moreover, fMRI revealed that breast cancer survivors showed hypoactivation in the dorsolateral prefrontal cortex during an executive function task and in the parahippocampal gyrus in an episodic memory task 10 years after the completion of chemotherapy. The latter phenomenon was associated with decreased attentional processing and recognition memory compared to the control group [21]. This research suggests that chemotherapy treatment is associated with long-term cognitive dysfunction. Another neuroimaging study evaluating grey matter showed that breast cancer patients had broad grey matter volume reductions one month following chemotherapy, and one year following treatment alterations were still observed among the frontal and temporal regions. The reductions in grey matter were associated with decreased cognitive functioning and processing speed [22]. Other studies have suggested that alterations in cerebral blood flow are associated with cognitive function [23]. For instance, chemotherapy treatment in breast cancer patients was associated with increased cerebral perfusion and negatively correlated with cognitive performance [23]. Finally, fMRI research has uncovered the important role of the default mode network (DMN) in CICD [24]. The DMN is a neuroimaging biomarker of disease- and age-related cognitive decline. The DMN reflects the intrinsic “resting state” network that shows *higher* activity during rest and *lower* correlations with activity in task-dependent networks. Analysis of the DMN incorporates widespread brain function, reflecting the high connectivity and integrated functional activity that is required for cognitive output [24]. In breast cancer patients, reductions in the DMN functional connectivity was associated with cognitive dysfunction [25].

In addition to fMRI, Kesler *et al.*, used diffusion tensor imaging (DTI) and graph theory to model neurodegeneration as a result of chemotherapy treatment [26]. First, the connectome of breast cancer survivors and controls were measured. Then, neurodegeneration was simulated

based on network attack analysis revealing that breast cancer survivors had a lower brain network tolerance to attacks on modeled brain regions and modeled brain connections. The lower brain network tolerance was associated with a lower fractional anisotropy, which suggests a loss of myelin integrity, and was associated with cognitive impairment [26]. This research suggests that chemotherapy treatment causes a decrease in network efficiency in response to simulated neurodegeneration as a result of decreased white matter organization and connectivity that may underlie cognitive dysfunction after breast cancer treatment.

Collectively, these studies suggest that there are multiple mechanisms underlying chemotherapy-induced cognitive dysfunction. Although the structural and functional changes that underlie CICD have been suggested, the mechanisms that permit these changes are less known.

### *1.1.5 Animal models of CICD*

Animal models are valuable tools that allow researchers to evaluate mechanisms underlying CICD. Benefitting from experimental control and study design, animal models allow for investigation into chemotherapy-specific mechanisms that are not possible in human imaging studies due to the commonality of combination chemotherapy regimens. Considering that the large number of chemotherapeutic agents used to treat cancer in humans each have unique mechanisms of action that induce apoptosis in cancer cells, a diverse body of literature regarding animal models of CICD exist. From this research, mechanisms underlying CICD have been identified including alteration of blood supply, cerebrospinal fluid composition, histone acetylation, myelination, cellular and dendritic morphology, neurogenesis and gliogenesis, glucose metabolism, neurotransmitter release, and the induction of neuroinflammation and oxidative stress [27]. For potential mechanisms underlying CICD in doxorubicin-treated animal models, see *1.2.6*.

Researchers have designed behavioral tests to evaluate cognition in animal models including the novel object/place recognition test (NORPT), the Morris water maze, matching- and nonmatching-to-sample learning tasks, the Y-maze, and the T-maze which evaluate hippocampal-dependent cognitive functions including episodic, spatial, and temporal memory. In addition, the puzzle box test has been employed to evaluate CICD which evaluates hippocampal-dependent spatial and short-term memory, as well as prefrontal cortex-dependent executive function in the form of a problem solving task [28, 29]. These tests allow researchers to quantify the severity and type of cognitive deficits following chemotherapy that can later be correlated with biological alterations in tissues from the specimens.

Downsides of animal models exist, the most significant of which is dosing. Considering that rodent metabolism is different from human metabolism, the difference in pharmacokinetics between rodents and humans means data from animal models do not translate to humans one-to-one [30]. In addition, animal research often utilizes intraperitoneal (i.p.) injections while humans are treated intravenously. Regarding dosing, some studies utilize a large bolus dose of chemotherapy as opposed to a dosing regimen that is typical of human cancer treatment. In these studies, the data gathered may not accurately reflect the phenotype in humans that contributes to CICD. It is preferable to employ tumor models to define a therapeutic dosing schedule that reduces tumor growth in animal models. Researchers may also use behavioral tests to ensure the dose is not too large: The evaluation of locomotor activity, such as the open field test or total interaction time in NOPRT, ensures that behavioral deficits are not due to reduced exploratory behavior and/or fatigue. In addition, animal models are typically used to evaluate the induction of CICD and short-term CICD, while CICD in humans may last for years following completion of treatment. Therefore, mechanisms that permit sustained cognitive

dysfunction after chemotherapy are often neglected in animal research. Finally, comorbidities that may influence CICD in humans such as obesity, preexisting health conditions, preexisting cognitive dysfunction, and age are often omitted in animal research. Nevertheless, animal models are important tools to evaluate chemotherapy-specific mechanisms underlying CICD.

### *1.1.6 Candidate mechanisms underlying CICD*

Cognitive dysfunction as a result of chemotherapy treatment is case- and chemotherapeutic-specific. Therefore, candidate mechanisms that may underlie CICD as a result of a variety of chemotherapy regimens will be discussed generally. Candidate mechanisms that trigger the initiation of CICD include a loss of blood-brain barrier (BBB) integrity, hormonal alterations, white matter damage, changes in neurogenesis, DNA damage, oxidative stress, genetic susceptibility, and cytokine dysregulation [20]. Firstly, chemotherapy and cancer itself can compromise the BBB by inducing endothelial cell dysfunction that results in increased BBB permeability [31]. As a result, increased levels of chemotherapeutic agents can enter the brain to cause neuronal cell death and decreased neurogenesis. Secondly, reductions in testosterone and estrogen as a result of chemotherapy treatment have been shown to influence cognitive functioning, particularly working memory [20]. Research has suggested that testosterone and estrogen can be neuroprotective and act as an antioxidant. Therefore, hormonal changes as a result of chemotherapy treatment may play a role in CICD. Thirdly, DNA damage and oxidative stress may lead to CICD. DNA damage and apoptosis caused by chemotherapy lead to oxidative stress, and oxidative stress is a common cause of DNA damage in neuronal cells [32]. It is important to note that the brain is particularly susceptible to oxidative stress considering its high energetic requirements and oxygen consumption by mitochondria – more than 20% of total oxygen metabolism in humans [33]. High oxidative stress in the CNS may lead to misfolded proteins,

mitochondrial dysfunction, lipid peroxidation, altered neuronal signaling, and neuronal apoptosis [34]. Considering the lipid-enriched milieu of myelinated fibers that facilitate signal transduction in the CNS, white matter is highly susceptible to damage from increased ROS as a result of chemotherapy treatment. Loss of white matter integrity is frequently observed in breast cancer survivors who received chemotherapy [35]. Taken together, dysregulation of the prooxidant-antioxidant balance may contribute to CICD. Fourthly, variations in genetic susceptibility to CICD exist [20]. Research has elucidated a relationship between genetic variability and cognitive function with an emphasis on brain-derived neurotrophic factor (BDNF), catechol-O-methyltransferase (COMT), and dopamine receptor genes [36]. Variations in dopamine metabolism and degradation can modify executive function and memory mediated by the prefrontal cortex, and BDNF is required for neuronal survival, repair, dendritic growth, and long-term potentiation (LTP). A study also identified that breast cancer patients with the ε4 variant of the *APOE* gene had higher susceptibility to chemotherapy-related cognitive impairment and development of Alzheimer's disease [37]. Lastly, cytokine dysregulation is strongly associated with neurotoxicity and cognitive dysfunction in a variety of neurodegenerative disorders including multiple sclerosis, Alzheimer's disease, and Parkinson's disease [38]. Cytokines play an important role in physiological CNS function including neurotransmitter metabolism, glial cell and neuronal functioning, neuronal repair, and cell-to-cell signaling. Systemic chemotherapy treatment has the potential to influence cytokines in the CNS. This can occur by peripheral cytokines migrating across the BBB, by activation of endothelial and glial cells to stimulate cytokine production in the CNS, and by stimulation of the peripheral vagus nerve to release cytokines in the CNS [20, 39]. Increased cytokines in the CNS can cause DNA damage, oxidative stress, and maintain neuroinflammation in a feed-forward cycle. Since pro-inflammatory cytokine

release in the brain is mediated by the activation of glial cells, most notably astrocytes and microglia, it is highly likely that glial cell activity contributes to CICD. Taken together, a variety of chemotherapy treatment-induced mechanisms may play a role in the initiation and sustaining of CICD.

### *1.1.7 Candidate interventions for CICD*

Although no FDA-approved therapies to alleviate CICD exist, a variety of interventions have been proposed. They range from behavioral interference, dietary treatments, stem cell therapy, and pharmacological interventions [40]. In terms of behavioral interference, physical activity has been shown to improve functioning in Alzheimer's disease, depression, and aging [41]. Exercise has been shown to reduce oxidative stress, inflammation, and increase BDNF and hippocampal volume. Therefore, exercise has been proposed as an intervention to mitigate CICD [42]. Dietary treatments proposed to alleviate CICD include foods high in naturally occurring antioxidants, including rutin and curcumin. Antioxidants have been shown to reduce levels of pro-inflammatory cytokines and may help to normalize the prooxidant-antioxidant balance that is disturbed by chemotherapy treatment [34]. However, considering that the mechanism underlying tumor control of some chemotherapies is the production of free radicals, antioxidant interventions are controversial. Stem cell therapy has recently been shown to be effective in reversing CICD in animal models. In a mouse model of cyclophosphamide-induced cognitive dysfunction, stem cell transplantation in the hippocampus lead to a reduction in microglia activation and neuroinflammation, and subsequently resolved cognitive impairments [43]. Moreover, the action of stem cell therapy has been suggested to be mediated by mitochondrial transfer. In a model of cisplatin-induced cognitive dysfunction, intranasal administration of mitochondrial isolated from mesenchymal stem cells transferred to endogenous neural stem cells, reducing the neurotoxic

effects of cisplatin [44]. The same group showed evidence that a similar intranasal administration of mitochondria successfully reversed CICD [45]. Taken together, stem cell therapies have the potential to improve CICD in cancer survivors. Finally, pharmacological interventions represent a much more conventional approach to alleviating sickness behavior, including CICD. A variety of pharmaceutical interventions have been evaluated to treat CICD including antidepressants, anti-inflammatory medication, stimulants, lithium, N-methyl-D-aspartate (NMDA) receptor antagonists, phosphodiesterase inhibitors, and novel pharmaceutical compounds including $A_3$ adenosine receptor agonists and HDAC6 inhibitors. Antidepressants have been shown to improve CICD by restoring cytokine balance via the p38/MAPK/JNK pathway, increasing BDNF expression in neurons, and protecting against mitochondrial membrane damage [46]. NMDA receptor antagonists act on excitatory neurotransmitter signaling pathways, such as dopaminergic and glutamatergic pathways, to improve learning and memory function in the hippocampus [47]. Phosphodiesterase inhibitors are thought to act through cyclic adenosine monophosphate signaling to modulate downstream pathways such as glucose metabolism and microtubule dynamics [48]. Finally, novel pharmaceutical compounds are currently being evaluated for their effectiveness in alleviating CICD. One class of compounds are inhibitors of HDAC6 (see *1.5*).

## 1.2 Doxorubicin

Doxorubicin is one of the most effective chemotherapy treatments for breast cancer patients[7]. In this section, I will discuss the history and mechanism of action of doxorubicin in cancer treatment. Then, I will provide an overview of side effects as a result of doxorubicin treatment including peripheral and central toxicities. Lastly, I will discuss cognitive dysfunction as a result of doxorubicin treatment including potential mechanisms and interventions identified in animal studies.

### *1.2.1 History*

Doxorubicin belongs to the anthracycline class of chemotherapeutic agents, a class of drugs that are extracted from *Streptomyces peucetius* bacteria. Indeed, the first anthracycline was called daunorubicin and was initially isolated from a strain of *Streptomyces peucetius* bacteria in Italy in the early 1960s [49]. The bacteria were isolated from a soil sample taken near a 13$^{th}$-century castle called Castel del Monte, which currently appears on the Italian version of the one cent Euro coin and is a World Heritage Site. Shortly after, a group of French researchers discovered the same compound and together with the Italians they named it Daunorubicin. This is a portmanteau of *Dauni*, a pre-Roman tribe that occupied the area of Italy where the compound was discovered, and *rubis*, the French word for ruby that describes the color. Daunorubicin was successful in clinical trials for acute leukemia and lymphoma, but by 1967 it was discovered that daunorubicin could cause severe cardiotoxicity [50]. Therefore, researchers mutated a strain of *Streptomyces* to alter the structure of the compound and therefore the biological activity. They were successful, and a new red-colored chemical was isolated. It was initially named Adriamycin, after the Adriatic Sea, but is currently referred to as doxorubicin [51]. Doxorubicin differs from daunorubicin only by the addition of a single hydroxyl group, yet its activity against solid tumors is greatly improved. Still, severe dose-limiting side effects including cardiotoxicity remain. Doxorubicin is used to treat solid tumors and hematological cancers including breast, prostate, uterus, ovary, stomach, liver, and esophagus tumors as well as leukemias [52]. Although they were discovered more than 50 years ago, anthracyclines are still used in 32% of breast cancer patients, 57 to 70% of elderly lymphoma patients, and 50 to 60% of childhood cancer patients [7]. Currently, doxorubicin is on the World Health Organization's List of Essential Medicines.

### *1.2.2 Structure and mechanism of action*

Doxorubicin treatment leads to apoptosis of cells by 3 main mechanisms: 1) DNA intercalation, 2) topoisomerase II inhibition, and 3) the production of reactive oxygen species [52]. Doxorubicin enters a cell by simple diffusion and is subsequently transported to the nucleus. Briefly, doxorubicin binds to the proteasome with high affinity, forming a doxorubicin-proteasome complex that translocates to the nucleus via nuclear pores. Once in the nucleus, doxorubicin dissociates from the proteasome complex and binds to DNA due to a higher binding affinity for DNA than the proteasome. Doxorubicin intercalates between the adjacent base pairs of DNA, inhibiting RNA synthesis and subsequently blocking transcription, biosynthesis, and cellular replication [52]. In addition, doxorubicin inhibits the progression of topoisomerase II, which is responsible for relaxing supercoils in DNA transcription. After topoisomerase II has broken the DNA chain for replication, doxorubicin stabilizes the topoisomerase II-DNA complex. This prevents the DNA from being released and stops the process of replication [52]. Subsequently, the affected cell undergoes apoptosis. Finally, doxorubicin leads to the production of ROS that may contribute to cell death via two mechanisms. Firstly, the structure of doxorubicin itself is highly reactive and can produce free radicals via redox cycling of its quinone moiety (Figure 1.2). The quinone moiety is converted to a semiquinone moiety to produce free radicals that react with oxygen to create highly reactive superoxides, hydroxyl radicals, and peroxides [53]. Secondly, doxorubicin also has a high affinity for cardiolipin in the inner mitochondrial membrane, the binding of which leads to mitochondrial dysfunction and subsequent production of ROS that induce apoptosis [53].

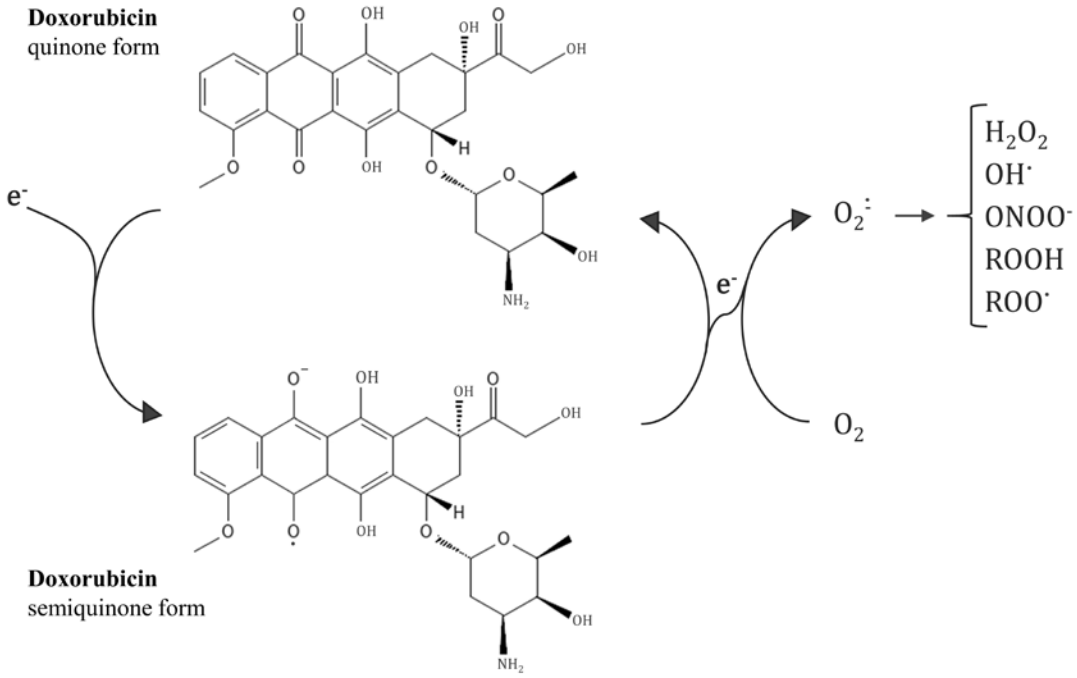



**Figure 1.1. Chemical structure of doxorubicin and redox cycling mechanism.**

### *1.2.3 Doxorubicin-induced peripheral toxicity*

Although doxorubicin is an extremely effective chemotherapeutic, its use is associated with severe side effects including cumulative cardiotoxicity, gastrointestinal disturbances, bone marrow aplasia, and inflammation[52]. The most well-documented of these side effects is cardiotoxicity. Indeed, cardiotoxicity has proven to be the most significant side effect that limits the dose and clinical use of anthracyclines. Risk is dependent on patient age, prior heart disease, and other cardiovascular risk factors that may increase the risk of heart failure as high as 3-5% with 400 mg/mg$^2$ of cumulative doxorubicin [54]. At the heart of the issue is the sensitivity of cardiomyocytes to ROS [55]. This is due in part to the high number of mitochondria per cardiomyocyte, occupying 50% of the cardiomyocyte mass due to the high energy requirements of the cell [53]. Heart tissue also has poor antioxidant activity because it lacks catalase [56]. In

order to reduce cardiac injury, liposomal formulations of doxorubicin (Doxil), which is doxorubicin encapsulated in phospholipid vesicles, have been developed as a drug delivery system. A side effect of these liposomal formulations is that they cross the BBB more easily via membrane fusion, causing behavioral side effects [57]. A second major side effect of doxorubicin treatment, which is common in most chemotherapeutic agents, is myelosuppression. Myelosuppression may increase the occurrence of infections, gingival bleeding, and delayed healing [52]. This is because chemotherapeutic agents target proliferative cells indiscriminately with the intention of causing apoptosis in cancer cells, but also affect normal adult stem cells in tissues such as bone marrow.

### *1.2.4 Doxorubicin-induced neurotoxicity*

Another significant concern of doxorubicin treatment, and the subject of this thesis, is doxorubicin-induced toxicities in the brain. Doxorubicin hydrochloride, which is the formulation primarily used to treat breast cancer, is generally thought to be blood-brain barrier impermeable [58]. While this is true in physiological conditions, doxorubicin in the blood interacts directly with endothelial cells that compose the BBB by making intercellular tight junctions that limit the passive diffusion of large molecules into the brain [59]. In the periphery, doxorubicin treatment and inflammation have been shown to cause endothelial cell dysfunction [60, 61]. In addition, pathological inflammation in the CNS is associated with increased BBB permeability and breakdown [62]. Thus, it is possible that doxorubicin may act on endothelial cells at the BBB, causing breakdown of the BBB and increasing permeability which may allow low amounts of doxorubicin to pass. Researchers detected doxorubicin hydrochloride at a level of 5 ng/g of fresh brain tissue in the cerebral hemispheres, cerebellum, and brainstem 1 hour after a 12 mg/kg intraperitoneal (i.p.) injection in rats [63]. Whether or not this level of doxorubicin is high enough

to induce neuronal apoptosis directly is not well known [63]. Additional research has provided evidence that doxorubicin is readily taken up by neural stem cells in the dentate gyrus (DG), which themselves have cellular processes associated with blood vessels [64]. These cellular processes are >30 nm in diameter and establish direct contact with endothelial cells in specialized niches in the hippocampus [64]. This uptake caused reduced NSC proliferation, which may help to explain anti-neurogenic effects of doxorubicin. While this research suggests that a direct mechanism may underlie doxorubicin-induced neurotoxicities, more research into the severity of this mechanism is required. The most well-accepted mechanism underlying doxorubicin-induced neurotoxicities is an indirect one that initiates in the periphery. Systemic doxorubicin treatment results in severe peripheral ROS production and inflammation in the form of circulating cytokines such as TNF-α [65]. Proinflammatory cytokines and activated immune cells are able to cross the BBB to interact with resident CNS immune cells, including microglia and astrocytes, to influence their reactivity and stimulate the production of additional proinflammatory cytokines [61, 65]. In aged mouse models, activation of the peripheral immune system induced neuroinflammation and oxidative stress in the brain [66]. Thus, the initiation of a feed-forward inflammatory cycle may begin in which microglia respond to pro-inflammatory cytokines by themselves becoming activated and producing additional pro-inflammatory cytokines. Microglia and astrocyte activation increases cellular energy requirements and the production of adenosine triphosphate (ATP) by mitochondria [61, 67]. Increased ATP production may then increase the presence of pathological ROS which can have detrimental effects by causing the oxidation of lipids, DNA, and proteins to influence cellular functions, protein conformations, and mitochondrial membranes [64]. ROS can also cause synaptic plasticity deficits and damage nucleic acids that can ultimately lead to

neurodegeneration, cellular apoptosis, and cognitive dysfunction [68]. Today, there is a clear association between doxorubicin treatment and cognitive dysfunction [1, 69].

### *1.2.5 Patient studies on doxorubicin-induced cognitive dysfunction*

In patient studies, evidence suggests that chemotherapy regimens that contain anthracyclines, including doxorubicin, have greater negative effects than regimens that do not include anthracyclines in regard to cognition and brain network connections [70]. However, it is difficult to parse the specific effects of doxorubicin treatment from human studies considering that patient-specific chemotherapy regimens often contain two or more chemotherapeutic drugs including doxorubicin, cyclophosphamide, paclitaxel, and fluorouracil. Therefore, the most compelling evidence regarding doxorubicin-specific mechanisms of CICD are uncovered with CICD animal models.

### *1.2.6 Pre-clinical models of doxorubicin-induced cognitive dysfunction*

Pre-clinical rodent models have been used to model doxorubicin-induced cognitive function (DICD). Through this research, a plethora of different mechanisms have been reported to initiate and sustain DICD. These mechanisms include neuroinflammation, oxidative stress and mitochondrial dysfunction, neurotransmitter alteration, glial cell dysfunction, DNA damage, and changes in apoptosis, autophagy, and neurogenesis [71]. Like many neurotoxic and neurodegenerative pathologies, the reality is that DICD is brain- and case-specific and likely the output of cognitive dysfunction is influenced by some or all of these mechanisms in an interrelated manner.

Although very little doxorubicin is thought to cross the BBB, doxorubicin in the blood can interact directly with endothelial cells at the BBB which act as active immunoregulatory cells to induce neuroinflammation [72]. However, doxorubicin has been shown to cross the

BBB via blood vessel-associated apical projections of neural stem cells in the DG of the hippocampus (see *1.2.4*) [64]. Therefore, direct mechanisms of neurotoxicity in DICD as assessed by *in vitro* experiments will briefly be discussed.

*In vitro* models have shown that doxorubicin can accumulate in cortical rat neurons and cause DNA double-strand breaks and DNA cross-linking, which leads to a decrease in neuronal survival [73]. More subtly, doxorubicin treatment led to damaged synapses and a decrease in neurite count indicated by decreased Synapsin fluorescence intensity [73]. In human neuroblastoma cells (IMR32), doxorubicin treatment decreased neurite outgrowth and increased intracellular ROS generation and apoptosis [74]. Doxorubicin treatment *in vitro* has also been shown to affect autophagy-lysosome systems [75]. Finally, in neurons exposed to doxorubicin, electron microscopy identified the accumulation of autophagosomes, lipid droplets, and mitochondria [75]. It is important to note, however, that the aforementioned *in vitro* experiments use doxorubicin doses that range from 0.01 – 1 μM. Primary cortical neurons treated with even the lowest dose of doxorubicin in this range (0.01 μM) resulted in a 50% reduction in synaptic density 2 days after treatment and death of the entire neuronal culture 7 days after treatment [73]. Therefore, the dose of doxorubicin exposed to neurons in these studies may be higher than doses that reach neurons after systemic treatment *in vivo*. Taken together, it is possible that minute amounts of doxorubicin passing through the BBB may influence neuronal survival, synaptic integrity, and neurite outgrowth which may lead to cognitive dysfunction. While *in vitro* studies on doxorubicin provide valuable information on the direct effects of doxorubicin on CNS cells, *in vivo* studies in rodents more accurately reflect the dosing schedule, presence of a BBB, and behavioral outputs that are required to accurately model DICD.

In rodent models, the wide-ranging consensus is that systemic doxorubicin treatment induces CNS neurotoxicity through indirect mechanisms that involve neuroinflammation and oxidative stress [52, 76–80]. Both neuroinflammation and oxidative stress are considered to be key factors that influence neurodegeneration and cognitive impairment, and indeed the two processes are tightly intertwined [81, 82]. For instance, *in vivo* studies have found that doxorubicin administration leads to the increase of both the pro-inflammatory cytokine TNF-α and oxidative stress in the brain [65, 83–86]. Increased oxidative stress is associated with increased reactive species including oxygen and hydroxyl free radicals as a result of mitochondrial dysfunction. Exposure to ROS can cause damage to macromolecules including lipids, proteins, and DNA to induce apoptotic cell death [33]. The brain has especially high energetic requirements, consumes large amounts of oxygen, and is enriched with lipids making the CNS highly vulnerable to oxidation-reduction imbalance [33, 67]. Indeed, neurodegenerative disorders including Huntington's disease, Parkinson's disease, and Alzheimer's disease are associated with oxidation-reduction imbalances [33, 34, 87]. Finally, the presence of excessive ROS in pathological brains is associated with decreased cognitive function [34]. Studies have identified an increase in oxidative stress following doxorubicin treatment as assessed by quantifying protein carbonyl levels and protein bound 4-hydroxynonenal (HNE) levels in both the hippocampus and plasma. Protein carbonyl levels and protein bound HNE levels are measures of protein oxidation and lipid oxidation, respectively [88]. Here, the increase in markers of oxidative stress was associated with decreased cognitive function in mice as assessed by the NOPRT [88]. In brain mitochondria, doxorubicin treatment was shown to decrease $Ca^{2+}$ retention capacity, and increase the permeability transition pore opening sensitivity and $H_2O_2$ emission *in vivo* [89]. A recent study highlighted that doxorubicin treatment induced altered mitochondrial dynamics as assessed by

membrane depolarization and mitochondrial swelling 5 days after the last dose of doxorubicin, and was associated with cognitive dysfunction [76]. This suggests that altered bioenergetics may be important for the initiation of CICD, but the role of mitochondrial dysfunction in sustaining long-term CICD is unknown [76]. In addition to direct analyses of mitochondrial function, doxorubicin treatment has been shown to decrease levels antioxidant enzymes glutathione and superoxide dismutase in the brain which suggests an increase in oxidative stress [77, 84]. This suggests a decreased capacity for the reduction of ROS in brains of doxorubicin-treated mice. The neurotoxic effects of oxidative stress following doxorubicin treatment have been further confirmed in studies that showed the concurrent application of molecules with antioxidant properties prevented protein oxidation, lipid peroxidation, and the loss cognitive function as a result of doxorubicin treatment [86, 88, 90, 91]. However, the clinical significance of antioxidant therapeutics in preventing CICD is controversial considering that one mechanism of action of doxorubicin treatment on cancer cells is DNA damage by free radical production. Therefore, concurrent application of an antioxidant therapeutic may interfere with the tumor control provided by doxorubicin treatment. Taken together, doxorubicin treatment has been shown to induce the generation of ROS by mitochondrial dysfunction and the loss of endogenous antioxidants that lead to subsequent protein, lipid, and DNA damage that contribute to doxorubicin-induced CNS damage and cognitive dysfunction.

In regards to neuroinflammation, Tangpong *et al.,* provided evidence that systemic bolus doxorubicin treatment induces TNF-α expression in the cortex and hippocampus that was associated with apoptosis in the brain as assessed by TUNEL+ staining [65, 92]. Later research, based on repeated dosing schedules to simulate to breast cancer treatment, confirmed that doxorubicin treatment was correlated with an increase in pro-inflammatory cytokines TNF-α, IL-

1β, and IL-6 in the brain [76–80]. In many of these studies, doxorubicin treatment led to cognitive dysfunction in the domains of spatial, temporal, and episodic memory as assessed by various behavioral tests including the NORPT, Morris water maze, and passive avoidance tests [76, 80, 90, 91]. Importantly, the release of pro-inflammatory cytokines in the hippocampus has been proposed to promote cognitive dysfunction [82]. The mechanisms underlying neuroinflammation and cognitive dysfunction are more easily understood when considering the cell types that mediate neuroinflammation. Pro-inflammatory cytokine release in the brain is mediated by the activation of glial cells, most notably astrocytes and microglia [93]. Briefly, astrocytes are important modulators of synaptic transmission and uptake neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA) at the synaptic cleft [94]. Astrocytes are also involved in energy homeostasis for neurons, storing glycogen that can be broken down to lactate and shuttled to neurons to facilitate neuronal oxidative phosphorylation [95]. Microglia are the primary immune cell in the brain, involved in brain maintenance in the form of phagocytosis of plaques or debris, synaptic maintenance, and responding to damage signals to facilitate repair (see *1.4*) [96]. In situations of pathological neuroinflammation, the dysregulated activation of astrocytes and microglia prevents these cells from performing their physiological functions. Indeed, reactive astrocytes exhibit abnormal regulation of glutamate transport, energy metabolism, and $Ca^{2+}$ responses leading to glutamate excitotoxicity, altered neuronal oxidative phosphorylation, and impaired synaptic plasticity [95, 97]. In addition, activated glial cells require higher energetic needs and further the oxidative stress on mitochondria, leading to additional ROS production and further CNS damage as discussed previously [67]. Finally, pathological activation of glial cells have been shown to induce enzymes that directly or indirectly phosphorylate tau in the brain, including NF-κβ and GSK-3β, leading to the development of, and association with, plaques,

clusters, and neurofibrillary tangles (NFTs) [98–100]. Indeed, a recent study showed that doxorubicin treatment increased astrocyte and microglia reactivity as assessed 5 days after the last dose of doxorubicin and was associated with cognitive dysfunction [76]. The cognitive deficits and glial cell reactivity were prevented by concurrent administration of donepezil, an Alzheimer's drug that binds reversibly to acetylcholinesterase to increase the availability of acetylcholine at the synapse and subsequent cholinergic transmission. This suggests that neuroinflammation mediated by reactive astrocytes and microglia play a role in short-term CICD. Another study identified broadly activated microglia in a model of doxorubicin-associated CICD 4 weeks after completion of treatment as assessed by CD68+ staining intensity and a NanoString mouse immunology panel [101]. Unfortunately, marker-based classifications may occlude the identification of more nuanced alternative microglia phenotypes that have recently been identified (see *1.4.6*). Doxorubicin has also been shown to alter acetylcholinesterase (AChE) activity in the hippocampus of rats which was associated with increased pro-inflammatory mediators TNF-α, PGE-2, COX-2, and cognitive dysfunction [102]. Considering that cholinergic neurons are involved in hippocampal-dependent learning and memory, and that dysregulated acetylcholine in the brain is associated with neurodegeneration, dysregulated AChE activity as a result of neuroinflammation likely contributes to CICD [103]. Taken together, the majority of animal research regarding CICD as a result of doxorubicin treatment consistently associate CICD with dysregulated neuroinflammation.

Lastly, pathological situations of neuroinflammation and oxidative stress can induce apoptosis. Indeed, repeated systemic dosing of doxorubicin has been shown to induce apoptosis in the CNS that correlated with cognitive dysfunction [102]. This was observed in several studies that quantified an increase in apoptotic markers including TUNEL+ staining and caspase-3 and

cytochrome c expression in the hippocampus *in vivo* following doxorubicin treatment [89, 102, 104]. These findings are substantial because neurons are post-mitotic cells, and the contribution of neurogenesis in reparative mechanisms in the adult CNS are not well understood. It is now known that neurogenesis occurs in the physiological brain throughout adulthood in specialized areas including the DG and subventricular zone [105]. Furthermore, evidence suggests that neurogenesis is altered following brain injury or neurodegeneration [106]. Still, more research is required to evaluate the extent to which neurogenesis contributes to CNS repair. In addition to inducing apoptosis, doxorubicin treatment was shown to decrease neurogenesis in the DG of the hippocampus as assessed by DCX+ and BrdU+ staining with concurrent impairments in short-term and spatial memory in the Morris water maze task [89]. A decrease in DCX+ cells in the hippocampus was recently shown to be correlated with decreased cognitive scores and functional interaction of synaptic proteins in humans [107]. Following doxorubicin treatment in rats, a decrease in DCX+ cells was associated with the decreased expression of neurotrophic factors BDNF and TrkB in the hippocampus. BDNF is a key molecule for synaptogenesis and dendritogenesis [108]. Doxorubicin was shown to decrease dendritic spine density and synaptic integrity as assessed by Golgi staining and PSD95 and Synapsin-1 immunofluorescence in the hippocampus, which was associated with cognitive dysfunction [80]. Here, an antioxidative protein called C-phycocyanin prevented cognitive dysfunction and synaptic damage as a result of doxorubicin treatment. These findings suggest that the induction of apoptosis and decrease in neurogenesis and synaptogenesis as a result of doxorubicin treatment likely contribute to CICD.

Taken together, increased neuroinflammation and oxidative stress are critical mechanisms underlying CICD in the brain of rodents treated with doxorubicin and may lead to synaptic damage and apoptosis. Neuroinflammation and oxidative stress are tightly interrelated

mechanistically and are primarily mediated by glial cells including astrocytes and microglia. Therefore, the identification of specific glial phenotypes that underlie CICD a result of doxorubicin treatment, as well as candidate pharmaceutical interventions that may attenuate neuroinflammation and oxidative stress, require further investigation.

## 1.3 Synaptic Integrity

Synaptic integrity is defined as a functional synaptic unit that facilitates unimpaired neuronal transmission [109]. In this section, I will provide an overview of the functional synaptic unit and the two synaptic proteins evaluated in this dissertation: synaptophysin and postsynaptic density protein 95 (PSD95). Then, I will discuss synaptic integrity in neurodegenerative disorders.

### *1.3.1 The functional synapse*

Functional synapses rely on the structural and functional integrity of both the presynaptic and postsynaptic boutons [110]. Functional synapses form the basis of synaptic transmission, the process by which neurotransmitters released from a presynaptic axon terminal bind to, and induce a change in, the appropriate receptor of a postsynaptic neuron. Modulation of synaptic strength, including long-term potentiation (LTP) and long-term depression (LTD), is a fundamental process that facilitates learning and memory [110]. In neurodegeneration, a loss of functional synapses strongly correlates with cognitive dysfunction [111, 112].

Briefly, presynaptic terminals contain clusters of synaptic vesicles packed with neurotransmitters. When an action potential occurs in the presynaptic neuron, voltage-gated calcium channels open to trigger $Ca^{2+}$-dependent exocytosis of synaptic vesicles and release of neurotransmitters into the synaptic cleft [110]. The neurotransmitters bind to and activate

postsynaptic receptors to induce a change in membrane potential in the postsynaptic neuron. In excitatory neurons, the postsynaptic terminal contains an electron-dense structure termed the post-synaptic density (PSD) which is made up of thousands of proteins including neurotransmitter receptors, scaffold proteins, cytoskeletal proteins, cell adhesion molecules, and membrane trafficking proteins that facilitate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and NMDA receptor anchoring, trafficking, and insertion into synapses [113]. Dysregulated PSD proteins is associated with neurodevelopmental disorders, neurodegeneration, and cognitive dysfunction [114].

In the literature, an accepted method of identifying functional synapses is by colocalization of presynaptic and postsynaptic proteins [115]. A loss of colocalization indicates a loss of functional synapses. However, research suggests that decreased expression of presynaptic or postsynaptic proteins also translates to a loss of functional synapses [116–120]. In this dissertation, I evaluated the expression of the presynaptic protein synaptophysin and the postsynaptic protein PSD95 as markers of functional pre- and postsynaptic terminals, respectively. Therefore, I will briefly discuss the roles of synaptophysin and PSD95 in synaptic transmission and their relationship to neurodegeneration.

### *1.3.2 Synaptophysin*

Synaptophysin is a synaptic vesicle glycoprotein that is localized to presynaptic vesicles [121]. It is one of the most well-characterized and specific markers for presynaptic terminals and is present in virtually all neurons in the brain that participate in synaptic transmission [122]. It participates in the docking and fusion of synaptic vesicles during exocytosis to facilitate the release of neurotransmitters into the synaptic cleft [123]. It is also required for endocytosis and synaptic vesicle recycling [120].

Evidence suggests that synaptophysin plays an integral role in maintaining functional synapses and synaptogenesis. In cultured hippocampal neurons, synaptophysin has been shown to regulate activity-dependent synapse formation [119]. It is one of the earliest synaptic proteins to be expressed at developing synapses *in vitro* and is expressed at high levels during synaptogenesis [124]. In Rett syndrome and transmissible spongiform encephalopathy, decreased synaptophysin expression correlated with dendritic spine pathology [125]. In addition, synaptophysin expression is significantly decreased in the hippocampus and temporal lobe of dementia patients [126, 127]. In cisplatin-induced models of CICD, decreased synaptophysin expression was correlated with cognitive dysfunction [28, 45, 128]. Synaptophysin expression was also significantly decreased in the hippocampus of patients with schizophrenia [129].

However, the research regarding synaptophysin expression and maintenance of a functional synapse is inconsistent. While there are clear associations with synaptophysin loss and neurodegeneration, studies have suggested that synaptophysin is not essential for neurotransmitter release [130]. One study identified impaired learning and memory in transgenic mice that lack synaptophysin, while another identified no phenotypic abnormalities [130, 131]. Research has suggested that redundancies between synaptic vesicle proteins may allow for functional synaptic transmission in transgenic mice that lack single synaptic vesicle proteins. One study identified redundancies between synaptophysin and synaptogyrin, another synaptic vesicle protein, that allow for unimpaired synaptic plasticity in synaptophysin or synaptogyrin knockout mice but not in synaptophysin and synaptogyrin double knockout mice [132]. Therefore, it is possible that studies identifying a loss of synaptophysin with a loss of synapse functionality failed to identify this redundant protein mechanism. Similarly, studies that

identified a loss of synaptophysin in neurodegenerative diseases may have failed to identify a concurrent loss of redundant proteins. However, a major study evaluating synapse loss in Alzheimer's disease patients confirmed that out of 7 synaptic proteins, synaptophysin was second most downregulated presynaptic protein behind rab 3A [127].

Taken together, synaptophysin is involved in functional synaptic transmission. Although redundancies exist that may compensate a loss of synaptic function as a result of decreased synaptophysin, synaptophysin loss still serves as a biomarker for neurodegeneration and is associated with cognitive decline.

### *1.3.3 PSD95*

PSD95 is a scaffolding protein that is expressed on the postsynaptic density of excitatory neurons. PSD95 is integral for maintaining cytoskeletal scaffolding to facilitate the clustering of receptors, ion channels, and associated signaling proteins. It is involved in the direct and indirect binding with NMDA receptors, glutamate receptors, AMPA receptors, and potassium ion channels to facilitate synaptic plasticity and long-term potentiation.

Evidence suggests that PSD95 is critical to maintain functional synapses. Through modulation of AMPA receptors at synapses, PSD95 alters neuronal excitability and synaptic strength to facilitate LTP and activity-dependent synaptic plasticity [133, 134]. Transgenic PSD95 knockout mice exhibit decreased AMPA-mediated excitatory postsynaptic currents (EPSCs) in the hippocampus [117, 135, 136]. Complementary to these findings, overexpression of PSD95 in the hippocampus increased AMPA-mediated EPSCs, occluded LTP, and enhanced LTD [137]. Interestingly, some studies have suggested that PSD95 is not required for the induction and early expression of LTP (35 minutes after pairing-induced LTP), but knockdown of PSD95 resulted in spines with a decreased density and altered morphology that were less

stable over longer time periods (3 days) [117]. Thus, it is possible that PSD95 may not be recruited during the induction of potentiation but is required for subsequent stabilization of synaptic changes and maintaining LTP. Nevertheless, synaptic plasticity is bidirectional and dependent on both LTP and LTD [138]. Considering that PSD95 knockout mice exhibit altered LTD, PSD95 expression is crucial for synaptic plasticity and therefore cognitive function [137]. This was validated in a PSD95 knockout mouse model which exhibited altered NMDA receptor and AMPA receptor expression in the prefrontal cortex which correlated with impaired performance in the NOPRT and T-maze task [116]. NMDA receptor-dependent LTD is a well-characterized mechanism that facilitates LTD [139]. Another study found that NMDA receptor expression was unaffected in the hippocampus of PSD95 knockout mice, and yet LTP and LTD were altered which correlated with a decreased performance in the Morris water maze [140]. Here, Migaud *et al.*, suggested that postsynaptic kinases and phosphatases downstream from NMDA receptors was modified in PSD95 mutants.

Research has also identified that PSD95 is required for Arc (activity-regulated cytoskeleton-associated protein) assembly into postsynaptic complexes and activity-dependent recruitment to excitatory synapses [141]. PSD95 knockout mice exhibit reduced Arc expression in the hippocampus [141]. Arc activation is required for synaptic plasticity and Arc dysregulation is associated with cognitive dysfunction [142].

Taken together, modulation of AMPA and NMDA receptors, alteration of kinases and phosphatases downstream of receptor activation, and loss of PSD95-Arc complexes may underlie cognitive dysfunction as a result of PSD95 loss. Regardless of the mechanism, research overwhelmingly suggests that a loss of PSD95 is associated with a loss of cognitive function [116, 118, 140, 143, 144].

### *1.3.4 Synaptic integrity and neurodegeneration*

A loss of synaptic integrity is a well-defined characteristic underlying cognitive dysfunction in neuropsychiatric diseases, neurodegeneration, and animal models of neuroinflammation [145, 146]. In Alzheimer's disease, synapse loss is a major correlate of cognitive dysfunction [147]. In Parkinson's disease, evidence indicates that the initial damage to dopaminergic neurons occurs at the synapse and contributes to cognitive dysfunction [148]. Decreased synaptophysin expression was identified in patients with schizophrenia [149]. Lastly, chemotherapy treatment in mice led to the decreased expression of synaptophysin and PSD95 that correlated with cognitive dysfunction [128].

Recently, the role of microglia in the loss of synaptic integrity during neurodegeneration has been explored. In aging and disease, evidence suggests the physiological role of microglia-mediated synapse removal becomes dysregulated leading to aberrant synapse elimination (see *1.4.2*). Microglia may also regulate synapse function indirectly through the release of soluble factors including BDNF [150]. Microglia activation has also been shown to enhance NMDA receptor activity in neurons via IFNγ binding and downstream signaling molecules [151]. As a result of increased NMDA receptor activity, increased glutamatergic signaling and $Ca^{2+}$ influx may stimulate the release of the chemokine CCL21 from neurons to further potentiate microglia activation [152]. One study showed that a NMDA receptor antagonist reduced lipopolysaccharide (LPS)-induced microglia activation and restored *Arc* expression in the cornu ammonis 3 (CA3) region of the hippocampus that correlated with restored performance in the Morris water maze [153]. Lastly, a loss of microglia homeostasis and perturbed neuro-glial support of synapses is associated with a synapse loss [154].

In view of the associations between the loss of synaptic integrity, neurodegeneration, and cognitive dysfunction, as well as the associations between microglia activity and synaptic integrity, this dissertation aims to elucidate potential neuro-glial mechanisms underlying cognitive function as a result of doxorubicin treatment.

## 1.4 Microglia

Microglia are the primary innate immune cells in the CNS and provide a variety of important functions related to overall brain maintenance. In this section, I will first describe the origin of microglia and the role of microglia in the physiological brain. Secondly, I will discuss the role of microglia in mediating neuroinflammation and brain repair. Thirdly, I will discuss the phenotype of activated microglia including its function, morphology, gene expression, and pathological consequences, as well as recently identified alternative microglia phenotypes. Finally, I will discuss the role of microglia in CICD.

### *1.4.1 History of microglia research and developmental overview*

Microglia are the primary innate immune cells in the CNS and comprise 10-15% of all cells in the brain. Once thought to be strictly regulators of the immune response, microglia are now recognized as multifunctional cells that interact with many cell types in the brain including astrocytes, oligodendrocytes, and neurons. Glia cells were first characterized by German pathologist Rudolf Virchow in 1856 to describe non-neuronal cells in the CNS such as astrocytes, oligodendrocytes, microglia, and more. It wasn't until 1919 that Pío del Río Hortega visually defined microglia as a distinct cell type. In the next 100 years and continuing today, the role of glial cells in development, immune surveillance, synaptic pruning, aging, and much more, was defined.

In rodent neonatal development, microglia originate from primitive macrophages, or erythromyeloid precursors, in the yolk sac and colonize the mesenchyme surrounding the neural tube using blood circulation around embryonic day 8 [155, 156]. At this stage, early microglia aid in axon guidance and phagocytize various products in the brain such as dying and dead cells, axons, and synaptic elements. Microglia rapidly proliferate in the CNS from embryonic day 8.5 to postnatal day 14, during which microglia promote cell genesis, myelination, and synaptic patterning to establish the neuronal architecture of the CNS [156]. At postnatal day 14, microglia numbers peak while regulation of synaptic patterning and promotion of myelination continues [157]. From P14 to P28, microglia either die back or migrate until a stable population is reached. In healthy brains, this microglia population will remain stable in most brain regions into adulthood while continuing to regulate synaptic formations, mediating the immune response in the brain, and maintaining homeostasis [157, 158]. Contributing to microglia's remarkable ability to surveil damage and respond to injury, microglia and newly-identified microglia subtypes adhere to strict spatial patterning and persist for long periods in the healthy adult brain: Studies reveal microglia have a proliferation rate of 1.3% in physiological conditions, have a median lifetime of well over 15 months in mice, and can be more than 20 years old in the human brain [159–163]. This long lifespan suggests that microglia-mediated CNS pathologies, such as pathological microglia phenotypes that influence neuroinflammation and oxidative stress, can persist long-term.

### *1.4.2 Microglia in the physiological brain*

Microglia have important functions in the CNS under physiological conditions. In healthy, intact brains, microglia play a role in the maintenance of synaptic structure via synaptic pruning wherein microglia engulf and remove superfluous synapses via phagocytosis [164]. Although synaptic pruning was once believed to be complete following sexual maturity, recent studies have

shown that synaptic pruning by microglia is necessary during synaptic plasticity to facilitate learning and memory in adults [164, 165]. In support of this, Appel *et al.* elegantly demonstrated that pathological phagocytosis of the postsynaptic protein PSD95 by microglia is associated with a decrease in dendritic spine density in the hippocampus of adult mice, and that this decrease was associated with impaired long-term memory [166]. As a result, dysregulated microglial homeostasis can have a negative impact on learning, memory, and general cognitive function. In addition to synaptic pruning, it has been suggested that microglia influence neuronal function and vice-versa: Microglia have been shown to respond to physiological neuronal activity to increase glutamate clearance in the synapse, suggesting a neuroprotective role against glutamate excitotoxicity [167]. Homeostatic microglia also participate in phagocytosis of cellular debris and apoptotic cells [168]. The non-inflammatory phagocytosis of apoptotic cells by microglia is called efferocytosis and is essential in maintaining CNS homeostasis [169]. Finally, microglia have been shown to promote neuronal survival and neurogenesis, and provide trophic support to neurons [164, 170]. The secretion of BDNF by microglia helps to facilitate learning-dependent synaptic plasticity [171]. In summary, microglia are necessary for maintaining neuronal function and CNS homeostasis under physiological conditions.

### *1.4.3 Physiological microglia activation*

The most well-known function of microglia is their regulation of neuroinflammation and promotion of repair following a variety of CNS pathologies including infection, injury, and disease. In the healthy adult brain, homeostatic microglia exist in a seemingly quiescent state but are constantly surveilling their surroundings with the highly motile extension and retraction of processes [168, 172]. Microglia surveillance is achieved by direct interaction with astrocytes, oligodendrocytes, and neurons as well as indirect diffusible factors including cytokines,

lipopolysaccharides, glutamate, extracellular vesicles, and changes in extracellular potassium and calcium [172, 173]. In response to injury and immunological stimuli, these diffusible factors can also indirectly influence signaling pathways including the phosphatidylinositol 3-kinase (PI3K)/Akt/ nuclear factor-κB (NF-κB) pathway to alter cell surface receptor expression and induce microglia activation (see *1.4.8*) [172, 174]. Microglia activation is also initiated directly through binding of pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs), and TLR4, among others [175, 176].

At the initiation of microglia activation, microglia undergo a graded transformation involving changes in gene expression, morphology, motility and migration, metabolism, secretome, phagocytosis, and cytokine release [177]. Contrary to earlier research that stereotyped this transformation as binary, where M1 microglia were described as activated and mediating the pro-inflammatory response while M2 microglia were believed to be responsible for resolution and repair, it is now known that microglia activation occurs as a continuum and is not “all-or-nothing” [177]. Indeed, microglia activation is variable and highly dependent on the type and severity of CNS pathology presented. In response to tissue damage, for example, microglia converge toward the site of CNS damage within minutes and transform to a reactive, ameboid form to phagocytose apoptotic neuronal cell bodies and cellular debris in addition to secreting growth factors and cytokines to modulate the extracellular matrix and ultimately promote repair [157]. In a less extreme example, even environmental and social stress has the capacity to mediate microglia activation: Restraint-induced stress was shown to alter microglia morphology and increase expression of the microglia activation marker CD11b in the hippocampus of Wistar rats, while mice exposed to repeated social defeat during adolescence presented a decrease in the number of microglia and an increase in microglia reactivity into adulthood [178–180]. Following repair and

clearance of the microglia activation signal, anti-inflammatory cytokines such as IL-10 suppress pro-inflammatory receptor expression to facilitate the resolution of microglia activation [181].

### *1.4.4 Microglia activation, inflammation, and ROS*

Although microglia activation is highly variable and pathology-dependent, activated microglia are typically associated with the increased production and secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-18 [172]. Importantly, these diffusible factors have the potential to influence the activation state of neighboring microglia and astrocytes to produce a cascade of pro-inflammatory signaling that results in a pro-inflammatory microenvironment [182]. In addition to an increase in cytokine release, microglia activation is closely associated with an increase in ROS production due to increased energetic requirements during migration, proliferation, and phagocytosis [183]. High levels are ROS are detrimental and can damage proteins, cell membranes, and DNA of nearby neurons. Excessive ROS production can also stimulate the production of pro-inflammatory cytokines by neighboring astrocytes and microglia to create a positive feedback system that further influences microglia activation and neuronal health [183]. Finally, a pro-inflammatory microenvironment and excessive ROS production have been shown to damage BBB integrity, increase BBB permeability, and allow for the entry of larger molecules, soluble factors, and peripheral immune cells [184].

### *1.4.5 Pathological microglia activation*

The evolutionary purpose of microglia activation is response to injury and promotion of repair. Indeed, acute microglia activation facilitates the clearance of debris, modulation of the extracellular matrix, and secretion of growth factors and cytokines which can promote remyelination, neuronal regeneration, and functional recovery after CNS injury [157, 185]. For example, in a cuprizone model of demyelination, microglia are involved in the phagocytosis of

myelin debris and apoptotic cells. Following phagocytosis, microglia help to activate and recruit oligodendrocyte precursor cells (OPCs) to the site of demyelination and offer trophic support [186]. In models of traumatic brain injury, microglia rapidly migrate toward DAMPs at the site of injury and clear cellular debris [176]. Following functional recovery, though, activated microglia have been shown to have detrimental effects on the CNS. Persistent microglia activation causes chronic low-grade inflammation that is associated with glial scarring, dysregulation of synapse formation, increased ROS production, decreased immune surveillance, suppression of neurogenesis, and cognitive impairment [185, 187, 188]. Persistent microglia activation is common in neurodegeneration and is indicative of a loss of homeostatic microglia function. In a mouse model of Alzheimer's disease, depletion of pathological microglia using CSF1R inhibition successfully reduced disease pathology and improved cognition[189]. In humans, persistent microglia activation was shown to predict the cognitive decline in the progression of Alzheimer's disease [190]. In multiple sclerosis patients, destruction of myelin in the CNS and subsequent cognitive impairment is associated with activated microglia and is considered a primary cause of disease pathogenesis [191]. Recent evidence also suggests that a variant of TREM2 expressed by microglia is associated with the progression of Parkinson's disease: Here, microglia activation correlates with Parkinson's disease progression, inducing dopaminergic neuron toxicity and death [192]. In the aging brain, primed microglia exist in an elevated inflammatory profile, exhibit a decreased surveillance capacity, exaggerated immune response, and a loss of homeostatic functions [193]. Indeed, aged mice injected with LPS exhibited an exacerbated inflammatory response and increase in sickness behavior compared to young mice [188]. Taken together, persistent microglia activation and concurrent loss of homeostatic functions can lead to detrimental outcomes regarding CNS homeostasis and cognitive function.

### *1.4.6 Alternative microglia phenotypes*

In addition to microglia activation, alternative microglia phenotypes have recently been identified in aging and neurodegenerative disorders that are characterized by heightened immune reactivity, altered phagocytosis and persistent activation [194–197]. These microglia phenotypes have been called “primed”, “dystrophic” or “disease-associated microglia” (DAMs) depending on disease models [196, 198, 199]. In general, these alternative microglia phenotypes are associated with neuroinflammation and neurodegeneration and reflect a loss of neuroprotective and reparative functions. They are characterized by an increased release of inflammatory cytokines, production of ROS, as well as alterations in phagocytosis, processes motility, and migration [200]. The prevalence of these alternative microglia phenotypes in aging is closely associated with microglia’s long life-span in the brain [195]. A consequence of these alternative microglia phenotypes is behavioral pathologies including stress, depression, and cognitive deficits [193]. This is, in part, related to the negative effects that long-term neuroinflammation has on neurogenesis, neural plasticity, LTP, and dendritic restructuring. These processes are required for the formation of memories and, therefore, cognitive function. Indeed, increased pro-inflammatory cytokines are associated with decreased neurotrophins, including BDNF, which negatively affects LTP [193]. Studies have shown that decreases in BDNF expression were observed in the cornu ammonis 1 (CA1) and CA3 in the hippocampus of aged rats [201]. Therefore, these alternative microglia phenotypes that are common in aging and neurodegenerative disorders may play a role in the initiation and sustaining of an exaggerated inflammatory profile in the CNS that leads to cognitive dysfunction.

The DAM phenotype was recently identified in a transgenic mouse model of Alzheimer’s disease aimed to evaluate microglia phenotype in the progression of the disease [199]. They

identified two stages of DAM activation that are characterized by distinct phenotypes. Stage 1 DAMs occur early microglia phenotype in the progression of Alzheimer's and are characterized by the TREM2-independent upregulation of *Apoe* expression without an increase in genes related to phagocytosis. The transition to stage 2 DAMs is TREM2-dependent and is characterized by an increase in phagocytosis genes thought to restrict the development of Alzheimer's disease.

### *1.4.7 Microglia morphology*

Microglia activation is closely associated with changes in microglia morphology. During normal physiological conditions, microglia display a hyper-ramified morphology with thin, highly branched processes that extend symmetrically from the cell soma [168]. In situations of CNS pathology, microglia become de-ramified in a stepwise manner toward an activated state characterized by shortened, bushy processes [202]. The result is a cellular morphology that exists on a continuum between a cell that can be described as less ramified with a swollen cell body and shorter, thick processes and a cell that can be described as rounded and ameboid with short or nonexistent processes [202]. In addition to canonical microglia activation, alternative microglia phenotypes observed in neurodegeneration and aging (primed microglia, senescent/dystrophic microglia and DAMs) are characterized by morphological features that overlap with those of canonical microglia activation [195]. Morphologically, these alternative microglia phenotypes are generally characterized by a deramified morphology with shorter projections and less branching, in addition to cellular hypertrophy, neuritic beading, and fragmented processes with a loss of fine branches [203]. Due to the morphological similarities between alternative microglia phenotypes and canonically activated microglia, distinguishing the two states based on morphology alone is difficult. Some researchers have even suggested that early studies on Alzheimer's disease have misidentified dystrophic microglia as active microglia prior to the phenotype being recognized

[198]. Indeed, much early research on microglia phenotypes used cellular morphology exclusively to identify microglia activation state [204]. Since morphology-based classifications may occlude the identification of more nuanced alternative microglia phenotypes, additional genetic or molecular signatures are required to accurately characterize and define the microglia phenotype.

### *1.4.8 Cell surface receptors in microglia activation*

In addition to morphological alterations, canonical microglia activation and alternative microglia phenotypes are characterized by changes in cell surface receptor expression. As a result, surface receptor expression is commonly used in conjunction with morphology to define microglia activation state [202]. Microglia homeostasis is sustained by the expression of inhibitory receptors such as C-X3-C motif chemokine receptor 1 ($CX_3CR1$), cluster of differentiation 200 receptor (CD200R), and signal regulatory protein α (SIRPα), in addition to purinergic receptors like purinergic receptor P2Y12 (P2RY12) [205]. The removal of these immune-suppressive signals induces microglia activation a loss of microglia homeostasis. Concurrently, an upregulation of surface receptors associated with the innate immune response such as major histocompatibility complex (MHC) and costimulatory molecules such as the cluster of differentiation molecule 11b (CD11b) promote microglia activation [183, 187].

Microglia are the only cells in the intact brain that express CD11b, however; following injury neutrophils and monocytes that also express CD11b may infiltrate the brain [206]. In order to differentiate infiltrating monocytes and microglia in applications like flow cytometry, cells require at least dual-staining such as CD11b (CD11b+ indicates infiltrating neutrophils and monocytes and resident microglia) and the common leukocyte antigen CD45 ($CD45^{low}$ identifies microglia while $CD45^{high}$ identifies invading monocytes) [207]. In contrast to MHC class II and CD11b activation markers, whose expression are dynamically regulated by microglia activation

and not expressed by quiescent microglia, ionized calcium-binding adapter molecule 1 (Iba1) is a microglia marker constitutively expressed on both quiescent and activated microglia and is well-suited for morphological characterization using immunohistochemical staining [202, 204]. Although it is not strictly an “activation marker”, Iba1 is involved in actin crosslinking which facilitates membrane ruffling in microglia. Since membrane ruffling is required for the morphological alterations following activation, alterations in Iba1 expression are associated with changes in microglia phenotype [208]. Although less described, senescent/dystrophic microglia also present a unique cell-surface expression profile characterized by changes in cell surface receptors, cell adhesions molecules, and genes involved in cytoskeletal dynamics [209]. In humans, aged microglia express decreased cell surface proteins including IL6R, IFNGR1, and TREML4 and increased CD163 and CXCR4 [209]. The most common method of identifying senescent microglia is an increase in $p16^{INK4a}$, p53, and senescence-associated β-galactosidase (SA-β-gal) expression associated with the senescence-associated secretory (SASP) phenotype characterized by increased secretion of interleukins, extracellular matrix components, chemokines, and metalloproteinases [210]. Regarding DAMs, the expression of TREM2 distinguishes the stage 1 and stage 2 phenotypes by absence and presence of TREM2, respectively [199]. Taken together, microglia cell surface markers help to identify the microglia phenotype in various CNS pathologies. However, the expression of cell surface markers may overlap between phenotypes and limit the characterization of nuanced alternative microglia phenotypes. The analysis of the microglia transcriptome, in addition to morphological and surface receptor expression, allows for accurate characterization of microglia phenotype.

### *1.4.9 Transcriptome of homeostatic and activated microglia*

RNA sequencing allows for analysis of disease-specific microglia gene expression. In tau transgenic mouse models of Alzheimer's disease, for example, genes involving the innate inflammatory pathways including NF-κB signaling, lysosome, phagosome, oxidative phosphorylation, and cytokine-cytokine receptor interactions were among the earliest upregulated [211]. Canonically activated microglia are characterized by a loss of microglia homeostasis genes *Tmem119, Mef2c, P2ry13, P2ry12, Siglech* and increase in classical pro-inflammatory genes *Ccl2, Tnf, Irf1, Gpr84*, and *Nfkbia* [212]. Analysis of microglia gene expression from a mouse demyelination model using lysolecithin injection revealed injury-responsive microglia with broad downregulation of the homeostatic microglia markers *P2ry12* and *Cx3cr1*. Importantly, variability in gene expression within these microglia indicated the presence of subpopulations defined by differential expression of proliferation markers, interferon response genes, and *Ccl4.* The significance of these findings is that microglia activation is a tailored, disease-specific response that is defined by both generalized and selective changes in gene expression [213]. Senescent/dystrophic microglia are characterized by increased expression of genes typically associated with senescence such as *Cdkn2a* (encodes $p16^{INK4a}$) and genes related to NF-κB signaling [210]. DAMs are characterized by the loss of microglia homeostasis genes such as *Cx3cr1*, *P2ry12*, *Tgfbr1*, *Slco2b1*, and *Tmem119* and the upregulation of neurodegeneration-associated genes such as *Apoe*, *Ttr*, *Ctsb*, *Cox6c*, *Clec7a*, *Itgax* and *Csf1* [199]. Stage 2 DAMs are distinguished from stage 1 DAMs by the upregulation of *Trem2* and genes related to phagocytosis including *Lpl* and *Cst7*. The DAM transcriptome is distinct from the transcriptome of classically activated microglia: After LPS-induced activation, only 12.1% of upregulated genes and 21.2% of downregulated genes were shared between DAMs and the LPS-activated microglia

[212]. Finally, primed microglia are characterized by similar genetic alterations including a loss of microglia homeostasis genes like *Slco2b1, Tgfbr1, P2ry12, Cx3cr1, Lrba,* and *Tmem119* and an upregulation of neurodegeneration-associated genes including *Apoe, Axl, Clec7a, Itgax, Lgals3, Gas7,* and *Cox6c*.

Evaluation of the microglia transcriptome is possible by single-cell RNA sequencing (scRNA-seq) as well as single-nuclear RNA sequencing (snRNA seq). scRNA-seq requires living microglia to be isolated from brain tissue that can subsequently be sequenced. snRNA-seq requires brain tissue to be immediately snap frozen, nuclei to be isolated, and subsequently sequenced. Both methods have their benefits and drawbacks. With scRNA-seq, the benefit is measurement of both cytoplasmic and nuclear transcripts, whereas snRNA-seq measures mainly nuclear transcripts and possibly transcripts attached to the rough endoplasmic reticulum [214]. However, the isolation of microglia requires extended incubations and processing that may induce isolation-based transcriptional artifacts. Microglia isolation often utilizes CD11b fluorescent tags or microbeads which has previously been shown to induce transcriptional artifacts and induce bias [215]. Furthermore, research suggests that snRNA-seq is sufficient to discriminate closely-related cell types [214]. Therefore, snRNA sequencing may be employed to avoid isolation-based transcriptional artifacts and identify nuanced microglia phenotypes.

Taken together, RNA sequencing of microglia allows for a deeper understanding of microglia phenotype in pathology compared to broad activation as defined by morphology and cell surface markers. To date, the changes in transcriptome that occur in microglia following chemotherapy treatment are not well known.

### *1.4.10 Microglia in chemotherapy-induced cognitive dysfunction*

The role that microglia play in the initiation and sustaining of chemotherapy-induced cognitive dysfunction (CICD) has been partly elucidated in recent years. Although the mechanism of action and resulting dysfunction is chemotherapy-specific, many chemotherapy treatments cause robust increases in both peripheral and central inflammation and production of ROS. These cytokines and ROS may compromise the integrity of the BBB, further increasing peripheral penetration of cytokines and chemotherapeutic drugs [216]. This, in turn, causes microglia activation which can then activate astrocytes, deplete OPCs, impair myelination, and lead to cognitive impairment. Indeed, in a methotrexate (MTX) model of CICD, a decrease in myelin sheath thickness and OPC proliferation was suggested to be mediated by microglia activation in the white matter of MTX-treated mice: Depletion of the activated microglia using the drug PLX5622 protected against MTX-induced demyelination and inhibition of OPC proliferation [217]. In rodents, doxorubicin treatment was shown to be correlated with an increase in pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in the hippocampus and prefrontal cortex immediately following treatment, suggesting microglia activation [78]. Importantly, the release of pro-inflammatory cytokines in the hippocampus has been proposed to mediate cognitive dysfunction [82, 218]. Indeed, repeated exposure to chemotherapeutic agents, which is standard in most cancer treatment schedules, can cause glial dysregulation and chronic neuroinflammation. As a consequence, altered synaptic pruning by dysregulated microglia can lead to disruptions in synaptic structure and function [219]. In support of this, previous research that found altered synaptic organization in the hippocampus of doxorubicin-treated mice [220]. Taken together, proposed microglia-mediated mechanisms underlying CICD are chemotherapy-specific but may

involve chronic neuroinflammation and production of ROS, impaired myelination, and synaptic dysregulation.

The specific microglia phenotype that underlies CICD as a result of doxorubicin treatment is not well known. In particular, the microglia phenotype that permits sustained, long-term CICD remains to be defined. Previously, Allen *et al.* showed that doxorubicin treatment caused microglia activation in the hippocampus of male mice 4 weeks after the final dose using CD68+ staining and a NanoString mouse immunology panel [101]. Although this methodology identifies broadly activated microglia, research suggests that the expression of CD68 varies between different stages of microglia activation and, like Iba1, is also expressed by resting microglia [221]. With the recent discovery of alternative microglia phenotypes such as primed, dystrophic and DAMs, further analysis of microglia following chemotherapy treatment is required. Although these microglia phenotypes are typically associated with aging and neurodegeneration, the latest research regarding CICD frequently draws comparison between CICD and aging and neurodegenerative brains [216]. Convergent mechanisms between CICD and neurodegeneration include increased inflammation, blood-brain barrier breakdown, reduced spines and dendrites, reduced neurogenesis and gliogenesis, altered white matter, and altered neurotransmitter balance [216]. Therefore, is possible that commonalities in microglia phenotype between aging/neurodegeneration and CICD exist.

## 1.5 HDAC6

Histone deacetylase 6 (HDAC6) is a unique member of the HDAC family in that it is localized in the cytoplasm and does not interact with histones directly. Recently, its role in neurodegeneration has been explored. In this section, I provide an overview of physiological HDAC6 structure and function. Next, I discuss the role of HDAC6 in cancer and

neurodegeneration. Finally, I discuss current research investigating HDAC6 inhibition as a pharmaceutical intervention for enhanced tumor control and neurodegenerative disorders.

### *1.5.1 Overview of HDAC6*

Classically, histone deacetylases (HDACs) are enzymes that remove the acetyl group from histone proteins on DNA, which in turn allows histones to wrap the DNA more tightly. As a consequence, transcription factors are less accessible and therefore transcription of tightly wrapped, condensed DNA segments is prevented [222]. This process is known as epigenetic modification and serves as a vital process that enables regulation of gene expression and phenotypic changes. Typically, levels of histone acetylation are regulated by histone acetyl transferases (HATs), which acetylate histone proteins, and HDACs. To date, 11 zinc-dependent HDAC isozymes have been identified and divided into four groups: class I, class IIa (nuclear), Class IIb (cytoplasmic) and Class IV. Class I contains HDACs 1-3 and 8; class IIa contains HDACs 4, 5, 7, and 9; class IIb contains HDACs 6 and 10; and class IV contains HDAC 11 [223]. Each HDAC varies in features ranging from subcellular localization to types of enzymatic activities. Of these, HDAC6 is a unique HDAC enzyme: HDAC6 is primarily localized in the cytoplasm of differentiated cells and does not interact directly with histones.

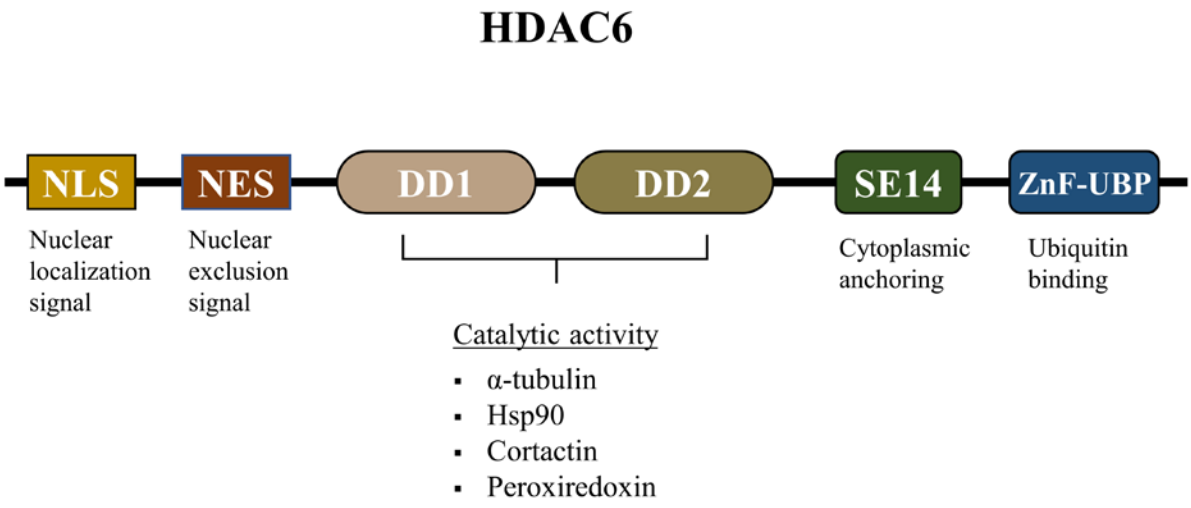



**Figure 1.2. HDAC6 functional domains.**

In addition to being primarily localized in the cytoplasm, HDAC6 is unique in that it is the only isoenzyme within the HDAC family that contains two tandem catalytic domains (DD1 and DD2, Figure 1.2) [224]. The deacetylase activity of HDAC6 is dependent on substrate interaction with *both* domains [225]. In addition, class II isoenzymes have both a nuclear localization signal (NLS, which is present on HDACs located primarily in the nucleus) and a nuclear export signal (NES, which is present on HDACs located primarily in the cytoplasm) [226]. The Ser-Glu-containing tetrapeptide (SE14) allows for anchorage in the cytoplasm in humans. Lastly, the ZnF-UBP domain facilitates high affinity ubiquitin binding.

The activity of HDAC6 is regulated by many post-translational modifications including kinases such as glycogen synthase kinase 3β (GSK3β), protein kinase C (PKC), extracellular signal-regulated kinase (ERK), epidermal growth factor receptor (EGFR), and others that lead to increased deacetylase activity [227]. Additional post-translational modifications include

ubiquitination and sumoylation which do not lead to degradation of HDAC6 like a typical ubiquitinated protein, or by acetylation which increases its deacetylase activity [227]. Although HDAC6 resides primarily in the cytoplasm of differentiated cells, in undifferentiated cells such as embryonic stem cells, neural stem cells, and in some cancer cells, HDAC6 resides in the nucleus. During differentiation, HDAC6 relocalizes into the cytoplasm due to the NES sequence [228]. Once in the cytoplasm, the NLS site is acetylated which inhibits the interaction of HDAC6 with a protein, importin-α, ensuring that HDAC6 remains in the cytoplasm. Within the HDAC6 promotor are binding sites for NF-κB, and the methylation status of its promotor determines the expression of *Hdac6*. Hypomethylation of the HDAC6 promotor, which typically correlates with a relaxed chromatin state and more transcriptionally active genes, causes overexpression of *Hdac6*.

### *1.5.2 Physiological roles of HDAC6*

HDAC6 plays important roles in regulating cellular polarity and shape, migration, cell division, directional movement of cells, protein degradation, aggresome formation, intracellular transport, and angiogenesis [224]. Due to its cytoplasmic localization, HDAC6 interacts with the cellular cytoskeleton to control cytoskeletal dynamics which consists mainly of microtubules (including α- and β-tubulin polymers), filamentous actins (F- and G-actin), and intermediate filaments (including fibrillar proteins). One of the most well-known substrates of HDAC6 is α-tubulin. Deacetylation of α-tubulin causes depolymerized microtubules. This modifies microtubule stability, causing an increase in microtubule dynamics that allows for a more flexible cytoskeleton, more cellular motility, and cell division [229]. In addition to cell division, microtube stability is important for guiding misfolded proteins to the microtubule-organizing center (MTOC) for degradation that is proteasome-independent. HDAC6 interacts with

polyubiquitinated proteins to facilitate retrograde transport toward the MTOC independently of its deacetylase activity. Once at the MTOC, the deacetylation of cortactin by HDAC6 then facilitates the formation of aggresomes around the misfolded protein [227]. The aggresomes then fuse with lysosomes and autophagosomes to degrade the misfolded proteins. In addition to proteasome-independent degradation, HDAC6 regulates autophagic degradation: HDAC6 deacetylates autophagy-related proteins and deacetylates LC3B-II, a key regulator of autophagy. HDAC6 is also involved in proteasome-dependent protein degradation [224]. Besides α-tubulin, another well-known substrate of HDAC6 is the chaperone heat shock protein 90α (HSP90). HSP90 and HDAC6 interact to allow proteins to fold properly and aid in the degradation of misfolded proteins in a proteasome-dependent manner [224]. Acetylation of HSP90 causes a loss of its chaperone activity and subsequent degradation of its client proteins. In addition to protein degradation, HSP90 is involved in the endocytosis and exocytosis of vesicles. This is accomplished through regulation of microtubule stability. One example is receptor internalization: When the EGFR receptor is bound to its ligand, it interacts with HDAC6 to inactive it by phosphorylation, leading to microtubule hyperacetylation and subsequent receptor internalization. HDAC6 is also involved in the remodeling of actin and formation of membrane ruffling to facilitate the formation and internalization of macropinosomes [227]. Macropinosomes are formed during micropinocytosis, a clathrin-independent endocytic mechanism, and eventually fuse with endosomes for degradation. Another biological process that HDAC6 is involved in is the regulation of apoptosis. The acetylation status of Ku70 is regulated by HDAC6 and controls its interaction with the proapoptotic protein BAX (Bcl-2-associated X protein) [227]. Acetylation of Ku70 causes decreased interaction with BAX, leading to apoptosis. Conversely, deacetylation of Ku70 by HDAC6 causes BAX to become sequestered and therefore inhibits apoptosis.

Importantly, HDAC6 is involved in the PI3K/AKT pathway, as well as the mitogen-activated protein kinase (MAPK)/ERK signaling pathway [227]. Inhibition of HDAC6 promotes AKT and ERK dephosphorylation which is associated with reduced cell proliferation and cancer cell death. One mechanism this is accomplished through is via HSP90 hyperacetylation, which leads to decreased phosphorylation of AKT and ERK levels. HDAC6 is also involved in antioxidant activity. Peroxiredoxin 1 and 2, antioxidant reducing enzymes, are substrates of HDAC6. HDAC6 inhibition increases the acetylation of peroxiredoxin, causing enhanced activity and subsequent decrease in oxidative stress. Although this may be beneficial in certain circumstances, peroxiredoxin 1 and 2 were found to be increased in many cancers and various neurodegenerative disorders and may confer resistance to chemotherapy [230]. In conclusion, HDAC6 is important for cell division, migration and angiogenesis, autophagic degradation, proteasome-dependent degradation, anti-apoptotic activity, PI3K/AKT – MAPK/ERK signaling pathways, and transcriptional repression in undifferentiated cells.

### *1.5.3 HDAC6 in cancer*

Dysregulated DNA methylation and histone modifications are common in cancer. As a result, HDAC inhibitors are currently being evaluated for enhanced tumor control in combination with chemotherapy. However, the role of HDAC6 in cancer is still being defined. For example, overexpression of HDAC6 has been associated with increased mobility, invasiveness of tumor cells, and increased tumorigenesis [224]. This is due to the role that HDAC6 plays in regulating microtubule stability via regulation of α-tubulin acetylation. Microtubules are also required for cell cycle division and therefore tumorigenesis. Indeed, increased HDAC6 expression has been correlated with increased tumor aggressiveness and advanced tumor stage in ovarian cancer, oral squamous cell carcinoma, and acute myeloid leukemia, among others [224]. At the same time,

higher expression levels of HDAC6 have been correlated with *better* survival prognosis in chronic lymphocytic leukemia, cutaneous T-cell lymphomas, and non-small-cell lung cancer. In this case, it has been hypothesized that loss of HDAC6 activity leads to decreased autophagy and decreased cell death [231]. Importantly, the expression of HDAC6 has been shown to be regulated by estrogen receptor α in breast cancer. In one study, overexpression of HDAC6 was correlated with small tumor size (less than 2 cm), better prognosis, and increased survival [224]. Conversely, HDAC6 overexpression was correlated with increased tumor invasion in MDA-MB-231 breast cancer cells. Therefore, more research into the role of HDAC6 expression in specific types and sub-types of cancer is required. Nevertheless, HDAC6 inhibitors in combination with chemotherapy are currently being evaluated in clinical trials NCT01997840, NCT02787369, and NCT01583283 for enhanced tumor control.

### *1.5.4 HDAC6 in neurodegenerative diseases*

Protein aggregates, dysregulated autophagy, neuroimmune dysfunction, dysregulated protein and mitochondrial transport, and neuronal oxidative stress are common underlying features in many neurodegenerative diseases. In healthy neurons, HDAC6 is involved in regulating the acetylation state of non-histone proteins that are linked to these features. In pathological conditions, dysregulated acetylation of non-histone proteins downstream of HDAC6 activity can lead to altered autophagy, protein transport and degradation, and synaptic function to cause neurodegeneration and cognitive dysfunction [232].

For example, HDAC6's ubiquitin binding domain facilitates misfolded protein binding, the formation of aggresomes, autophagy, and induction of heat shock proteins. In neurodegenerative disorders like Alzheimer's disease and Parkinson's disease, an accumulation of protein aggregates inhibits the ubiquitin-proteasome system and further protein degradation.

As a result, there is an increase in highly ubiquitinated misfolded proteins. In these neurodegenerative disorders, HDAC6 expression may worsen disease pathology. Mechanistically, this occurs because HDAC6 deacetylates HSP90, and deacetylated HSP90 increases its chaperone activity and the formation of additional proteins [233]. When HDAC6 is inhibited, HSP90 is hyperacetylated which inhibits its chaperone activity and leads to protein degradation.

In addition, increased oxidative stress causes damages to lipids, proteins, and DNA that can lead to mitochondrial dysfunction, excitotoxicity, and cell death. In neurons, HDAC6 is involved in deacetylating peroxiredoxin 1 and 2 enzymes that reduce peroxides. Increased acetylation of peroxiredoxin is associated with an increase in its activity, leading to a decrease in oxidative stress. Therefore, HDAC6 inhibition was shown to decrease oxidative stress in neurons [234].

Dysfunctional protein and mitochondrial transport are also common motifs in neurodegenerative diseases. HDAC6 has been shown to play a role in this mitochondrial transport: Miro1 is an HDAC6 substrate that links mitochondria with motor proteins to facilitate axon transport. Acetylation of its lysine 105 residue increases mitochondrial axonal transport. It was found that HDAC6 inhibition with Tubastatin A increased acetylation of lysine 105 and subsequently increased mitochondrial transport in hippocampal neurons [235]. In a model of cisplatin-induced cognitive dysfunction, HDAC6 inhibition was found to increase mitochondrial transport and subsequently restore cognitive function and reverse mechanical allodynia [28, 236]. In addition, HDAC6 interacts with the microtubule protein α-tubulin to regulates its acetylation, and the microtubule-associated protein tau to regulate its phosphorylation [28]. In Alzheimer's disease, hyperphosphorylated tau reduces its binding to microtubules, which causes disrupted

microtubule assembly and decreased axonal transport. Inhibition of HDAC6 increases α-tubulin acetylation, allowing for stabilization of microtubules and increased axonal transport [28]. In addition, tau hyperphosphorylation leads to β-amyloid (Aβ) and neurofibrillary tangles. In fact, one study found that the expression of HDAC6 protein in the hippocampus of Alzheimer's patients was increased by 91% [234]. Finally, HDAC6 inhibition improved learning, memory, and proper acetylation of α-tubulin in a mouse model of Alzheimer's disease [237].

HDAC6 has also been suggested to impact neurotransmission, memory regulation, and cognition at the level of synapses. In fruit flies, HDAC6 deacetylates Bruchpilot which enhances release of neurotransmitters. Mammalian equivalents of Bruchpilot, including CAST (cytomatrix at the active zone-associated structural protein)/ELK proteins, regulate short-term plasticity in the hippocampus. It is possible that altered acetylation levels of these synaptic proteins would similarly alter synaptic transmission in mammals, as was observed in flies [232]. In addition to neurotransmitter release, HDAC6 likely influences cognition/memory formation through regulation of cytoskeletal proteins and inflammatory microenvironments. For example, HDAC6 interacts with β-catenin, and β-catenin binds to PDZ (PSD-95/Discs Large/ZO-1) domains largely known for their scaffolding functions. Inhibition of HDAC6 has been shown to increase acetylation of β-catenin and its subsequent association with the PDZ domain to facilitate signaling, scaffolding functions, and synaptogenesis [238]. In addition, acetylation homeostasis is thought to be an important regulator of immune cell activation, including microglia. Indeed, HDAC6 inhibition was proven to be effective in decreasing innate and adaptive immune responses in a model of systemic lupus erythematosus, and neuroprotective in experimental models of multiple sclerosis [239, 240]. Also, studies have noted that HDAC6 activity is required for LPS-induced macrophage activation, and that HDAC6 inhibition prevented microglia

activation and dopaminergic neuronal degeneration in a mouse model of Parkinson's disease [241, 242]. Taken together, the role HDAC6 plays in neurotransmitter release, protein and mitochondrial transport, protein degradation, the modification of synaptic cytoskeletal elements, and immune cell activation help to explain the role HDAC6 plays in neurotransmission and cognition.

### *1.5.5 HDAC6 inhibition*

HDAC6 inhibitors have been shown to be beneficial in animal models of cancer and neurodegenerative disorders. HDAC6 inhibitors are largely within the chemical class of hydroxymates, although benzamides and other compounds have been shown to inhibit HDAC6 [227]. Well-known HDAC6 inhibitors include tubastatin A, tubacin, and ricolinostat. Initially, HDAC6 inhibitors were solely tested on cancer cell lines where they induced α-tubulin acetylation, antiproliferative activity, and promoted the death of cancer cells. *In vivo*, HDAC6 inhibitions shows anti-tumor activity in various cancer models including breast cancer [224]. Ricolinostat (ACY-1215), an HDAC6 inhibitor, is currently being tested in multiple clinical trials to evaluate its enhanced tumor control in combination with chemotherapy (NCT01997840, NCT02787369, and NCT01583283). In addition to cancer, HDAC6 inhibitors have shown promise in pre-clinical models of Alzheimer's, multiple sclerosis, and rheumatoid arthritis [237, 239, 243]. In Alzheimer's models, HDAC6 inhibition decreased tau phosphorylation and improved cognition [237, 244]. *In vitro*, HDAC6 inhibition blocked the centrosomal recruitment of parkin, as well as the formation of aggresomes-like bodies that interfere with autophagy [245, 246]. In addition, HDAC6 inhibition compensated for a transport deficit by mediating α-tubulin acetylation and offering neuroprotection in primary cortical neurons from a Huntington's disease mouse model [247]. HDAC6 inhibition has also offered improvements in mitochondrial

movement and neuroprotection from oxidative stress in neurons [230, 235]. In a model of multiple sclerosis, HDAC6 inhibition improved cognitive function [239]. In mouse models of Parkinson's, HDAC6 inhibition attenuated microglia activation and protected dopaminergic neurons [241]. In addition to neurodegenerative disorders, HDAC6 inhibition was shown to improve cognitive function in an anesthetic-induced model of cognitive dysfunction. Here, tubastatin A reversed decreases in synaptophysin and PSD95 expression [248]. Finally, it was recently demonstrated that ACY-1083, a brain-barrier permeable HDAC6 inhibitor, successfully reversed cognitive dysfunction in a cisplatin-induced model of CICD by reversing deficits in synaptosomal mitochondrial bioenergetics [28]. Considering the varying mechanisms of specific chemotherapeutic agents, and therefore the varying mechanisms underlying chemotherapy-induced side effects such as cognitive dysfunction, the possibility of HDAC6 inhibition to alleviate side effects of chemotherapy-specific models should be evaluated. One such group of chemotherapeutic agents are anthracyclines, including doxorubicin.

## Chapter 2: Materials and Methods

*Reproduced with permission from* McAlpin, B., Mahalingam, R., Sing, A.K., Dharmaraj, S., Chrisikos, T.T., Boukelmoune, N., Kavelaars, A., and Heijnen, C.J. (2021) HDAC6 inhibition reverses long-term doxorubicin-induced cognitive dysfunction by restoring microglia homeostasis and synaptic integrity. *Theranostics* 2022; 12(2):603-619. doi:10.7150/thno.67410.

### 2.1 Mice

Female C57BL/6 J mice (aged 12 weeks at the start of the experiment) were used exclusively due to the low occurrence of breast cancer in males (less than 1%) [5]. Mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in The University of Texas MD Anderson Cancer Center animal facility on a reversed 12 h light/dark cycle, with free access to food and water. Animals were randomly assigned to treatment groups. All procedures were consistent with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of M.D. Anderson Cancer Center. Analyses were performed by investigators blinded to treatment.

### 2.2 Orthotopic Mammary Tumor Model

MMTV-PyMT breast cancer cells were generously provided by Dr. Stephanie Watowich. MMTV-PyMT cells are a murine breast cancer cell line derived from the genetic MMTV-Polyoma Middle T (PyMT) mammary tumor mouse model on the C57BL/6 background, as previously described [249]. Briefly, MMTV-PyMT cells were cultured in Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were washed three times with PBS and resuspended in endotoxin-free PBS prior to injection into mice. Ten to twelve-week old female C57BL/6 mice received a unilateral injection of 2.5 × 105 MMTV-PyMT cells in the 4th mammary fat pad. Doxorubicin

hydrochloride (5 mg/kg/week, Pfizer, New York, NY) or PBS was administered intraperitoneally beginning 4 weeks after injection with MMTV-PyMT cells. Doxorubicin treatment occurred weekly for 4 weeks, for a total of 20 mg/kg. Tumor length and width were measured biweekly with electronic calipers. Mice were euthanized when tumors reached 15 mm in any direction or when ulceration >2 mm occurred.

### 2.3 Drug Administration

Mice were treated with doxorubicin hydrochloride (5 mg/kg/week, Pfizer, New York, NY) or PBS intraperitoneally for 4 weeks, followed by 1 week of rest. Mice were then treated with the blood-brain barrier permeable HDAC6 inhibitor ACY-1083 (10 mg/kg/day, Regenacy Pharmaceuticals, Waltham, MA) or vehicle intraperitoneally daily for 2 weeks. ACY-1083 was dissolved in 20% 2-hydroxypropyl-B-cyclodextrin (Sigma-Aldrich, St. Louis, MO) + 0.5% hydroxypropyl methylcellulose (Spectrum Chemical, Gardena, CA) in milliQ water.

### 2.4 Behavioral Testing

Cognitive function in female mice (n = 8-16 mice/group) was evaluated using the puzzle box test (PBT) and novel object/place recognition tests (NOPRT) [29, 250]. The tests were performed starting 4 weeks after the final dose of ACY-1083 in order to allow for rest, habituation and to evaluate long-term cognitive function. Behavioral data obtained is used with permission from Dr. Anand K Singh, Ph.D. who performed the behavioral experiments.

The PBT was performed as previously described with slight modifications [29]. The testing area consisted of a white box divided into two compartments by a black barrier: a brightly lit start zone (58 cm × 28 cm) and a smaller, dark goal box (15 cm × 28 cm) was connected by a 4 cm-wide tunnel. The test consisted of 11 trials at 3 levels of difficulty over the course of 4 consecutive days. In the first easy level of difficulty (training, days 1-2 and trials 1-4) mice were introduced

to the brightly lit start box with a connecting tunnel to the dark goal box. In the intermediate level of difficulty (training, days 2-3 and trials 5-7), the tunnel was obstructed with bedding that the mice must burrow through to reach the goal box. In the difficult trials (testing, days 3-4 and trials 8-11), the tunnel was blocked by a cardboard plug that the mice must manipulate to complete the task. Each trial began when the mouse was placed in the start box, and the duration of time it took for the mouse to reach the goal box was recorded with a maximum of 4 minutes during the testing phase. This task utilizes both spatial and short-term working memory regarding the position of the tunnel and recognition of the plug, as well as complex problem-solving in order to remove the plug using their teeth and front paws.

The NOPRT evaluates spatial working memory and was performed as previously described [250]. A testing arena (46.99 cm × 25.4 cm) was set up with two identical objects placed on the same side of the arena. In the training phase, mice were placed in the testing arena for 5 minutes and then returned to their home cage. Thirty minutes later, mice were transferred back to the arena that now contains one familiar object in the same location, and one novel object placed on the opposite end of the arena for the testing phase. Investigative behavior was defined as nose point within 1 cm of the object. The time (T) of investigative behavior toward either object during the 5 min testing phase was evaluated using EthoVision XT 10.1 video tracking software (Noldus Information Technology Inc., Leesburg, VA). Discrimination index was determined as (TNovel − TFamiliar)/(TNovel + TFamiliar).

### 2.5 Immunofluorescence

Mice were euthanized 6 weeks after the last dose of ACY-1083 by CO2 euthanasia and perfused intracardially with ice-cold PBS. The skull was removed to allow access to the brain. Brains were removed and transferred to 4% paraformaldehyde. Brains were post-fixed in 4%

paraformaldehyde for 24 h, cryoprotected in sucrose, and frozen in optimal cutting temperature compound (Sakura Finetek, Torrance, CA). Coronal brain sections (8 or 20 μm) were blocked with blocking buffer (10% normal goat serum, 2% bovine serum albumin and 0.1% saponin in PBS), followed by incubation with rabbit anti-synaptophysin (1:1000, MilliporeSigma (AB9272), Burlington, MA, n = 7-9 mice/group), rabbit anti-PSD95 (1:1000; Abcam (ab18258) Cambridge, UK, n = 9-14 mice/group), rabbit anti-GFAP (K39) (1:1000; OriGene (AP32987SU-N), Rockville, MD, n = 4 mice/group) or rabbit anti-Iba1 (1:1000; Wako (019-19741), Richmond, VA, n = 5-10 mice/group for 20 μm sections and n = 4 mice/group for 8 μm sections) diluted in antibody buffer (2% normal goat serum, 2% bovine serum albumin and 0.1% saponin in PBS) at 4 °C overnight. Slides were then washed three times with PBS, followed by incubation with Alexa-488 goat anti-rabbit (1:500; Invitrogen (A-21206), Carlsbad, CA) or Alexa-647 goat anti-rabbit (1:500; Invitrogen (A-21245)) at room temperature for 2 h. For negative control sections, primary antibody was omitted. After antibody staining, slides were washed three times with PBS, followed by incubation with DAPI (1:5000; Sigma-Aldrich) for 5 min. Slides were then washed three times with PBS, and sealed with FluorSave Reagent (MilliporeSigma). Regions of interest (ROIs) in the hippocampal CA1 and CA3 regions were imaged using a 40x objective with Nikon A1R Confocal Microscope (Nikon Instruments Inc., Melville, NY, USA). The mean fluorescence intensity in each ROI was quantified using Nikon NIS-Elements Advanced Research (Nikon Instruments Inc.).

### 2.6 Synaptosome Isolation and Mitochondrial Bioenergetics Analysis

Synaptosomes were isolated as previously described [28]. Briefly, one hemisphere of the brain (n = 9 mice/group) was homogenized (10% w/v) into 0.32 M sucrose solution in HEPES buffer using a glass Dounce homogenizer. The lysate was centrifuged at 1000 ×g for 10 min at 4

°C. The supernatant was mixed with equal volume of 1.3 M sucrose in HEPES buffer and centrifuged at 20,000 ×g for 30 min at 4°C. The synaptosomal pellet was then resuspended in XF media (Agilent Technologies, Santa Clara, CA) supplemented with 5.5 mM glucose, 0.5 mM sodium pyruvate, and 1 mM glutamine. Oxygen consumption rate (OCR) was measured with an XF24 Flux Analyzer (Agilent Technologies). Oligomycin (6 μM), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 6 μM), and rotenone/antimycin A (2 μM each) (Sigma-Aldrich) were injected sequentially during the assay. An assay cycle of 2 min mix, 2 min wait, and 2 min measure was repeated three times for baseline rates and after each port injection. Basal respiration, ATP-linked respiration, maximal and spare respiratory capacity were determined as previously described [28].

### 2.7 Electron Microscopy

For transmission electron microscopy, synaptosomes were fixed in 2% glutaraldehyde + 2% paraformaldehyde in PBS for over 24 h. Samples were then processed as previously described [18]. The samples were polymerized in a 60 °C oven for approximately 3 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica Microsystems, Wetzlar, Germany), stained with uranyl acetate and lead citrate in a Leica EM Stainer (Leica Microsystems), and examined in a JEM 1010 transmission electron microscope (JEOL USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA). In total, 26–34 mitochondria were quantified from each group (4 mice/group, with 5 images/mouse). Atypical mitochondria were identified by 2-fold increases in diameter and/or excessive vacuolization (more than 50% translucent). The percentage of atypical mitochondria was calculated for each group.

## 2.8 Black Gold II Staining

Black gold staining was performed as previous described with slight modifications [251]. Mice were euthanized 6 weeks after the last dose of ACY-1083 by CO2 euthanasia and perfused intracardially with ice-cold PBS. The skull was removed to allow access to the brain. Brains were removed and transferred to 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for 24 h, cryoprotected in sucrose, and cut at 25 μm in the coronal plane on a sliding microtome. For each animal, 4 sections were used for Black Gold II (Millipore, #AG105) staining according to manufacturer's instructions. Briefly, 25 μm sections were mounted onto glass slides and dried at room temperature overnight. The following day, the slides were rehydrated in $ddH_2O$ before immersion in Black Gold II solution at 60 °C for 15 min. After washing in ddH2O, slides were incubated in pre-warmed 1% sodium thiosulfate solution at 60 °C for 3 min. Slides were then rinsed with $ddH_2O$ and dehydrated through a series of ethanol and xylene and sealed with Permount (Thermo Fisher Scientific). Bright field images were taken using the EVOS® FL Auto microscope and percent area was quantified using ImageJ.

## 2.9 Microglia Modeling

Hippocampal microglia were visualized by means of immunohistochemical staining of 20 μm-thick coronal brain sections with rabbit anti-Iba1 (1:1000; Wako (019-19741)) as described above. Z-stack images of the hippocampus were acquired with the Nikon A1R Confocal Microscope (Nikon Instruments Inc.) at 1 μm slice intervals at 40x objective. Images were transformed into 3D models using the image analysis software Imaris 9.0 using the filament tracer function (Bitplane, Concord, MA). Briefly, filament starting points and seed point thresholds were defined by soma diameter size and distal filament diameter, respectively. Only microglia whose entire soma and projections were within the field of view were selected. Microglia associated with

the granular layer of the hippocampus were omitted. After selecting somas, the signal threshold for the detection of filaments was adjusted based on filament diameter size and the 3D rendering was performed. Thresholds for starting points, seed points and signal were not altered between images once defined. After 3D modeling, various parameters were measured: total microglia projection length, full branch level, and Sholl analysis. Total microglia projection length is defined as the sum of the length of each projection from the soma of a microglia. The full branch level is a numerical value that begins at the beginning of a projection at the soma with a value of 1. At each branching point, the dendrite segment with the smaller diameter sequentially increases branch level while the dendrite with a larger diameter maintains the same branch level. Finally, Sholl analysis is a valuable tool to visualize and quantify cellular morphology in a 3D realm. In Sholl analysis, the number of filament intersections per concentric spherical shells are quantified beginning at the soma and distanced at a radius of 1 μm apart. The area under the curve was computed with GraphPad Prism 8 (GraphPad, San Diego, CA).

### 2.10 Single-Nucleus RNA-seq Analysis

Nuclei isolation and single-nucleus RNA sequencing were performed by Singulomics Corporation (Singulomics.com). In summary, bilateral hippocampi from 4 mice/group was flash-frozen and combined. Tissue was then homogenized and lysed with Triton X-100 in RNase-free water for nuclei isolation. The isolated nuclei were purified, centrifuged, resuspended in PBS with RNase Inhibitor, and diluted to 700 nuclei/μl for standardized 10x capture and library preparation protocol using 10x Genomics Chromium Next GEM 3’ Single Cell Reagent kits v3.1 (10x Genomics, Pleasanton, CA). The libraries were sequenced with Illumina NovaSeq 6000 (Illumina, San Diego, CA).

Preprocessing and quality control: The raw sequencing files were processed with CellRanger 6.0 (10x Genomics). The sequencing was mapped to the mouse genome (mm10) with the option include-introns and generated gene count matrices. The downstream analysis was performed with the R package Seurat 4.0 [252]. For all the samples, we filtered out the nuclei with <200 genes and >5% mitochondrial genes. The final filtered matrix for 3 samples contained 26823 nuclei and 18961 genes.

Cell type clustering and dimensionality reduction: Each sample was processed separately and then integrated to identify the cell types. The filtered matrix was log-normalized and identified the top 2000 variable genes for each sample using the FindVariableFeatures function. The integration anchors were identified through FindIntegrationAnchors function with the parameter dims = 1:20. To integrate all the samples, the IntegrateData function was applied with parameter dims = 1:20. The integrated data was scaled, and clusters were identified with a resolution of 0.4. The differential expression genes for each cluster were identified using the Wilcoxon rank-sum test which is available in the FindAllMarkers function. We assigned the cell type based on the expression of the known markers which yielded 11 clusters. Microglia cell type was extracted using the Subset function and analyzed for differential gene expression of PBS vs. doxorubicin and doxorubicin vs. doxorubicin + ACY-1083 comparisons. The pathway enrichment analysis was performed using the Ingenuity Pathway Analysis tool (IPA; Qiagen Inc.). Data is used with permission from Dr. Rajasekaran Mahalingam, Ph.D. who performed the preprocessing, quality control, cell type clustering, pathway enrichment analysis, and dimensionality reduction.

## 2.11 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad). Error bars indicate SEM and statistical significance was assessed by either unpaired t-test or two-way ANOVA with Tukey's post-hoc analysis.

## Chapter 3: Results

*Reproduced with permission from* McAlpin, B., Mahalingam, R., Sing, A.K., Dharmaraj, S., Chrisikos, T.T., Boukelmoune, N., Kavelaars, A., and Heijnen, C.J. (2021) HDAC6 inhibition reverses long-term doxorubicin-induced cognitive dysfunction by restoring microglia homeostasis and synaptic integrity. *Theranostics* 2022; 12(2):603-619. doi:10.7150/thno.67410.

### 3.1 Doxorubicin induces cognitive dysfunction in a therapeutic dosing model that is reversed by HDAC6 inhibition

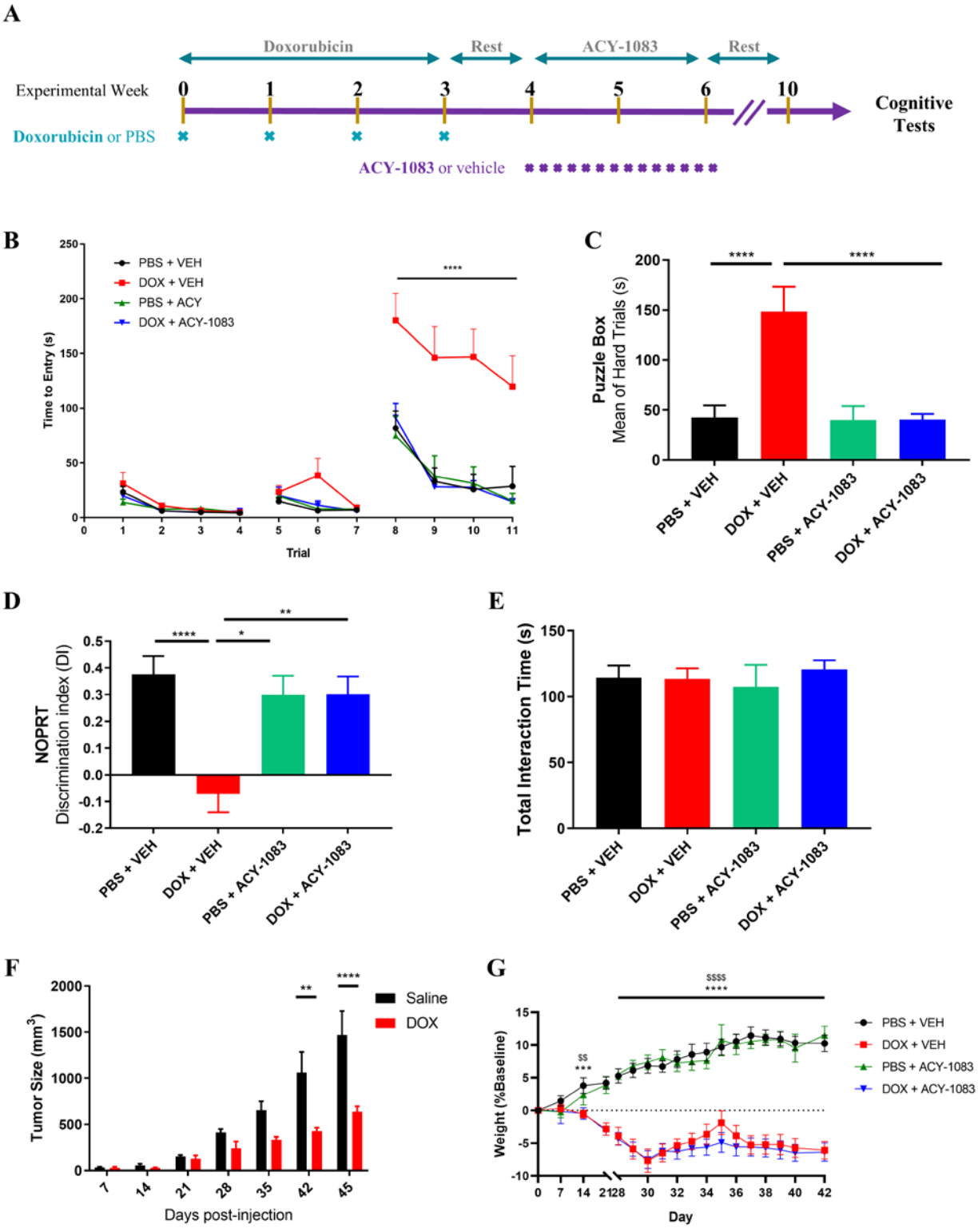
To assess the effects of doxorubicin on cognitive function, doxorubicin hydrochloride (5 mg/kg) was administered intraperitoneally weekly for 4 weeks to female mice (cumulative dose 20 mg/kg). This cumulative dose was shown to effectively reduce tumor growth in a MMTV-PyMT breast cancer mouse model (Figure 3.1F). Following the final dose of doxorubicin, mice were given 1 week of rest. Then, to test the reversal potential of HDAC6 inhibition on cognitive dysfunction, the HDAC6 inhibitor ACY-1083 (10mg/kg) was administered intraperitoneally daily for a total of 14 days (cumulative dose 140 mg/kg; Figure 3.1A). This dose was shown to be effective in reversing cognitive dysfunction in a cisplatin-associated model of CICD and did not affect the body weight of mice (Figure 3.1G) [28].

Behavioral testing to evaluate cognitive function began 4 weeks after the last dose of ACY-1083 to allow for rest, habituation and to evaluate the long-term effects of doxorubicin treatment. Mice were first tested using the puzzle box test, a problem-solving task designed to evaluate executive function and spatial memory [29]. In brief, mice were introduced to a brightly lit start box with a connecting tunnel to a dark goal box. Based on light/dark motivation, the time taken to enter the dark goal box was recorded. After 3 days of training with both an unobstructed tunnel (days 1-2, trials 1-4) and a tunnel obstructed with bedding (days 2-3, trials 5-7), mice were

tested during the difficult trials (day 3-4, trials 8-11) in which the tunnel was blocked with a cardboard plug that the mice must manipulate to complete the task. Detailed information can be found in the materials and methods section.

During all 4 testing trials (trials 8-11), doxorubicin-treated mice required significantly more time to escape compared to control mice (Figure 3.1B). Treatment with ACY-1083 completely restored the deficits in spatial memory and executive function observed in doxorubicin-treated mice (Figure 3.1B). Summation of hard trial averages allows for a clearer representation of doxorubicin-induced deficits in spatial memory and executive function, as well as reversal by treatment with ACY-1083 (Figure 3.1C).

Next, mice were tested using the novel object/place recognition test which assesses spatial working memory in mice based on their innate preference for novelty [250]. Control mice showed a clear preference for the novel object in a novel location, whereas doxorubicin-treated mice did not show a preference for novelty. (Figure 3.1D). ACY-1083 treatment restored the preference for novelty in doxorubicin-treated mice, indicating a restoration of spatial and working memory (Figure 3.1D). Total interaction time spent with the both novel and familiar objects was unchanged between groups (Figure 3.1E). These behavioral results indicate that doxorubicin treatment impaired executive function, spatial memory and working memory in mice long-term. These impairments were completely reversed by the HDAC6 inhibitor ACY-1083.


A
Doxorubicin
Rest
ACY-1083
Rest
Experimental Week
0
1
2
3
4
5
6
10
Cognitive Tests
Doxorubicin or PBS
ACY-1083 or vehicle
B
PBS + VEH
DOX + VEH
PBS + ACY
DOX + ACY-1083
Time to Entry (s)
Trial
****
C
Puzzle Box
Mean of Hard Trials (s)
****
****
PBS + VEH
DOX + VEH
PBS + ACY-1083
DOX + ACY-1083
D
NOPRT
Discrimination index (DI)
****
*
**
PBS + VEH
DOX + VEH
PBS + ACY-1083
DOX + ACY-1083
E
Total Interaction Time (s)
PBS + VEH
DOX + VEH
PBS + ACY-1083
DOX + ACY-1083
F
Tumor Size (mm3)
Saline
DOX
**
****
Days post-injection
G
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Weight (%Baseline)
PBS + VEH
DOX + VEH
PBS + ACY-1083
DOX + ACY-1083
Day

**Figure 3.1. HDAC6 inhibition with ACY-1083 reverses doxorubicin-induced deficits in executive functioning, working and spatial memory. (A)** Female mice were intraperitoneally treated with doxorubicin (5mg/kg) or PBS, followed by 14 daily administrations of ACY-1083 (10mg/kg) or vehicle. Cognitive function was tested 4 weeks after the final dose of ACY-1083. **(B)** Performance in the puzzle box test with 3 levels of complexity: An open tunnel (easy trials 1–4), a bedding-covered tunnel (intermediate trials 5–7), and a cardboard-plugged tunnel (difficult trials 8–11). Time taken to enter the goal box is recorded. **(C)** The average time taken to enter the goal box during the cardboard-plugged trials (difficult trials 8-11). **(D)** Performance in the NOPRT. The time mice spent interacting with each object was tracked. The discrimination index was calculated as $(T_{Novel} - T_{Familiar}) / (T_{Novel} + T_{Familiar})$. **(E)** Total interaction time with both novel and familiar objects in the novel object place recognition test. **(F)** Doxorubicin inhibition of tumor *growth in vivo* in a PyMT breast cancer model. On day 0, 2.5 x $10^5$ PyMTcells were injected in the $4^{th}$ mammary fat pad. Doxorubicin treatment began at day 28. **(G)** Percentage of baseline body weight was recorded. Behavioral results are expressed as mean ± SEM; n = 8-16 mice/group; Two-way ANOVA with Tukey's post hoc analysis *$p \leq 0.05$; **$p \leq 0.01$; **** $p \leq 0.0001$; Behavioral data obtained is used with permission from Dr. Anand K Singh, Ph.D. who performed the behavioral experiments. Tumor size results are expressed as mean ±SEM; n = 4 mice/group; Unpaired t test **$p \leq 0.01$; **** $p \leq 0.0001$. Body weight results are expressed as means ±SEM; n = 5-11 mice/group; Two-way ANOVA with Tukey's post hoc analysis; ***$p \leq 0.001$; **** $p \leq 0.0001$ PBS/VEH vs. DOX/VEH and PBS/VEH vs DOX/ACY-1083; $$$p \leq 0.01$; $$$$$p \leq 0.0001$ PBS/ACY-1083 vs. DOX/VEH and PBS/ACY-1083 vs. DOX/ACY-1083.

## 3.2 Doxorubicin treatment does not alter mitochondrial function or morphology in synaptosomes in a long-term CICD model

It has previously been shown that mitochondrial dysfunction in neuronal synaptosomes is an underlying mechanism in a cisplatin-induced model of cognitive impairment [253]. In addition, doxorubicin treatment has previously been shown to alter mitochondrial dynamics 5 days after treatment in rats [76]. Therefore, I evaluated CNS mitochondrial function and morphology in neuronal synaptosomes isolated from doxorubicin-treated mice. Using the Seahorse XF24 extracellular flux analyzer, I measured the oxygen consumption rate (OCR) to calculate basal, spare and maximum respiratory capacity of mitochondria. Surprisingly, basal, spare and maximum OCR was unaffected by doxorubicin treatment, indicating that long-term doxorubicin-associated CICD is not sustained by impaired mitochondrial function in neuronal synaptosomes (Figure 3.2A-C). Furthermore, the percentage of atypical mitochondrial morphology, characterized by swollen and/or abnormal cristae structure, was not significantly affected by doxorubicin treatment (Figure 3.2D-E).

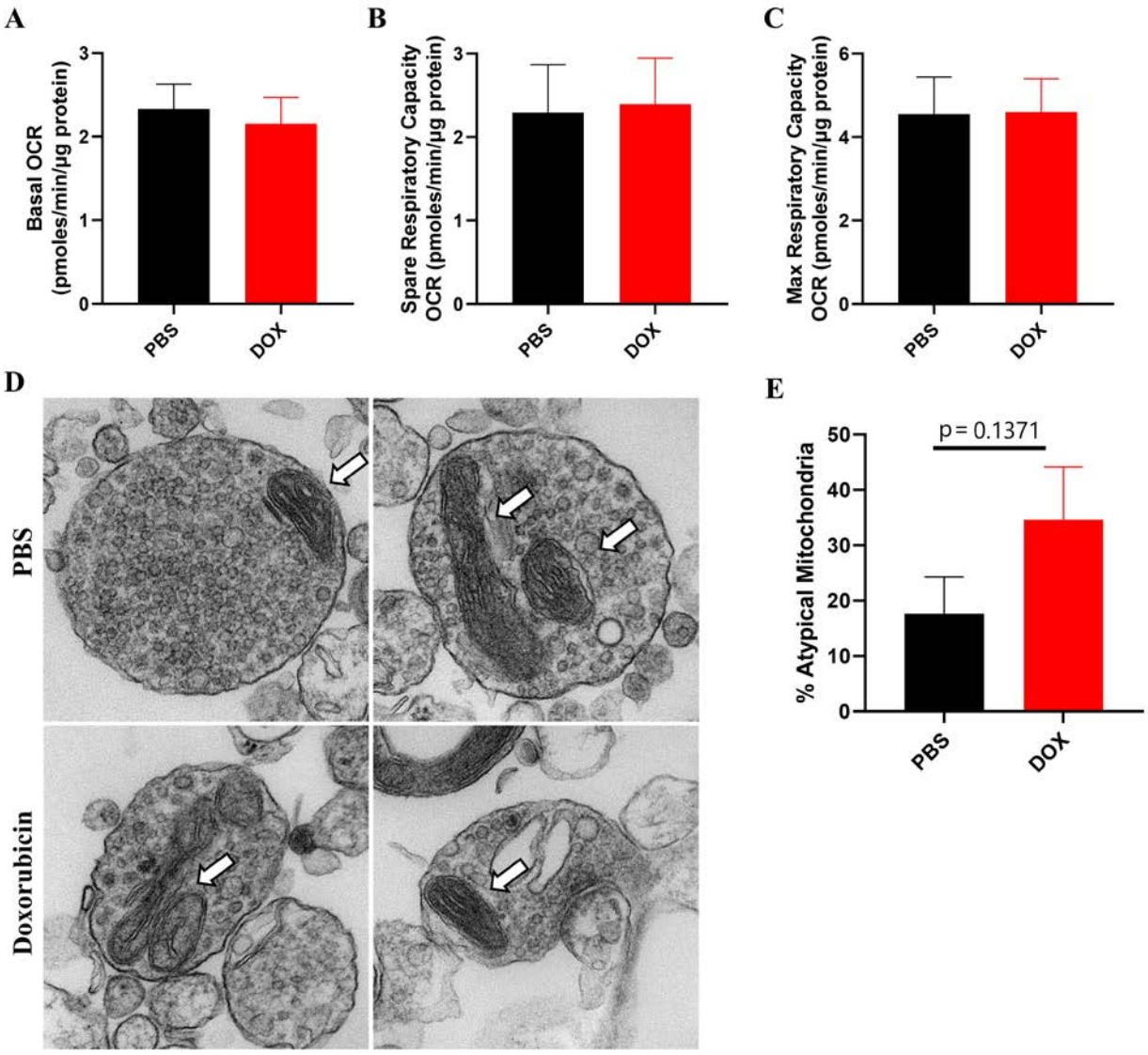



**Figure 3.2. Doxorubicin treatment does not significantly alter mitochondrial function or morphology in neuronal synaptosomes.** After completion of cognitive tests, synaptosomes were isolated from the brains of mice treated with doxorubicin or PBS. Oxygen consumption rates (OCR) were analyzed in isolated synaptosomes using the Seahorse XFe24 Flux Analyzer. **(A)** Basal respiration, **(B)** spare respiratory capacity, and **(C)** maximum respiratory capacity results are shown. **(D)** Mitochondrial morphology in synaptosomes was assessed using transmission electron microscopy. Arrows indicate mitochondria. **(E)** Percentage of atypical

mitochondria was quantified. OCR results are expressed as mean ± SEM; n = 9 mice/group; Unpaired t test. Mitochondrial morphology results are expressed as mean ± SEM; n = 4 mice (26-34 mitochondria)/group; Unpaired t test.

### 3.3 Doxorubicin treatment does not alter GFAP+ expression in a long-term CICD model

A proposed mechanism underlying short-term CICD as a result of doxorubicin treatment is an increase in astrocyte reactivity. Recent research identified reactive astrocytes in the hippocampus as assessed 5 days after the last dose of doxorubicin [76]. To evaluate whether astrocyte reactivity is involved in sustaining long-term CICD, I stained hippocampal sections with the astrocyte marker GFAP to quantify changes in GFAP expression and astrocyte reactivity. The results indicated no alteration in GFAP expression or astrocyte morphology following doxorubicin treatment, suggesting that astrocyte reactivity is not involved in sustaining long-term CICD (Figure 3.3).

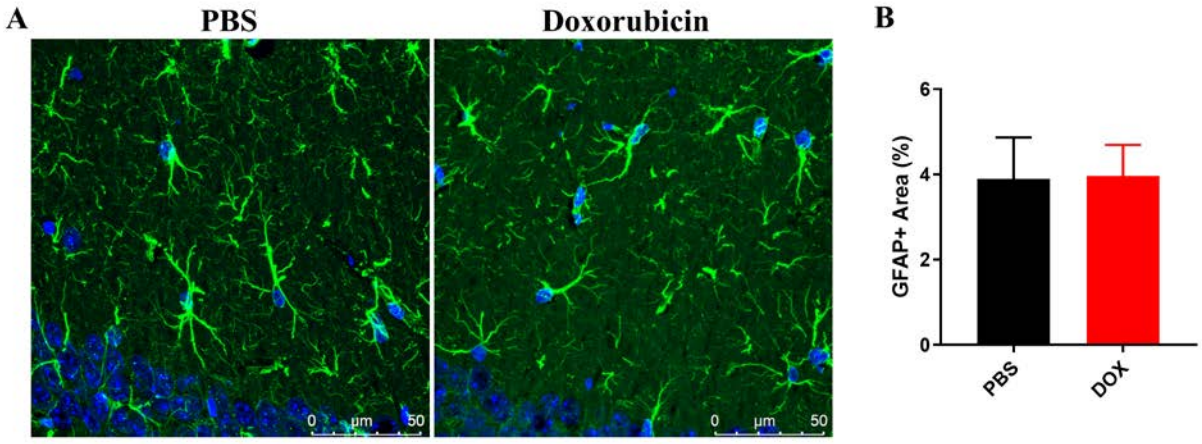



**Figure 3.3. Doxorubicin treatment does not alter GFAP+ expression in a long-term CICD model. (A)** 8 µm-thick mouse hippocampal sections stained with GFAP to visualize astrocyte morphology; scale bars 50 µm. **(B)** Percentage of GFAP+ staining area was unchanged following doxorubicin treatment. Results are expressed as mean ± SEM; n = 4 mice/group; Unpaired t test *$p \leq 0.05$.

## 3.4 Doxorubicin treatment does not alter myelin coherency in the cingulate cortex in a long-term CICD model

Previous studies have identified the alteration of choline-containing compounds following bolus doxorubicin treatment that may suggest decreased membrane synthesis and demyelination [88]. The cingulate cortex is a key node in the default mode network and has been implicated in cognitive processes, notably executive function [254]. To evaluate whether alterations in myelination play a role in long-term CICD, I stained coronal mouse sections of the cingulate cortex with Black Gold II, a lipophilic dye with a high affinity for myelin. The results indicated that doxorubicin treatment did not alter the intensity of Black Gold II staining in the cingulate cortex, suggesting that myelination was unaffected in the long-term CICD model (Figure 3.4).

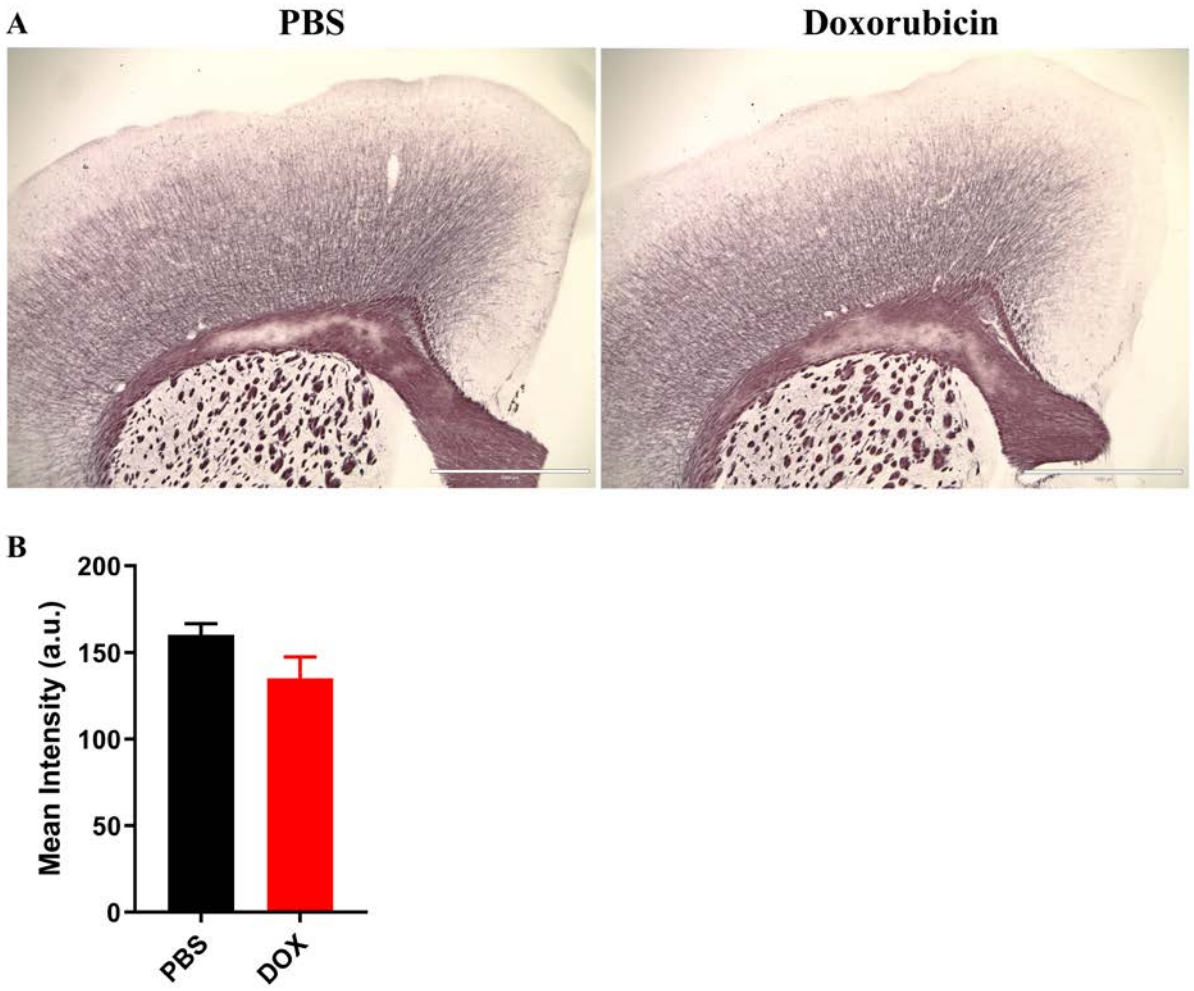



**Figure 3.4. Doxorubicin does not significantly affect myelin staining in the cingulate cortex of mice. (A)** Representative images of Black Gold II staining for myelin in the sensorimotor cortex of mice; scale bars = 1 mm; 4x magnification. **(B)** Mean intensity of Black Gold II staining was measured in the sensorimotor cortex. Intensity is expressed as a pixel value from 0-255 where 0 represents white and 255 represents black. Results are expressed as mean ± SEM; n = 4 mice/group; Unpaired t test.

### 3.5 HDAC6 inhibition restores expression of postsynaptic marker PSD95 in doxorubicin-treated mice

Synaptic protein expression is necessary for proper synaptic transmission, synaptic plasticity, and learning and memory formation [255]. To evaluate the effects of doxorubicin and ACY-1083 on the expression of synaptic proteins, hippocampal sections were stained with the presynaptic marker synaptophysin and the postsynaptic marker PSD95. The expression level of each marker was quantified in the CA1 and CA3 of the hippocampus.

In mice treated with doxorubicin, PSD95 expression was significantly decreased in the CA3 (Figure 3.5A-C) but not the CA1 (Figure 3.6A-C) of doxorubicin-treated mice compared to the control. This suggests that a decrease in postsynaptic integrity may play a role in sustaining CICD. Treatment with ACY-1083 restored PSD95 expression in the CA3 region of doxorubicin-treated mice, indicating the reversal of postsynaptic density loss (Figure 3.5A-C). In contrast, there was no significant change in the expression of the presynaptic marker synaptophysin in either the CA3 or CA1 (Figure 3.7A-D) of the hippocampus following doxorubicin treatment.

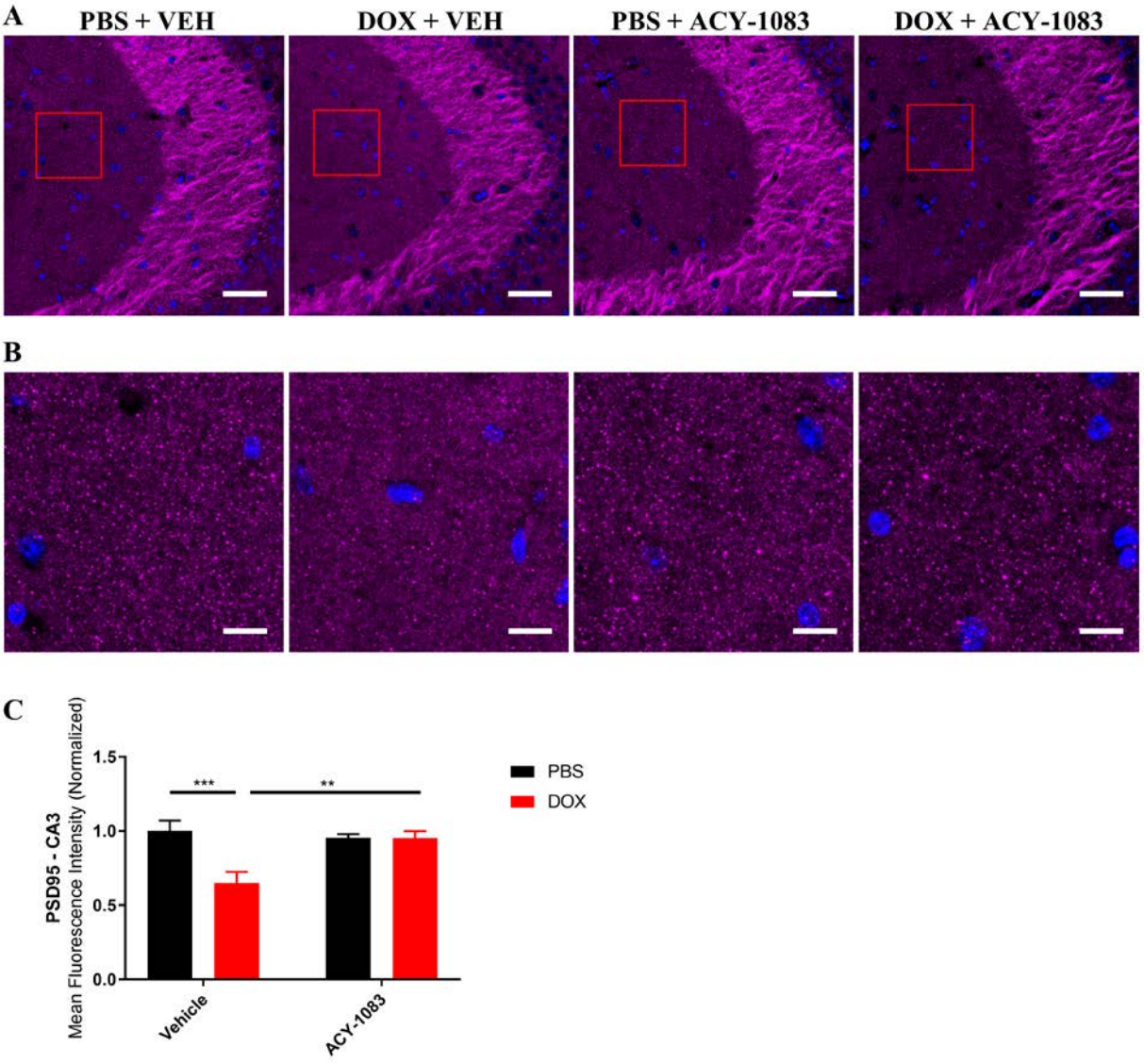



**Figure 3.5. HDAC6 inhibition with ACY-1083 restores expression of postsynaptic marker PSD95 in the CA3 region of the hippocampus in doxorubicin-treated mice. (A)** Mouse CA3 hippocampal region stained with PSD95 for different treatment groups; scale bars 50 µm; magnification 40x. **(B)** Higher magnification ROI reveals PSD95+ synaptic puncta; scale bars 10 µm. **(C)** Quantification of the mean fluorescence intensity of PSD95+ puncta. Results are expressed as mean ± SEM; n = 9-14 mice/group; Two-way ANOVA with Tukey's post hoc analysis **$p \leq 0.01$; *** $p \leq 0.001$.

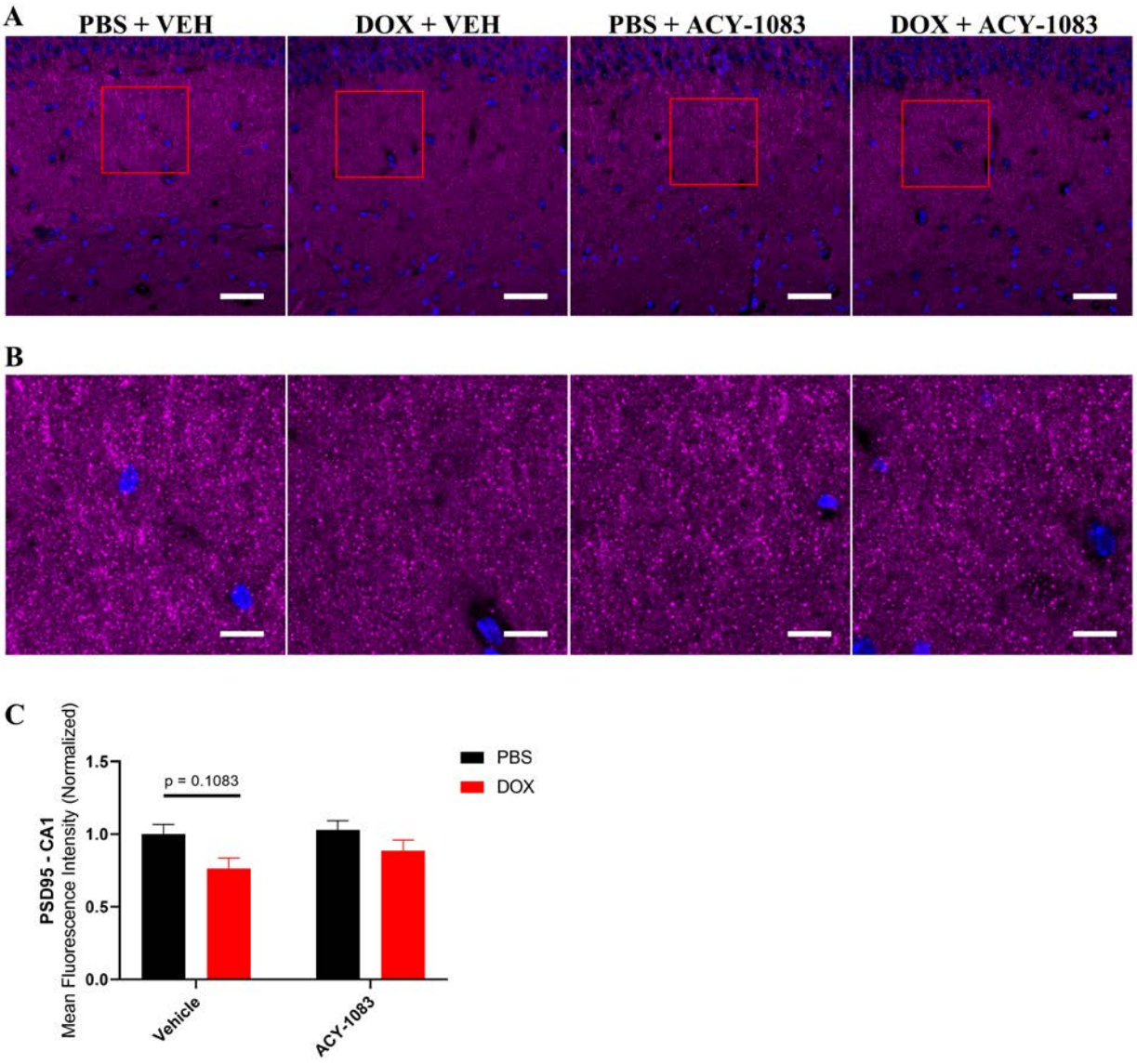



**Figure 3.6. Doxorubicin treatment did not significantly reduce the postsynaptic marker PSD95 in CA1 region of the hippocampus. (A)** Mouse CA1 hippocampal region stained with PSD95 for different treatment groups; scale bars 50 µm; magnification 40x. **(B)** Higher magnification ROI reveals PSD95+ synaptic puncta; scale bars 10 µm. **(C)** Quantification of the mean fluorescence intensity of PSD95+ puncta. Results are expressed as mean ± SEM; n = 9-14 mice/group; Two-way ANOVA with Tukey's post hoc analysis.

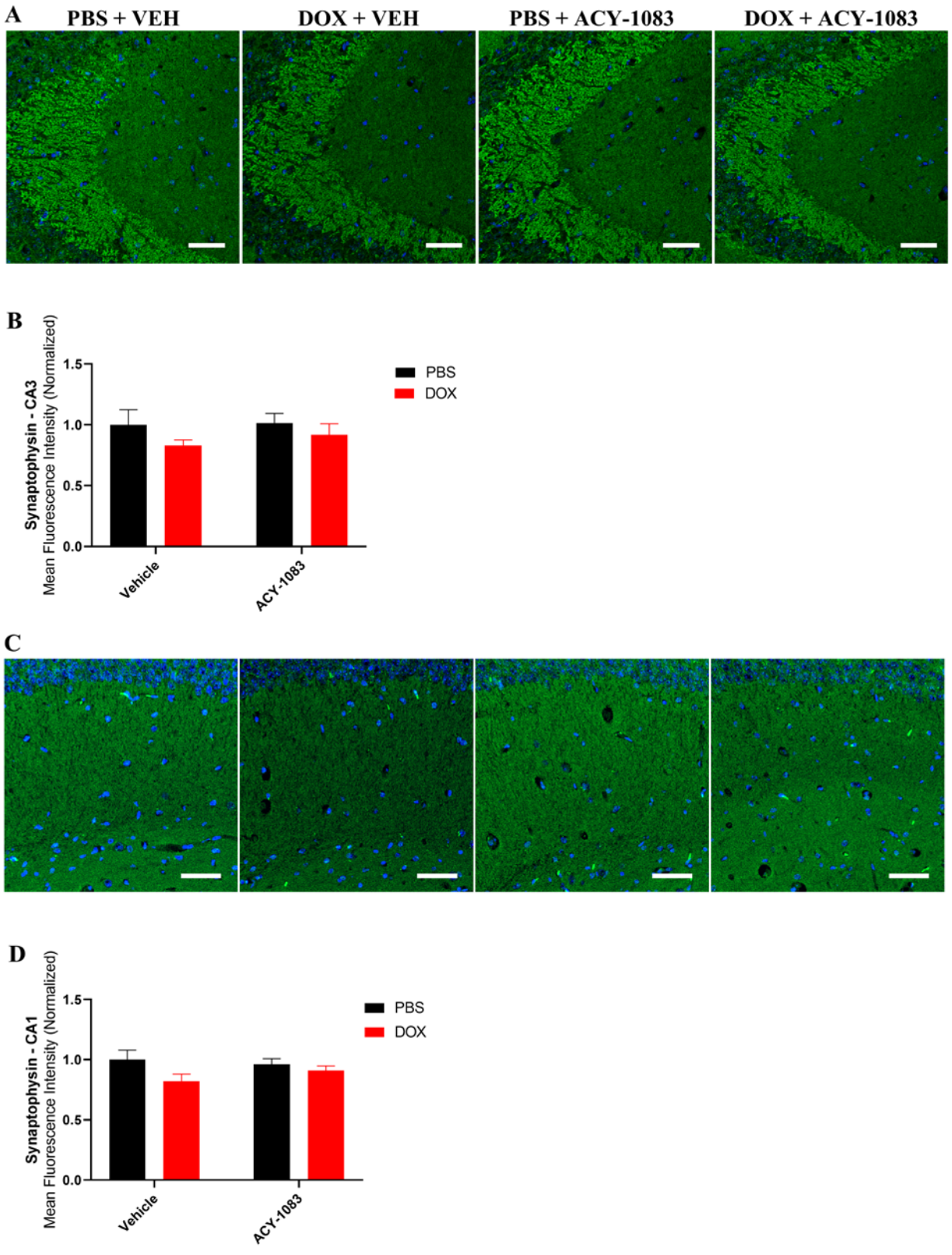

A
PBS + VEH
DOX + VEH
PBS + ACY-1083
DOX + ACY-1083
B
Synaptophysin - CA3
Mean Fluorescence Intensity (Normalized)
1.5
1.0
0.5
0.0
PBS
DOX
Vehicle
ACY-1083
C
D
Synaptophysin - CA1
Mean Fluorescence Intensity (Normalized)
1.5
1.0
0.5
0.0
PBS
DOX
Vehicle
ACY-1083

**Figure 3.7. Doxorubicin does not significantly affect the expression of presynaptic marker synaptophysin. (A)** Mouse CA3 hippocampal region stained with synaptophysin for different treatment groups; scale bars 50 µm; magnification 40x. **(B)** Quantification of the mean fluorescence intensity of CA3 synaptophysin staining. **(C)** Mouse CA1 hippocampal region stained with synaptophysin for different treatment groups; scale bars 50 µm; magnification 40x. **(D)** Quantification of the mean fluorescence intensity of CA1 synaptophysin staining. Results are expressed as mean ± SEM; n = 7-9 mice/group; Two-way ANOVA with Tukey's post hoc analysis.

### 3.6 HDAC6 inhibition reverses doxorubicin-induced reductions in microglial ramification in the hippocampus

In mouse models, large systemic bolus doses of doxorubicin have been shown to induce pro-inflammatory cytokine expression in the CNS, but no study has evaluated therapeutic dosing of doxorubicin, neuroinflammation and its association with long-term CICD [65]. Considering the important role microglia play in mediating neuroinflammation and synaptic pruning, I subsequently stained 8 μm-thick hippocampal sections with Iba1 to quantify Iba1 expression and visualize alterations in microglia [93]. Iba1 expression and the length of microglia projections was significantly decreased in the CA1 of the hippocampus following doxorubicin treatment (Figure 3.8A-D). In order to more accurately define the microglia phenotype in our long-term CICD model, I quantified microglia morphology using 3D cellular modelling and tested whether HDAC6 inhibition with ACY-1083 had the potential to reverse these alterations in conjunction with cognitive dysfunction. Using 20 μm-thick coronal sections, hippocampal microglia were visualized by immunohistochemical staining with an antibody to Iba1. Then, sections were then imaged at 1 μm slice intervals and combined to form a focused, Z-stack image. These images were transformed into 3D models using the image analysis software Imaris (Figure 3.9), and various parameters were measured: total microglia projection length, full branch level, and Sholl analysis as described in the methods.

In the CA3 of the hippocampus, the total microglial projection length and the full branch level were significantly decreased in doxorubicin-treated mice indicating a change in morphology that may be indicative of microglia activation. Treatment with ACY-1083 significantly reversed the shortened projection length and decrease in full branch level (Figure 3.10A-B). Sholl analysis revealed a robust decrease in the number of intersections in doxorubicin-treated mice compared

to controls, indicating reduced microglia ramification (Figure 3.10D). Overall differences between groups were determined using the area under the Sholl curve for each animal, as previously described [188]. In the CA3 of doxorubicin-treated animals, the area under the Sholl curve was significantly smaller compared to controls (Figure 3.10E). Treatment with ACY-1083 significantly increased the area under the Sholl curve, indicating a reversal of altered microglia morphology (Figure 3.10E). Although reduced microglial ramification was more severe in the CA3 of the hippocampus, similar alterations in microglia morphology were observed in the CA1 region following doxorubicin treatment and reversed with ACY-1083 (Figure 3.11).

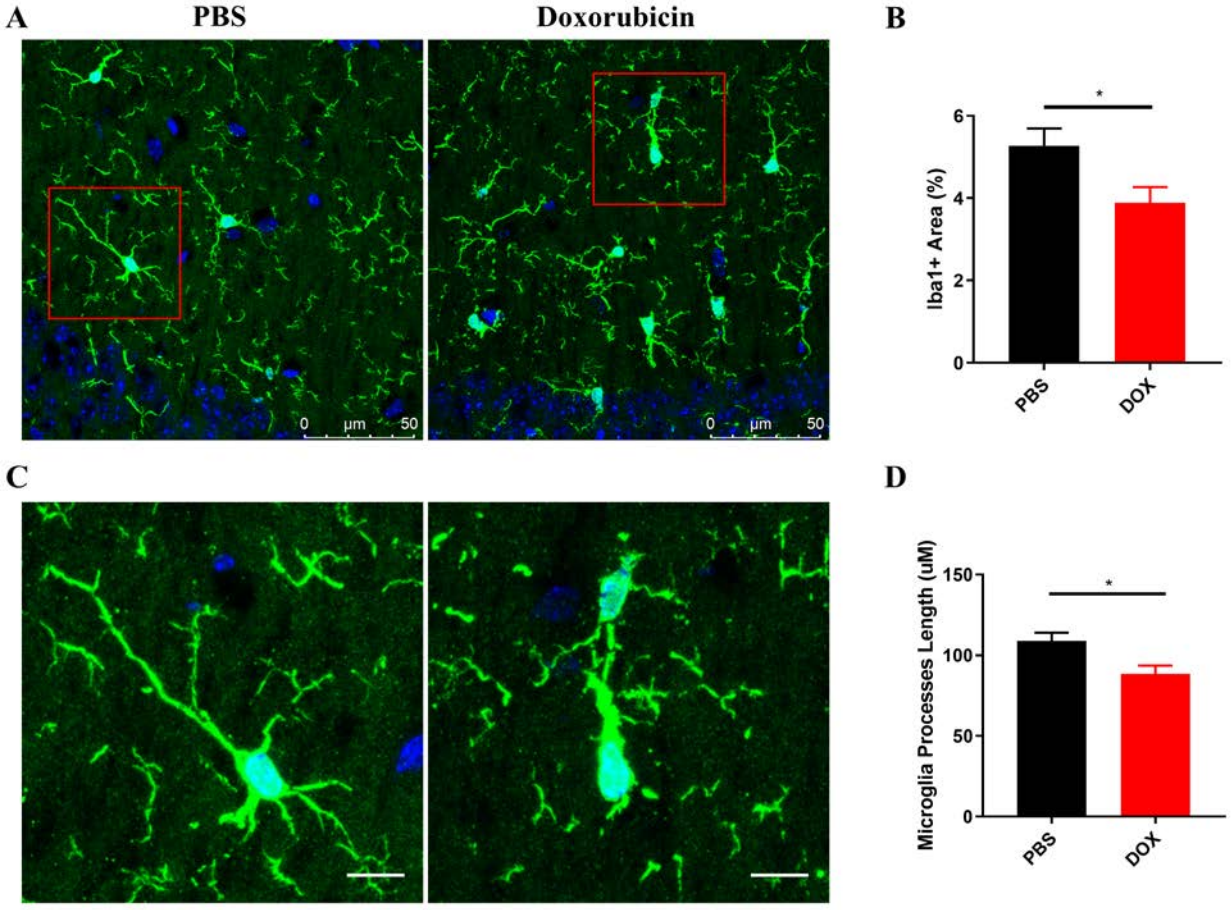



**Figure 3.8. Doxorubicin treatment reduces Iba1 expression and alters microglia morphology in the hippocampus. (A)** 8 µm-thick mouse hippocampal sections stained with Iba1 to visualize microglia morphology; scale bars 50 µm; magnification 40x. **(B)** Percentage of Iba1+ staining area was decreased following doxorubicin treatment. **(C)** Higher magnification ROI reveals an altered microglia morphology and **(D)** significant decrease in microglia projection length; scale bars 10 µm. Results are expressed as mean ± SEM; n = 4 mice/group; Unpaired t test *$p \leq 0.05$.

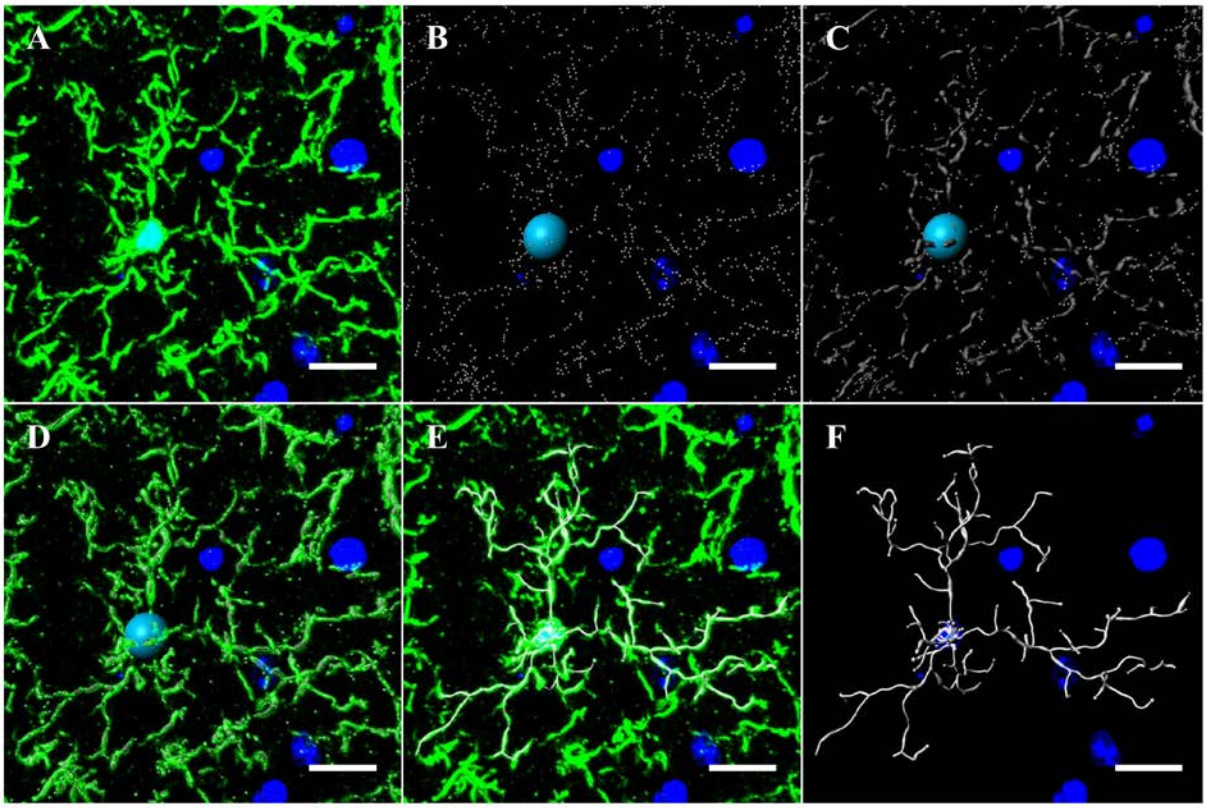



**Figure 3.9. Microglia modeling workflow using Imaris. (A)** 20 µm-thick mouse hippocampal sections stained with Iba1 were imaged at 1 µm slice intervals at 40x magnification and combined to form a focused, Z-stack image. The isolated Iba1+ GFP channel was 3D modeled using Imaris' filament tracer function. **(B)** Briefly, filament starting points and seed point thresholds were defined by soma diameter size and distal filament diameter, respectively. **(C)** The signal threshold for the detection of filaments was adjusted based on filament diameter size. **(D)** Iba1+ GFP channel overlaid with threshold detection and seed point representations as seen in B and C. **(E)** Iba1+ GFP channel overlaid with 3D filament render. **(F)** Final 3D rendering of Iba1+ microglia, allowing for automated quantification of filament length, branching level and Sholl analysis. Scale bars = 10 µm.

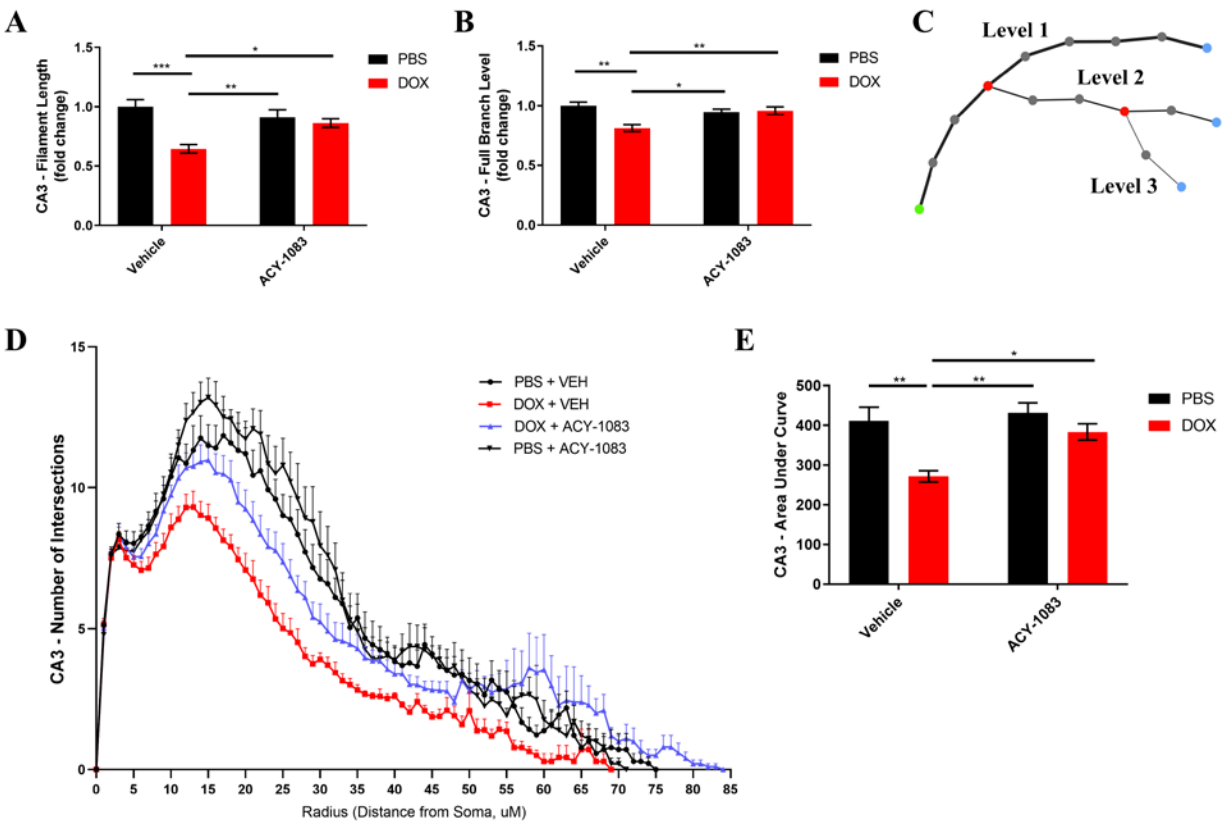



**Figure 3.10. HDAC6 inhibition with ACY-1083 reverses doxorubicin-induced reductions in microglial ramification in the CA3 region hippocampus. (A)** Filament length is defined as the sum of the length of each projection from the soma of a microglia. **(B)** Full branch level is the sum of the average branch level of each projection from the soma of a microglia. Branch level is a numerical value that begins at the beginning of a projection at the soma with a value of 1. At each branching point, the filament segment with the smaller diameter sequentially increases branch level while the dendrite with a larger diameter maintains the same branch level. **(C)** Schematic illustration visualizing increasing branch levels. The green dot represents the soma. **(D)** Sholl analysis quantifies the number of projection intersections per concentric

spherical shell beginning at the soma and distanced at a radius of 1 μm apart. **(E)** Quantification of the area under the Sholl curve from D. Results are expressed as mean ± SEM; n = 5-10 mice/group; Two-way ANOVA with Tukey's post hoc analysis *$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$.

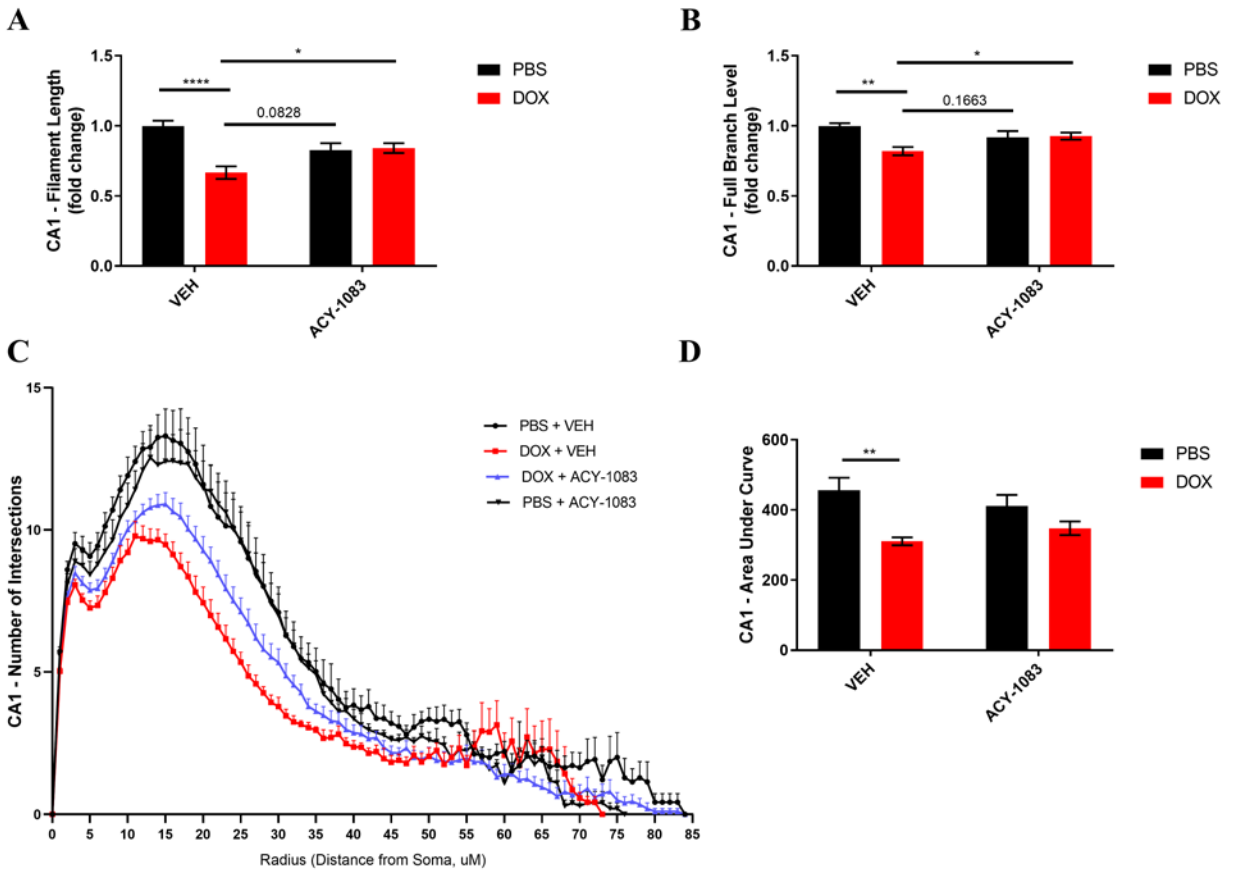



**Figure 3.11. HDAC6 inhibition with ACY-1083 reverses doxorubicin-induced reductions in microglial ramification in the CA1 region of hippocampus. (A)** Filament length is defined as the sum of the length of each projection from the soma of a microglia. **(B)** Full branch level is the sum of the average branch level of each projection from the soma of a microglia. **(C)** Sholl analysis quantifies the number of projection intersections per concentric spherical shell beginning at the soma and distanced at a radius of 1 μm apart. **(D)** Quantification of the area under the Sholl curve from D. Results are expressed as mean ± SEM; n = 5-10 mice/group; Two-way ANOVA with Tukey's post hoc analysis *$p \leq 0.05$; **$p \leq 0.01$; *** $p \leq 0.001$.

## 3.7 HDAC6 inhibition reverses doxorubicin-induced transcriptomic alterations in the nucleus of hippocampal microglia

While microglia modeling provides evidence of a robust decrease in microglia ramification, it lacks the potential to distinguish between canonical microglia activation and alternative microglia activation phenotypes (primed, dystrophic, DAMs) due to the morphological similarities between the phenotypes. RNA sequencing allows for an unbiased and in-depth analysis of gene expression that helps to identify cellular phenotypes while avoiding marker-based classifications that limit the identification of nuanced phenotypes. Therefore, I employed single-nucleus RNA sequencing of hippocampal tissue 6 weeks after the final dose of ACY-1083 to evaluate the transcriptome and clearly define microglia phenotype. After identifying 11 cell populations (Figure 3.12A), the microglia population was extracted from the integrated data and the differential gene expression of control, doxorubicin-treated and doxorubicin/ACY-1083-treated mice was calculated. Then, genes that were differentially expressed between doxorubicin-treated and control mice, named DOX Gene Set (362 genes) were identified. Genes that were differentially expressed between doxorubicin-treated and doxorubicin/ACY-1083-treated mice were named ACY-1083 Gene Set (3327 genes). After quality control, data from a total of 436 microglia nuclei was analyzed using Seurat 4.0, resulting in the quantification of 3689 expressed genes. Finally, in order to identify genes that may contribute to the altered microglia phenotype caused by doxorubicin treatment and subsequent reversal by HDAC6 inhibition, I compared both gene sets to identify common genes that were differentially expressed in both the DOX Gene Set and ACY-1083 Gene Set. I identified the differential regulation of 135 common genes expressed by microglia in both gene sets. Of the 135 common genes, 113 genes altered by doxorubicin treatment were reversed following treatment with ACY-1083. Genes upregulated following

doxorubicin treatment were associated with neurodegeneration and cellular stress including *Apoe*, *Ttr*, *Cox6c*, *Col6a1*, *Crym*, and *Ntng1*, all of which were downregulated following treatment with ACY-1083. Conversely, doxorubicin treatment led to the downregulation of genes related to microglia homeostasis and synaptic organization including *Slco2b1*, *Tgfbr1*, *Fermt3*, *Prickle2*, *Dock3*, *Msn*, *Scamp5*, *Lrp1b*, *Rhoa*, *Fut8*, *Cadm2*, and *Pdlim5*, all of which were upregulated following treatment with ACY-1083. Interestingly, canonical microglia homeostasis genes that were not affected by doxorubicin treatment but were upregulated by ACY-1083 included *Cx3cr1*, *Tmem119*, *Fcrls*, *Hexb*, *Tgfbr2*, *P2ry12*, *Foxo3*, *Sall1* and *Gpr34*. A subset of these genes is visualized in Figure 3.12B. These results indicate that HDAC6 inhibition reverses the altered genetic signature in microglia, while also enhancing homeostatic microglia gene expression, in the hippocampus of doxorubicin-treated mice in our long-term CICD model.

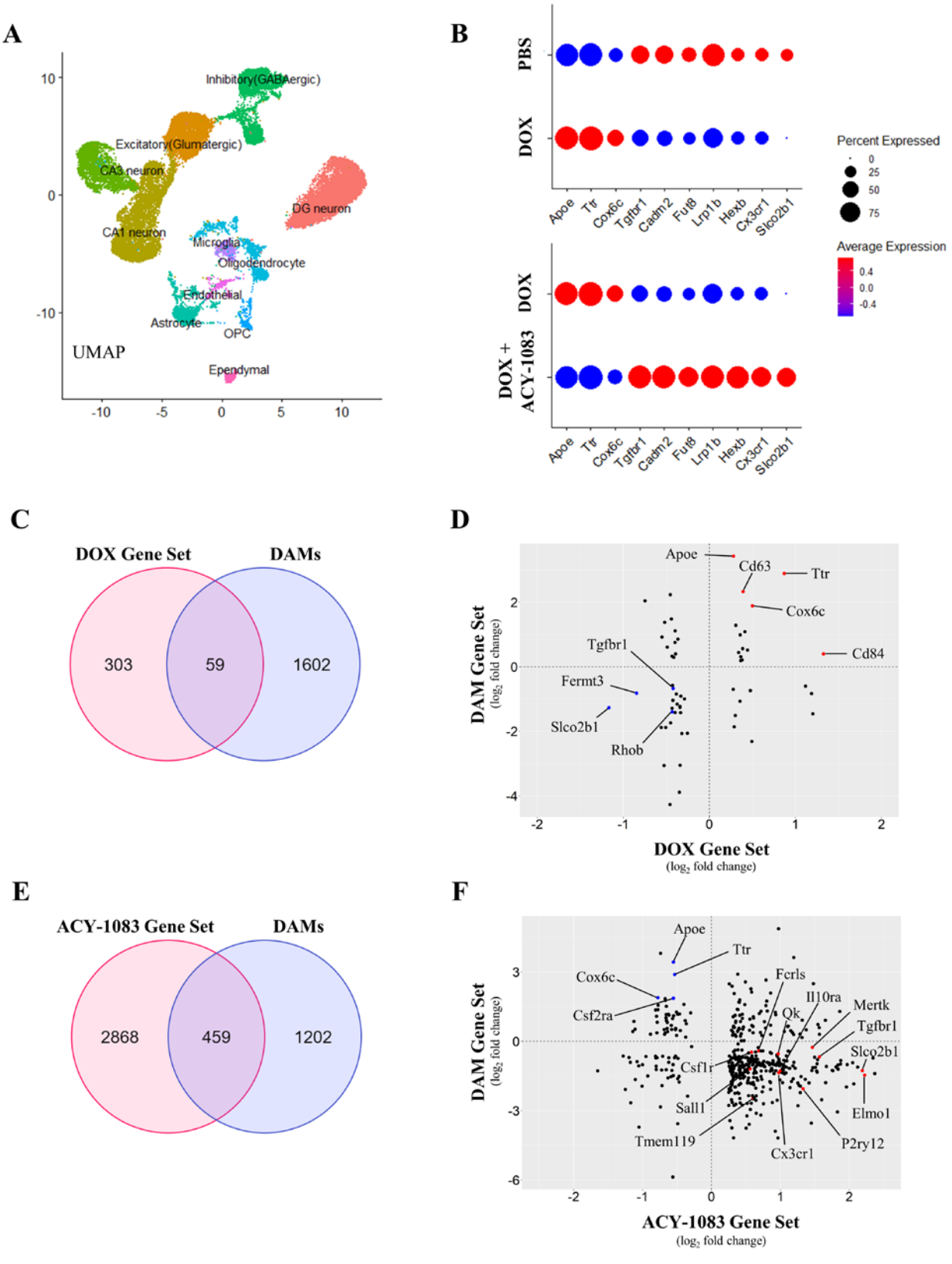
In order to define the microglia phenotype in our long-term CICD model, I compared the DOX Gene Set and ACY-1083 Gene Set to published microglia transcripts from healthy mice and disease models. I found strong similarities between microglia from our long-term CICD model and a unique microglia phenotype recently discovered in Alzheimer's disease transgenic mouse brains, termed disease-associated microglia (DAMs) [199]. I compared the DOX Gene Set and ACY-1083 Gene Set with genes that were differentially expressed between homeostatic microglia and DAMs (Keren-Shaul, *et al*., 2017, Table S3; 1661 genes with $p<0.05$) [199]. An overlap of 59 common genes was identified between DAMs and the DOX Gene Set, of which 38 correlated with the expression I observed following doxorubicin treatment (Figure 3.12C-D). This indicates that microglia-expressed genes that were upregulated after doxorubicin treatment were also upregulated in DAM microglia, and vice versa. Notably, I observed a downregulation of microglial homeostasis genes *Tgfbr1*, *Rhob*, *Slco2b1* and *Fermt3*. I saw an increase in DAM genes

associated with oxidative stress and neurodegeneration including *Apoe, Cd63, Cd84, Cd34, Cox6c, Ttr,* and *Ifnar1* but not phagocytic pathway genes. Following HDAC6 inhibition with ACY-1083, I identified an overlap of 459 genes common to DAMs and the ACY-1083 Gene Set (Figure 3.12E-F). Of these, 337 genes inversely correlated with the expression I observed in our long-term CICD model, indicating a reversal of gene expression and restoration of homeostatic microglia. Notably, I saw an upregulation of the microglial homeostasis genes that were downregulated after doxorubicin treatment: *Tgfbr1, Slco2b1* and *Fermt3*. I also observed an increase in microglia homeostasis genes that had not been affected by doxorubicin including *Cx3cr1, Tmem119, Csf1r, Fcrls, Mef2a, Sall1, Maf, Gpr34, Lrba, Med12l,* as well as genes that are typically associated with resolution of inflammation, microglia migration or purinergic signaling including *Ifngr1, Il10ra, Tnfaip8, Il6ra, Pde3b, Mef2c, Ifngr1, Plxna4, Selplg, Filip1l, Pdlim5, P2ry12* and *Entpd1*. I saw robust increases in 8 genes from the PI3K pathway whose activation was shown to promote recovery after injury [175]. Interestingly, after HDAC6 inhibition, I saw a large increase in phagocytic pathway genes including *Qk, Siglech, Elmo1, Cd33, Itgam, Inpp4b, Abi3,* and *Nckap1l* which may indicate promotion of repair by active clearance of debris. In addition, I saw an increase in *Mertk,* an engulfment receptor required for the unique non-inflammatory clearance of apoptotic cells called efferocytosis [169]. Finally, I saw an upregulation of 22 ubiquitin system genes that play a role in autophagy and phagosome maturation [256]. After ACY-1083 treatment, I observed the downregulation of genes that were upregulated following doxorubicin treatment and in DAMs, including neurodegeneration and inflammation-associated genes such as *Apoe, Csf2ra, Jun, Cox6c, Tlr7,* and *Ttr*. These results suggest that doxorubicin treatment induces a DAM-like microglia phenotype in our long-term

CICD model, which is restored to a homeostatic microglia phenotype following HDAC6 inhibition as illustrated in Figure 3.13.

Importantly, Keren-Shaul, et al. showed that DAM activation follows a two-step process: a TREM2-independent state, stage 1 DAMs, that are characterized by a loss of microglia homeostasis genes and increase in *Apoe* expression but no increase in phagocytic pathway genes, and a TREM2-dependent transition to stage 2 DAMs in which phagocytic pathway genes are upregulated [199]. The lack of *Trem2* expression in our model indicate that *Apoe* upregulation occurred independently of TREM2 signaling which, in addition to the lack of phagocytic pathway gene expression, suggests our microglial phenotype after doxorubicin treatment strongly overlaps with the stage 1 DAM phenotype.

The entire gene dataset from this dissertation, including the DOX Gene Set, the ACY-1083 Gene Set, and the list of genes common between both these gene sets and stage 1 DAMs, can be found in the open-source supplementary files from McAlpin *et al.*, 2021 (*Theranostics*. doi:10.7150/thno.67410).


A
Inhibitory(GABAergic)
Excitatory(Glumatergic)
CA3 neuron
DG neuron
CA1 neuron
Microglia
Oligodendrocyte
Endothelial
Astrocyte
OPC
Ependymal
UMAP
B
PBS
DOX
DOX + ACY-1083
Apoe
Ttr
Cox6c
Tgfbr1
Cadm2
Fut8
Lrp1b
Hexb
Cx3cr1
Slco2b1
Percent Expressed
Average Expression
C
DOX Gene Set
DAMs
303
59
1602
D
DAM Gene Set
(log2 fold change)
DOX Gene Set
(log2 fold change)
Apoe
Cd63
Ttr
Cox6c
Cd84
Tgfbr1
Fermt3
Slco2b1
Rhob
E
ACY-1083 Gene Set
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2868
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Apoe
Ttr
Cox6c
Fcrls
Il10ra
Csf2ra
Qk
Mertk
Tgfbr1
Slco2b1
Csf1r
Sall1
Elmo1
Tmem119
P2ry12
Cx3cr1

**Figure 3.12. HDAC6 inhibition with ACY-1083 reverses doxorubicin-induced transcriptomic alterations in the nucleus of hippocampal microglia. (A)** Uniform Manifold Approximation and Projection (UMAP) plot showing 11 cell populations identified from single-nuclear transcriptomes isolated from the hippocampus. **(B)** Dot plot depicting selected microglia-expressed genes associated with neurodegeneration and microglia homeostasis. Dot size encodes percentage of nuclei expressing the gene, while color encodes the average gene expression level per nuclei. **(C)** Venn diagram of genes that were differentially expressed between the DOX Gene Set and DAMs. **(D)** Scatterplot matrix depicting differential expression of microglia-expressed genes in the DOX Gene Set and DAMs. Blue dots indicate selected downregulated genes while red dots indicate selected upregulated genes. **(E)** Venn diagram of genes that were differentially expressed between the ACY-1083 Gene Set and DAMs. **(F)** Scatterplot matrix depicting differential expression of microglia-expressed genes in the ACY-1083 Gene Set and DAMs. Blue dots indicate selected downregulated genes while red dots indicate selected upregulated genes. Data is used with permission from Dr. Rajasekaran Mahalingam, Ph.D. who performed the preprocessing, quality control, cell type clustering, and dimensionality reduction.

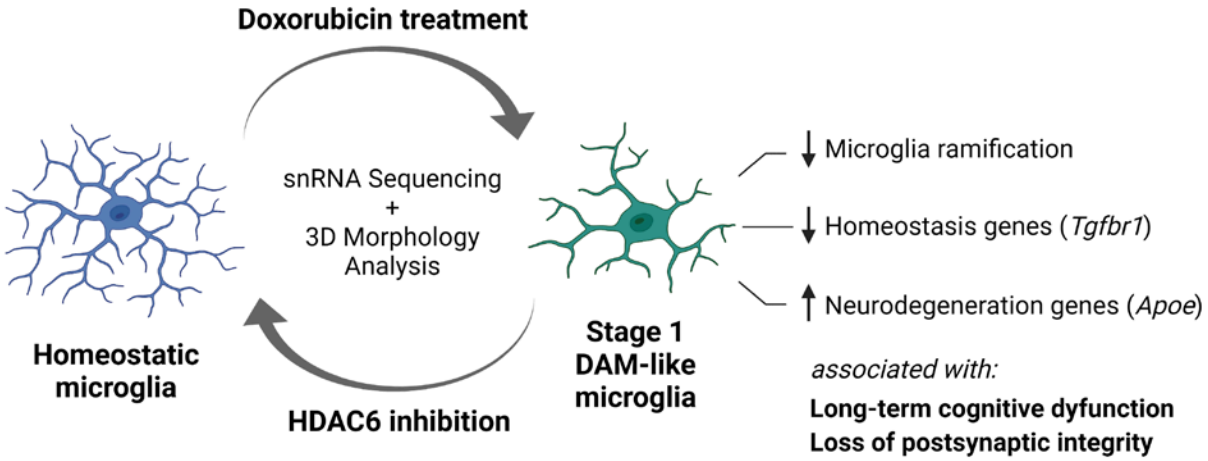


**Figure 3.13. Schematic illustrating the loss of microglia homeostasis that underlies long-term CICD and reversal by HDAC6 inhibition.** Doxorubicin treatment induces a neurodegenerative microglia phenotype closely resembling stage 1 DAM microglia. This microglia phenotype is characterized by decreased microglia ramification, a decrease in homeostasis genes, and an increase in neurodegeneration genes that are associated with long-term CICD and loss of postsynaptic integrity. Following HDAC6 inhibition, the restored microglia ramification, increased homeostasis genes, and decreased neurodegeneration genes were associated with restored cognitive function and postsynaptic integrity. Illustration created with BioRender.com.

# Chapter 4: Discussion and Conclusion

In this dissertation I have provided evidence that a therapeutic dosing schedule of doxorubicin induces long-term cognitive dysfunction in female mice. Additionally, I showed that long-term CICD is associated with a neurodegenerative microglia phenotype similar to stage 1 DAMs in the hippocampus as defined by cellular morphology and nuclear genome. I have also provided evidence that long-term CICD and a neurodegenerative microglia phenotype are associated with postsynaptic degeneration in the hippocampus as evidenced by a loss in PSD95 expression. Finally, I showed that pharmacological inhibition of HDAC6 using the blood-brain barrier permeable HDAC6 inhibitor, ACY-1083, successfully reverses long-term CICD, restores microglia homeostasis, and restores PSD95 expression. My present findings highlight persistent mechanisms that sustain CICD long-term, as well as a candidate pharmaceutical intervention that restores cognitive functioning following the cessation of chemotherapy treatment.

## 4.1 Relationship between microglia phenotype and cognitive function

The current dissertation adds to the vast amount of literature that draws a close association between pathological microglia phenotypes and cognitive dysfunction. Indeed, dysregulated microglia phenotypes have been correlated with cognitive dysfunction in a variety of disease models including Alzheimer's disease, Parkinson's disease, Huntington's disease, traumatic brain injury, and stroke [190, 217, 257–260]. While the consequences of dysregulated microglia are disease-specific, a loss of microglia homeostasis can cause persistent neuroinflammation, increased oxidative stress, dysregulated synapse maintenance, apoptosis, decreased immune surveillance, and suppression of neurogenesis, all of which may influence the cognitive state of an individual [261]. The results presented in this dissertation support the idea that restoring

microglia homeostasis in a disease state likely restores proper functioning of microglia including phagocytosis of cellular debris, efferocytosis, synaptic maintenance, and regulation of repair. I provide evidence that a restoration of microglia homeostasis was associated with a restoration of PSD95 expression in the hippocampus that coincided with restored cognitive function long after the cessation of doxorubicin treatment.

The microglia phenotype identified in this dissertation overlaps with that of stage 1 DAMs, a microglia phenotype recently identified in a mouse model of Alzheimer's disease [199]. The stage 1 DAM phenotype is defined by a loss of microglia homeostasis genes and increase in neurodegeneration-associated genes without alteration of phagocytosis genes. Similarly, in the long-term CICD model I identified a loss of homeostasis genes following doxorubicin treatment including *Slco2b1* and *Tgfbr1* which are involved in microglia surveillance and the resolution of inflammation, respectively [262, 263]. *Slco2b1* expression is implicated in the microglial "sensome" that facilitates endogenous ligand recognition and its expression is decreased during aging [263]. TGF-β1 has been implicated in maintaining neuronal integrity in part by regulation of microglia activation [264]. In addition, altered TGF-β signaling has been implicated in the pathogenesis of cognitive disorders [265].

I also identified an increase in neurodegeneration-associated genes including *Apoe* and *Ttr* whose upregulation are biomarkers for glial cell dysfunction and oxidative stress, respectively [266, 267]. *Apoe* overexpression is a genetic risk factor for Alzheimer's disease and was shown to mediate microglia activation and neurodegeneration in a tauopathy mouse model [268]. *Ttr* encodes transthyretin, a transport protein whose expression correlates well with ROS and is upregulated in microglia from aged mice [266, 269]. Following HDAC6 inhibition, I identified the robust restoration of these microglia homeostasis genes and decrease in

neurodegeneration genes that suggest a restoration of microglia homeostasis that coincided with restored cognitive function.

Interestingly, canonical microglia homeostasis genes that were not significantly downregulated after doxorubicin treatment were upregulated by HDAC6 inhibition including *Cx3cr1* and *P2ry12*. CX3CR1 is expressed by microglia and interacts with a ligand expressed by neurons, CX3CL1, to inhibit microglia activation [270]. P2RY12 is a purinergic receptor that mediates ATP-evoked membrane ruffling to facilitate microglia activation. It is stably expressed in resting microglia and decreased upon microglia activation [271]. The fact that some microglia homeostasis genes that were not significantly downregulated by doxorubicin treatment were also upregulated by HDAC6 inhibition suggests that the induction of the stage 1 DAM phenotype by doxorubicin and subsequent restoration of microglia homeostasis by HDAC6 inhibition may be mechanistically distinct. That is, the beneficial effects of HDAC6 inhibition on microglia phenotype may not be dependent on previous doxorubicin treatment and may also be beneficial in other models.

After HDAC6 inhibition, I also observed a robust increase in genes related to phagocytosis including *Qk* and *Elmo1*, 8 genes from the PI3K pathway whose activation was shown to promote recovery after injury, and 22 ubiquitin system genes that play a role in in autophagy and phagosome maturation [175, 256]. Importantly, HDAC6 inhibition also upregulated *Mertk*, a receptor critical for the unique non-inflammatory clearance of apoptotic cells called efferocytosis [169]. Therefore, the robust increase in phagocytosis genes may indicate a promotion of repair by active debris or apoptotic cell clearance that was restricted in microglia as a result of doxorubicin treatment. It is important to note that the stage 2 DAM phenotype, highly enriched in genes regulated related to phagocytosis, was shown to restrict

neurodegeneration in Alzheimer's disease transgenic mice [199]. In the Alzheimer's disease transgenic mice, transition to stage 2 DAMs was TREM2-dependent. The lack of *Trem2* expression after HDAC6 inhibition in the long-term CICD model indicate that HDAC6 inhibition did not induce a true stage 2 DAM phenotype. Nevertheless, the upregulation of phagocytosis genes after HDAC6 inhibition likely indicates similar neuroprotective functions of microglia in the long-term CICD model.

I also identified the upregulation of *Sirpa*, a gene recently identified to be a negative regulator of synaptic pruning (see *4.4*). It is possible that increased *Sirpa* expression following HDAC6 inhibition indicates a restoration of functional synaptic maintenance by microglia which would be permissive to the restoration of cognitive function. Distinct mechanisms that underlie the phagocytosis of synaptic elements, phagocytosis of debris, and efferocytosis may explain how an increase in genes related to phagocytosis correlated with an increase in PSD95 expression in the hippocampus and the restoration of cognitive function.

Taken together, it is likely that decreased microglia homeostasis genes and increased neurodegeneration-associated genes reflect a neurodegenerative microglia phenotype whose immune surveillance, phagocytic capacity, and synaptic maintenance functions are compromised as a result of doxorubicin treatment and contribute to cognitive dysfunction. Following HDAC6 inhibition, the robust decrease in neurodegeneration-associated genes and increase in genes related to microglia homeostasis and phagocytosis likely reflect the restoration of a homeostatic microglia phenotype and function that contribute to the reversal of cognitive dysfunction long after the cessation of doxorubicin treatment.

## 4.2 Relationship between PSD95 expression and cognitive function

PSD95 expression is integral for maintaining cytoskeletal scaffolding at the postsynaptic density of excitatory neurons to facilitate the clustering of receptors, ion channels, and associated signaling proteins. It is involved in the direct and indirect binding with NMDA receptors, glutamate receptors, AMPA receptors, and potassium ion channels to facilitate synaptic plasticity and long-term potentiation. The loss of PSD95 was shown to alter NMDA- and AMPA-receptor function and impair learning and memory [272]. In addition, altered PSD95 expression is common in aging and neurodegenerative diseases [273–276]. The importance of PSD95 in learning and memory was validated in a PSD95 knockout mouse model which exhibited altered NMDAR and AMPAR expression which correlated with impaired performance in the NOPRT and T-maze task [116]. Consistent with prior research, I provide evidence here that decreased PSD95 expression is correlated with impaired performance in the NOPRT in the long-term CICD model. Therefore, it is possible that decreased hippocampal PSD95 expression in the long-term CICD model impairs NMDA- and AMPA-mediated LTP to impair synaptic plasticity and induce cognitive dysfunction.

Considering the close association between the hippocampus and learning and memory, postsynaptic integrity in the hippocampus is integral for proper cognitive functioning. The current dissertation confirms this by demonstrating that a loss of PSD95 expression is associated with decreased performance in hippocampal-dependent behavioral tasks including the PBT and NOPRT. In addition, the restoration of PSD95 expression in the hippocampus correlated with restored performance in these tasks. These results are in agreement with previous research that identified behavioral deficits in the PBT in mice with dysregulated AMPA receptor function and hippocampal lesions [29]. The alterations in microglia phenotype and PSD95 expression were

consistently more robust in the CA3 region of the hippocampus, whereas PSD95 loss in the CA1 region was not statistically significant. The discrepancy between the CA3 and CA1 regions is consistent with the functional differences between the two regions. Functionally, research has suggested that the CA1 mediates temporal associations while the CA3 is fundamental in the consolidation of spatial memory [277, 278]. In support of this, previous research found that loss of NMDA receptors in the CA3 region caused decreased performance in the Morris water maze task, while the basic cellular properties of CA1 pyramidal cells including firing rate, spike width, and place field size were unchanged [279]. In addition, optogenetic inhibition of the CA3 region, but not the CA1, led to deficits in the NOPRT [280]. The current dissertation supports these findings with results that indicate a significant decrease of PSD95 expression in the CA3, but not the CA1, was associated with impaired NOPRT performance. Therefore, a loss of PSD95 expression in the CA3 region may reflect dysregulated AMPA- and NMDA-receptor function that may lead to the learning and memory deficits observed in our long-term CICD model.

### 4.3 Potential mechanisms underlying the loss of microglia homeostasis by doxorubicin treatment and restoration by HDAC6 inhibition

This dissertation adds to previous research that has identified broadly activated microglia 4 weeks after completion of doxorubicin treatment as assessed by CD68+ staining intensity and a NanoString mouse immunology panel [101]. Unfortunately, marker-based classifications limit the identification of nuanced microglia phenotypes. Using advanced imaging techniques and unbiased single-nucleus RNA sequencing, I have provided more depth to the understanding of the nuanced microglia phenotype that occurs in long-term CICD as a result of doxorubicin treatment. This dissertation provides valuable evidence that microglia activation is not a simple

phenotypic “switch” that is all-or-nothing, and in fact alternative microglia phenotypes that are not suggestive of canonical activation may underlie cognitive dysfunction in other models of neurodegeneration.

Generally, systemic doxorubicin treatment has been associated with neuroinflammation and oxidative stress that are indicative of canonical microglia activation (see *1.2.6*). This is likely due to a variety of mechanisms including endothelial cell dysfunction at the BBB and activation of the peripheral immune system which can induce microglia activation in the brain [39]. Much of the prior research on CICD, however, evaluates the microglia phenotype shortly after the cessation of doxorubicin treatment. While it is likely that canonical microglia activation did occur early in the doxorubicin dosing schedule, which was previously shown as assessed 5 days after cessation of doxorubicin treatment, the time point selected for evaluation of long-term CICD occludes these results [76]. Research has suggested that repeated inflammatory insults can induce long-term alterations in microglia phenotype that are common in neurodegeneration and aging and are associated with cognitive dysfunction [196]. Repeated exposure to systemic inflammatory insults induces a “primed” microglia phenotype that is distinct from canonical activation and characterized by a higher baseline of inflammatory markers, lower threshold for activation, altered protein synthesis, an exaggerated inflammatory response following immune activation, and increased phagocytosis that persist long-term [196, 281, 282]. In this dissertation, the therapeutic doxorubicin dosing scheduled used to induce long-term CICD is 4 doses over the course of 4 weeks, which likely represents 4 distinct systemic inflammatory insults in treated mice. Thus, the microglia phenotype identified here likely indicates a sustained shift in microglia homeostasis, as a result of repeated doxorubicin treatment, that reflects the loss of homeostasis similarly observed in neurodegeneration and

aging. However, unlike the primed microglia phenotype, microglia both in the long-term CICD model and stage 1 DAMs are not characterized by an increase in genes related to phagocytosis. In addition, the overlap between genetic transcripts was higher in DAMs than primed microglia (see *4.5*). Therefore, the neurodegenerative microglia phenotype identified in long-term CICD is phenotypically more similar to stage 1 DAMs than the primed phenotype.

The exact mechanism underlying the restoration of a homeostatic microglia phenotype after treatment with ACY-1083 in mice previously treated with doxorubicin remains to be determined. HDAC6 mainly deacetylates cytosolic proteins such as HSP90, peroxiredoxins that regulate the response to oxidative stress, and α-tubulin [229, 230, 283]. Changes in α-tubulin acetylation can affect multiple cellular processes including phagocytosis, cell morphology and migration, and intracellular transport of organelles that could all contribute to the restoration of brain function and microglia homeostasis as a result of HDAC6 inhibition [227]. In addition, the beneficial effects of HDAC6 inhibition on reversing cisplatin-induced peripheral neuropathy are IL-10-dependent (Zhang *et al.*, submitted). IL-10 expression is involved in the resolution of inflammation and has been shown to prevent pathological microglia phenotypes [181].

Although the molecular mechanism underlying HDAC6 inhibition and the restoration of microglia homeostasis following doxorubicin treatment is unknown, the nuclear genetic transcript of microglia gives us insight into possible signaling pathways that may be involved in the process. Previous research showed that HDAC6 inhibition with Tubastatin A significantly increased LPS-induced IL-10 production *in vivo* that was associated with decreased inflammation and was dependent on α-tubulin acetylation [284]. HDAC6 inhibition with ACY-1083 was previously shown to increase α-tubulin acetylation in the brain [28]. Furthermore, α-tubulin acetylation was shown to be increased by GSK3β inhibition via the Akt-GSK3β

signaling pathway [235]. Moreover, GSK3β has been shown to negatively regulate IL-10 expression by microglia [285]. Finally, IL-10 production is positively regulated by the PTEN/PI3K/Akt signaling pathway and several transcription factors including NF-κB [285, 286]. Thus, HDAC6 inhibition and subsequent α-tubulin acetylation may modulate GSK3β signaling and IL-10 production to attenuate pathological microglia phenotypes and restore cognitive function.

In support of this potential pathway, following treatment with ACY-1083 I observed an upregulation of *Gsk3b, Pten, Akt3* and *Il10ra* that coincided with a restoration of microglia homeostasis. In addition, I observed an upregulation of *Nfkb1* and downregulation of *Nfkb2* following HDAC6 inhibition. I also observed the upregulation of 8 genes from the PI3K pathway after HDAC6 inhibition. The activity of the GSK3β, PTEN/PI3K/Akt and NF-κB pathways are, however, dependent on post-translational modifications such as phosphorylation and are therefore not quantifiable with RNA sequencing. As such, additional research at a molecular level is required to define the exact mechanism underlying the restoration of microglia homeostasis by HDAC6 inhibition. Nevertheless, this dissertation provides evidence that HDAC6 inhibition has the potential to attenuate pathological microglia phenotypes and restore microglia homeostasis.

### 4.4 Potential mechanisms underlying the loss of PSD95 expression by doxorubicin treatment and restoration by HDAC6 inhibition

In this dissertation I provided evidence that a restoration of microglia homeostasis was associated with a restoration of PSD95 expression in the hippocampus that coincided with restored cognitive function long after the cessation of doxorubicin treatment. It is likely that the neurodegenerative microglia phenotype identified following doxorubicin treatment is associated

with the loss of PSD95 in the hippocampus. Conversely, it is likely that the restoration of microglia homeostasis is integral to the restoration of PSD95 expression, potentially as a result of the restoration of proper synaptic maintenance by microglia. This potential mechanism is supported by previous research showing that synaptic pruning by microglia is necessary for postnatal development and synaptic maturation in mice [287]. Indeed, microglia have been shown to directly colocalize with, and engulf, postsynaptic PSD95 in the hippocampus [287]. In addition, associations have been made between altered microglial phagocytosis and PSD95 loss in the hippocampus [166]. This microglia-mediated mechanism is supported by the snRNA sequencing results which showed an upregulation of *Sirpa* expression following HDAC6 inhibition that correlated with the restoration of PSD95. *Sirpa* encodes SIRPα, a transmembrane protein that was recently shown to be a negative regulator of synaptic pruning in microglia [288]. One study showed that a loss of *Sirpa* expression in microglia increased synaptic elimination via phagocytosis and was associated with decreased PSD95 expression in the hippocampus and cognitive impairment. Therefore, it is likely that HDAC6 inhibition restored microglia homeostasis and proper synaptic maintenance which contributed to the restoration of cognitive function.

However, other studies have suggested that PSD95 and microglia colocalization does not prove the phagocytosis of postsynaptic material by microglia, but is actually representative of non-phagocytic membrane exchange from microglia-derived PSD95[289]. Indeed, microglia have been shown to synthesize PSD95 during development, and this synthesis was shown to be modulated by inflammation [290]. In fact, gene network analysis has identified DLG4, the human protein equivalent of PSD95, as a hub protein in the microglial inflammatory response [290]. Hub proteins are defined as critical proteins with high connectivity in protein-protein

interaction networks [291]. Thus, it is possible that microglia in the adult CNS similarly synthesize PSD95 with modulation by inflammation. Therefore, the loss of PSD95 expression in the long-term CICD model may reflect a loss of PSD95 synthesis by microglia that was restored following HDAC6 inhibition.

Lastly, it is possible that the neurodegenerative microglia phenotype identified may promote neuroinflammation and oxidative stress. Oxidative stress has previously been shown to impair PSD95 expression in the hippocampus of mice [292]. In addition, oxidative stress was reported to alter PSD95 function by modifying a voltage-gated potassium channel at the postsynaptic density [293]. In support of this, I showed that a gene that encodes a marker of oxidative stress, *Ttr*, was upregulated in microglia after doxorubicin treatment [266]. Taken together, it is possible that the neurodegenerative microglia phenotype identified in long-term CICD reduces PSD95 expression as a result of increased oxidative stress in the hippocampus.

In addition to the microglia-mediated mechanism, doxorubicin treatment has been reported to impair glutamate receptors and cause neuronal toxicity [294, 295]. Considering that PSD95 is highly enriched at glutamatergic synapses, it is possible that early glutamatergic insults as a result of doxorubicin treatment were sustained long-term due to impaired repair mechanisms in the neurodegenerative microglia phenotype. Recent research showed that PSD95-associated complexes in the CA1 and CA3 regions represent two distinct neuronal populations [296]. The study showed that glutamatergic receptor pathways are over-represented in PSD95-associated complexes in the CA3 region. Therefore, the CA3 region of the hippocampus may be more vulnerable to doxorubicin-induced damage than the CA1 as was observed in our long-term CICD model. Lastly, researchers have suggested that the CA3 region is also more susceptible to neurodegeneration than the CA1, but other studies have presented

contradictory results [297–299]. Therefore, alterations in glutamatergic signaling and excitotoxicity as a result of doxorubicin treatment might explain the preferential decrease of PSD95 in the CA3 region of the hippocampus.

HDAC6 inhibition may also rescue PSD95 expression independently of microglia function. Previous studies have found associations between HDAC6 and neurotransmission, memory regulation, and cognition at the level of synapses. Indeed, HDAC6 inhibition has been shown to increase acetylation of β-catenin and its subsequent association with the PDZ (PSD-95/Discs Large/ZO-1) domain to facilitate signaling, scaffolding functions, and synaptogenesis [238]. Therefore, HDAC6 inhibition may influence the acetylation state of proteins related to synaptic function to restore PSD95 expression and cognitive function.

Taken together, it is likely that the neurodegenerative microglia phenotype identified in the long-term CICD model contributed to the loss of PSD95 expression due to dysregulated synaptic maintenance, altered PSD95 synthesis, and the promotion of neuroinflammation and oxidative stress. It is likely that HDAC6 inhibition contributed to the restoration of PSD95 by restoring microglia homeostasis and functions and also by modifying protein acetylation which promoted restored postsynaptic function.

### 4.5 Genetic overlap with primed microglia phenotype

In addition to DAMs, the DOX Gene Set and ACY-1083 Gene Set also overlap with the genetic signature of a recent meta-analysis that combined the expression profile of 5 neurodegenerative and aging mouse models to define an important consensus “primed” microglia phenotype (Holtman, *et al.*, 2015, Table S5; 295 upregulated genes and 205 downregulated genes) [197]. This primed phenotype is associated with long-term heightened reactivity and morphological alterations, particularly in aging and neurodegenerative brains

[196]. Indeed, after doxorubicin treatment 5 upregulated genes that are common to consensus primed microglia were identified: *Apoe, Cox6c, Cd63, Cd84* and *Gas7*. In addition, 6 downregulated genes that are common to doxorubicin-treated and consensus primed microglia were identified: *Tgfbr1, Rhob, Trim26, Tppp, Scamp5* and *Slco2b1*. Then, I identified 9 genes that were downregulated following ACY-1083 and upregulated in the consensus primed microglia genetic signature including *Apoe, Cox6c, Gnptg, Galc* and *Nfkb2*. I also identified 93 genes that were downregulated in the consensus primed microglia genetic signature and upregulated following ACY-1083, including genes related to microglia homeostasis and efferocytosis such as *Slco2b1, Tgfbr1, P2ry12, Cx3cr1, Lrba, Tmem119, Csf1r, Ifngr1, Maf,* and *Mertk.* Although the consensus primed microglia and doxorubicin-treated microglia overlap in genes related to neurodegeneration and microglia homeostasis, a clear distinction exists: Consensus primed microglia, like stage 2 DAMs, are characterized by a robust increase in pathways related to phagocytosis, while doxorubicin-treated microglia, like stage 1 DAMs, are not [197, 199]. Therefore, the current study provides evidence that doxorubicin-treatment induces a stage 1 DAM-like microglia phenotype, and that the restoration of a homeostatic microglia phenotype has the potential to reverse cognitive decline following cancer therapy.

### 4.6 Dentate gyrus neuron nuclear transcriptome

The current dissertation utilized single-nuclear RNA sequencing as an unbiased approach to evaluate the microglia transcriptome without the presence of cell isolation-based transcriptional artifacts. As a result, I obtained transcriptomic data in 11 cell populations that is the subject of further study at the time of writing and will not be discussed in its entirety in this dissertation. In order to further support my findings that HDAC6 inhibition after doxorubicin

treatment promotes a reversal of disease state, I will briefly discuss the nuclear transcriptome of neurons isolated from the DG.

A total of 7,195 nuclei from DG neurons were sequenced. Compared to microglia (436 microglia nuclei), the large number of nuclei from DG neurons allowed for identification of 6 distinct subclusters (Figure 3.14A). After doxorubicin treatment, a robust enrichment in subcluster 5 neurons correlated with cognitive dysfunction. Subcluster 5 neurons are characterized by genes related to neurodegeneration and oxidative stress including *Ttr*, *Apoe*, *Cst3*, *Cox6c*, *Ndufa4*, and *Ubb*. Pathway analysis identified pathways related to Alzheimer's disease, Parkinson's disease, and oxidative phosphorylation which indicate an increase in neurodegeneration and oxidative stress (Figure 3.14B). This data supports the loss of postsynaptic integrity and the presence of a neurodegenerative microglia phenotype that I identified in the hippocampus as a result of doxorubicin treatment. Research suggests the DG acts as the input region of the hippocampus and is a "preprocessor" of incoming information that sends excitatory output to the CA3 region [300]. In addition, researchers have hypothesized that glutamatergic signaling in the DG can influence synaptic strength and neuronal activity in the CA3 [301]. Although I did not evaluate microglia morphology and PSD95 expression in the DG in this dissertation, it is possible that the DG neuron nuclear genotype that occurs as a result of doxorubicin treatment may influence the CA3.

After restoration of cognitive function with HDAC6 inhibition, subcluster 5 was drastically depopulated and subcluster 3 became enriched with neurons that were virtually absent in mice treated with doxorubicin only. Subcluster 3 neurons are characterized by genes related to axonogenesis, synaptogenesis, and voltage-gated ion channels including *Cadm2*, *Kcnip41*, *Lrrtm4*, *Rbfox1*, *Lsamp*, and *Fgfr2*. Pathway analysis identified pathways related to

chemical synapse transmission, axon guidance, and LTP which may indicate increased synaptic plasticity that promoted the restoration of cognitive function (Figure 3.14C). This data supports the restoration of postsynaptic integrity and microglia homeostasis that occurred as a result of HDAC6 inhibition after previous treatment with doxorubicin.

In addition, subclusters 3 and 5 are largely absent in control mice. Similarly to microglia, this suggests that the induction of a pathological phenotype by doxorubicin and induction of a regenerative phenotype by ACY-1083 are mechanistically distinct. Therefore, the beneficial effects of HDAC6 inhibition on DG neurons may not be dependent on previous treatment with doxorubicin and may extend to other disease models.

Taken together, the snRNA-seq data from DG neurons further supports the reversal of disease state that occurs after HDAC6 inhibition in mice previously treated with doxorubicin. The enrichment of pathways related to LTP and synaptogenesis likely reflect an increase in synaptic plasticity that facilitates the restoration of cognitive function.

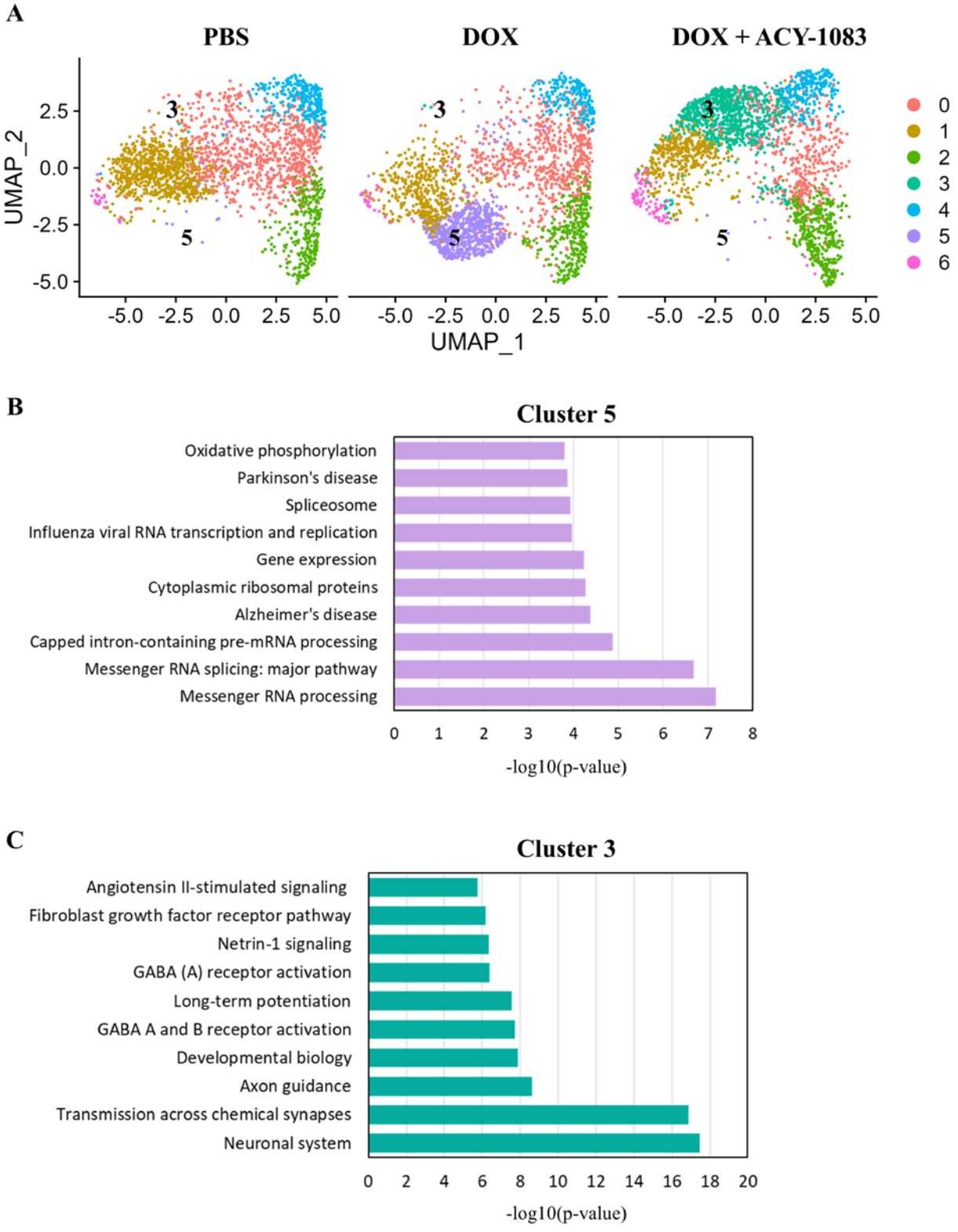
A
PBS
DOX
DOX + ACY-1083
UMAP_2
UMAP_1
3
5
0
1
2
3
4
5
6
B
Cluster 5
Oxidative phosphorylation
Parkinson's disease
Spliceosome
Influenza viral RNA transcription and replication
Gene expression
Cytoplasmic ribosomal proteins
Alzheimer's disease
Capped intron-containing pre-mRNA processing
Messenger RNA splicing: major pathway
Messenger RNA processing
-log10(p-value)
C
Cluster 3
Angiotensin II-stimulated signaling
Fibroblast growth factor receptor pathway
Netrin-1 signaling
GABA (A) receptor activation
Long-term potentiation
GABA A and B receptor activation
Developmental biology
Axon guidance
Transmission across chemical synapses
Neuronal system
-log10(p-value)

**Figure 3.14. Subpopulations of DG neurons are differentially enriched after doxorubicin treatment and HDAC6 inhibition with ACY-1083. (A)** Uniform Manifold Approximation and Projection (UMAP) showing 6 subclusters identified from single-nuclear transcriptomes of DG neurons in the hippocampus of control, doxorubicin-treated and doxorubicin/ACY-1083-treated mice. **(B)** Ingenuity pathway analysis of DG neurons in subcluster 5 that is enriched as a result of doxorubicin treatment. **(C)** Ingenuity pathway analysis of DG neurons in subcluster that is enriched as a result of HDAC6 inhibition.

### 4.7 Implications of mitochondrial function, astrocytes, synaptophysin, and myelin data

Interestingly, doxorubicin treatment was not associated with a significant reduction in synaptophysin expression 9 weeks after the final dose which may be related to altered glutamatergic signaling as a result of doxorubicin (see *4.4*). Additionally, a mouse model of Alzheimer's disease showed that synapse loss early in the progression of the disease was specific to PSD95+ puncta, but not synaptophysin+ puncta, in the hippocampus and mediated by excessive synaptic pruning by microglia [273]. Stage 1 DAMs were identified early in the course of Alzheimer's disease progression and are more similar to homeostatic microglia than stage 2 DAMs [199]. Therefore, it is possible that the preferential loss of PSD95 in the long-term CICD model is a result of a microglia phenotype that is consistent with the two previously mentioned studies.

Contrary to research that showed altered mitochondrial dynamics and astrocyte reactivity as assessed 5 days after the cessation of doxorubicin treatment, I did not identify altered mitochondrial function or morphology in synaptosomes or altered GFAP+ expression as assessed 9 weeks after the final dose of doxorubicin [76]. In this dissertation I evaluated mitochondrial function in synaptosomes, whereas Ongnok *et al.*, quantified mitochondrial dynamics in whole brain tissue. It is possible that regional differences between mitochondrial function exist in long-term CICD, and that mitochondrial dysfunction is present in specific cell types in long-term CICD. It is also likely that mitochondrial dysfunction is involved in the initiation of CICD but may not be required for sustaining long-term CICD. Similarly, considering the important role that astrocytes play in the regulation of neuroinflammation it is likely that astrocytes are involved in the initiation of CICD. However, the results of this

dissertation suggest that astrocyte reactivity may not be not integral in sustaining long-term CICD.

Lastly, white matter damage has been implicated in promoting CICD in humans. In this dissertation, white matter staining as assessed by Black Gold II staining was not significantly altered by doxorubicin treatment. Black Gold II is a lipophilic dye with a high affinity for myelin and can be used to visualize myelin loss. However, it is possible that a loss of myelin integrity in the form of myelin decompaction may have occurred without drastic demyelination. In cases of acute myelin decompaction, electron microscopy levels of resolution may be used to quantify the extent of damage [251]. Therefore, it is possible that myelin decompaction occurred in the long-term CICD model that was not quantifiable at the resolution of Black Gold II staining.

### 4.8 Future directions

While this dissertation provides evidence that doxorubicin treatment induces a neurodegenerative microglia phenotype that persists long-term as assessed by microglia morphology and nuclear genome, it lacks functional assays that could further define the microglia activity in long-term CICD. Future studies could utilize primary microglia cultures isolated from the hippocampus of treated mice to quantify microglia metabolism (Seahorse XFe96 Extracellular Flux Analyzer), phagocytic activity (FluoSpheres or fluorescently labeled myelin), migratory capacity (time-lapse migration), and activation in response to proinflammatory activators (LPS). Microglia engulfment of synaptic elements could be quantified *in vitro* by primary co-cultures of neurons and microglia isolated from treated mice to identify whether aberrant synaptic pruning underlies PSD95 loss. The risks associated with these experiments is that manipulation of CNS tissue and microglia isolation, which often

requires CD11b fluorescent tags or microbeads, may lead to tissue injury and microglia activation. *In vivo* synaptic pruning could also be evaluated by quantifying the engulfment of synaptic elements with immunofluorescence staining and advanced imaging techniques. In addition, synapse density could be quantified by colocalization of presynaptic and postsynaptic markers as opposed to individual markers of presynaptic and postsynaptic integrity.

Future studies could also evaluate changes in active synaptic transmission such as NMDA- and AMPA-mediated LTP and LTD by evaluating AMPA and NMDA receptor expression. Whole-cell patch clamp recordings of *ex vivo* brain slices to measure miniature and evoked EPSCs would give insight into synaptic transmission.

Importantly, the role of IL-10 in the restoration of microglia homeostasis could be evaluated by testing whether IL-10 knockout mice who are treated with doxorubicin and ACY-1083 also exhibit restoration of microglia homeostasis.

Finally, it is important to note that HDAC6 inhibition induced the expression of microglia homeostasis genes that were not significantly affected by doxorubicin treatment. This suggests that the beneficial effects of HDAC6 inhibition on microglia gene expression identified in this dissertation may not be dependent on previous treatment with doxorubicin, and the regenerative actions of HDAC6 inhibition independent of doxorubicin treatment should be investigated in more detail. Therefore, HDAC6 inhibition may have the potential to restore microglia homeostasis in other CNS disorders that are characterized by microglia dysfunction. Future directions should focus on the beneficial effects of HDAC6 inhibition in broader neurodegenerative disease.

## 4.9 Concluding remarks

In conclusion, the results of this dissertation provide evidence that pharmacological inhibition of HDAC6 using the blood-brain barrier permeable drug ACY-1083 successfully reverses long-term CICD as a result of doxorubicin treatment. I provide evidence that a therapeutic doxorubicin dosing schedule leads to CICD that is associated with alterations in microglial transcriptome and morphology that suggests a neurodegenerative microglia phenotype closely resembling the stage 1 DAM phenotype, as well as decreased postsynaptic integrity. Inhibition of HDAC6 reversed CICD and restored microglial homeostasis, which likely contributes to the restoration of postsynaptic integrity. Taken together, the novel findings in this dissertation indicate that treatment with a blood-brain barrier permeable HDAC6 inhibitor shows promise as an efficacious pharmaceutical intervention to reverse the microglia-mediated neurotoxic effects of doxorubicin treatment in association with a reversal of CICD. This study has implications in reversing cognitive dysfunction, and therefore improving the quality of life, of breast cancer survivors globally.

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## Vita

Blake Robert McAlpin was born in Michigan, the son of Stuart and Pamela McAlpin. After graduating from Lahser High School in 2010 he entered University of Michigan in Ann Arbor, Michigan. He received a Bachelor of Science degree with a major in Neuroscience in 2014 while working as a Research Assistant in both the Tooth Organogenesis and Regeneration Laboratory and the Laboratory of Oral, Head and Neck Cancer Invasion and Metastasis. He then traveled to Paris, France to complete a Master of Research (MRes) degree at the Université Paris Diderot (Paris 7) in Cell Biology, Physiology, and Pathology. In August of 2015, he entered The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences.