Using a toxic aging coin to assess hexavalent chromium-induced neurotoxicity.

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USING A TOXIC AGING COIN TO ASSESS HEXAVALENT CHROMIUM-INDUCED NEUROTOXICITY

By
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B.S., University of Alabama, 2021

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DEDICATION

This thesis is dedicated to my parents,

Tom and Diane Vielee,

whose love, support, and motivation

have provided me the opportunities

to pursue my dreams and aspirations.
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I would like to thank my mentors, Dr. John P. Wise, Jr. and Dr. John Pierce Wise, Sr., for the unwavering support, inhuman patience, and continual guidance they have shown me throughout this project. I would not be in the position I am today without their mentorship.

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ABSTRACT

USING A TOXIC AGING COIN TO ASSESS HEXAVALENT CHROMIUM-INDUCED NEUROTOXICITY

Samuel Thomas Vielee

June 30, 2023

We are facing an aging crisis, with 20% of the U.S. population projected to be geriatric (65+) by 2030 and live another 40+ years. Age-related diseases accompany a growing geriatric population, emphasizing the need to understand their etiology. Environmental pollutants compound this crisis by: 1) geriatrics are more susceptible, exacerbating age-related diseases and comorbidities, and 2) they accelerate biological aging, inducing age-related diseases at younger ages. We address this crisis using a ‘toxic aging coin’ approach; heads examines how age impacts toxicity, tails examines how chemicals accelerate aging. This thesis applies the heads side for Cr(VI)-induced neurotoxicity across ages. We hypothesize low concentrations of Cr(VI) in drinking water induces distinct age-, sex-, and region-specific neurotoxicity in rats, with geriatrics exhibiting the strongest effects. We observed Cr accumulation in the hippocampus but not frontal cortex, altered neurobehaviors (frailty, activity, spatial memory), and neurodegeneration, with age, sex, and regional differences.
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1. INTRODUCTION

1.1 Overview

Geriatric populations are growing rapidly around the globe. These populations are expected to make up 20% of the U.S. population by 2030 and live another 20+ years as geriatrics [1]. We are faced with an aging crisis, as this augmenting geriatric population will result in higher prevalence of aging-associated diseases and comorbidities.

Compounding this issue is ubiquitous environmental pollution. Nearly all environmental pollutants are associated with age-related diseases, but we do not understand how geriatric populations are distinctly vulnerable to these pollutants [2-3]. More concerning, environmental pollutants may act as gerontogens – i.e., chemicals that accelerate aging at a biological level.

We employ a “toxic aging coin” approach to address this intersection between aging and toxicology [4]. The heads side of our coin considers how age impacts toxic outcomes, while the tails side of our coin describes how chemicals accelerate aging. Our work utilizes this approach to study brain aging and neurotoxic outcomes that occur at different life stages.

Alzheimer’s disease (AD), Parkinson’s disease (PD), and amyotrophic lateral sclerosis (ALS) are significant burdens to human health and are linked to environmental pollution exposures. These diseases are widespread; AD impacted 5.5 million Americans in 2018, PD impacted 8.5 million people in 2019, and ALS affected 32,000 people in
Interactions between brain aging and environmental pollution are critical to understanding these diseases, as geriatric populations continue to grow and prevalence of neurodegenerative diseases continues to increase.

This thesis will focus on the heads side of our toxic aging coin, examining how age alters neurotoxic outcomes following exposure to environmentally relevant levels of hexavalent chromium [Cr(VI)] in drinking water. To put this project in context, I will first describe the aging crisis, brain aging, the role of environmental pollution in exacerbating the aging crisis, the role of heavy metals in neurodegenerative diseases, and the known neurotoxic effects of Cr(VI) in humans, animals, and cell culture models.

1.2 The Aging Crisis

We are currently facing an aging crisis. Modern medicine and advancements in healthcare have enabled the global population to reach geriatric ages in droves. Current estimates predict 20% of the U.S. population will be at least 65 years of age by the year 2030 [1].

Our growing geriatric population poses a significant threat to healthcare infrastructure due to the increased cost of age-related diseases. These age-related diseases constitute a variety of disorders that affect all organ systems, including neurodegenerative diseases, metabolic disorders, cancers, osteoporosis, etc. Neurodegenerative diseases are among the most debilitating age-related diseases. Most neurodegenerative diseases lack effective treatment options and generate significant expenses due to the amount of specialized care required for affected individuals. AD and related dementias affected
approximately 5.5 million Americans in 2018 [5]. The risk of developing these neurodegenerative diseases increases exponentially with age, with risk for developing AD reaching 13.9% for Americans aged 71 years or older [8]. In addition to age differences in AD diagnosis, there are drastic sex differences in AD affliction. Roughly two thirds of individuals diagnosed with AD are women [5]. A study from 2013 estimates approximately 8.4 million Americans will be diagnosed with AD or a related dementia by the year 2030, and these diseases will affect 13.8 million geriatric Americans by 2050 [9].

Increasing incidence of these diseases creates a heightened demand for specialized healthcare professionals and medical devices, placing significant stress on healthcare infrastructure. Further, estimates predict individuals will continue to live another 20+ years after becoming geriatric, demanding greater attention to healthspan – or the duration an individual lives a healthy life [10]. Changes to both lifespan and healthspan will lead to an enlarged population requiring long-term care for age-related diseases. The cost of long-term care for individuals suffering from age-related diseases was $849 billion in 2018 [11]. Projections show this cost will rise significantly, reaching $2.5 trillion by 2030 [11]. AD alone imposed an economic burden of $259 billion in 2017, but the Alzheimer’s Association predicts increasing incidence of AD-related long-term care will cost $1.1 trillion in 2050 [12]. The disease and economic burden of the aging crisis is exacerbated by widespread and persistent environmental pollution. Environmental pollution directly contributes to the development of age-related diseases, including neurodegenerative diseases [13-14].
Evidence that environmental pollution contributes to neurodegenerative diseases in geriatric populations emphasizes the need for improved environmental regulations, as these regulations rarely consider the distinct vulnerability of geriatric populations. Overlooking protections for geriatrics poses an imminent threat to public health as aged populations continue to grow at an unprecedented rate. Biological contexts change across lifespan and may alter toxic outcomes, meaning regulations should consider how toxicants influence people of all ages. The National Institute for Environmental Health Sciences (NIEHS) recognizes this point, stating in Theme 1 for the *Strategic Plan for Advancing Environmental Health* that research needs to consider impacts at “…all stages of lifespan” [15].

Increased susceptibility of geriatrics is a prevalent factor in assessing age-related changes in health across a wide spectrum of insults. Geriatrics suffer from increased susceptibility to various diseases, as demonstrated during the COVID-19 pandemic [16]. This susceptibility extends beyond infectious diseases and includes susceptibility to environmental factors [17]. For example, geriatrics suffer increased susceptibility to health issues such as air pollution-induced stroke and myocardial infarctions from a high heat index [18-19]. Complicating increased susceptibility in aged populations, geriatric individuals interact with an increasingly complex exposome. Some factors contributing to this complex exposome include lifelong accumulation of toxicants, re-exposure to stored toxicants (e.g., due to weight loss or osteoporosis), polypharmacy, and increased use of medical devices.

The growing use of medical devices and implants by geriatric individuals is particularly concerning as they present unique chemical exposures. Medical professionals
are using metal-on-metal prostheses at increasing rates and these professionals frequently use materials with cobalt-chromium alloys. One projection from New Zealand predicts the use of total hip replacements and total knee replacements will increase by 84% and 183%, respectively, from 2001 to 2026 [20]. An increase in hip and knee replacement surgeries is worrisome as metal-on-metal hip prostheses fail at significantly greater rates than prostheses made from other materials [21]. Shockingly, one metal-on-metal hip replacement was found to fail at a 50% rate over a 6-year period [21]. Multiple studies report failure of cobalt-chromium alloy implants induced metallosis contributing to neurological disorders [22-23].

Our rapidly growing geriatric population poses serious knowledge gaps, such as lack of consideration for geriatrics in environmental regulations, a deficit of toxicological data for aged individuals, and the need to assess unique geriatric exposomes. Environmental pollution exacerbates age-related diseases, making geriatric populations distinctly vulnerable. The lack of consideration for geriatric vulnerability in environmental regulations directly influences the societal and economic burden of disease. The prevalence of medical devices used by geriatrics concomitantly increased and has drastically increased their exposure to mixtures of toxicants, such as heavy metals, often interacting with geriatric polypharmacy. We lack an adequate understanding of how environmental pollution impacts geriatric populations distinctly from younger populations and how the unique exposome of geriatric populations complicates an assessment of toxic outcomes. Heavy metals are significant factors of environmental pollution and the geriatric exposome, but the impact of heavy metals on the geriatric brain is not fully elucidated.
1.3 The Aging Brain

Research into brain aging spans the last 40 years, but still has limited links to toxicology. Recently, 9 hallmarks of brain aging were identified to guide brain aging studies. These hallmarks include: 1) oxidative damage, 2) mitochondrial dysfunction, 3) impaired molecular waste disposal, 4) impaired DNA repair, 5) aberrant neuronal network activity, 6) stem cell exhaustion, 7) glial cell activation and inflammation, 8) impaired adaptive stress response signaling, 9) and dysregulated neuronal calcium homeostasis [24]. Mattson and Arumugam (2018) suggest telomere damage and cellular senescence may be hallmarks of brain aging, and multiple studies suggest cellular senescence plays significant roles in brain aging [25-26]. While not yet confirmed to be hallmarks of brain aging, telomere attrition and cellular senescence are considered hallmarks of aging in peripheral tissues and are often reported increased in aging brain tissues [24, 26-28]. The hallmarks of aging may affect specific cell types or tissues, meaning aging of cells and tissues can occur in a very localized manner.

Age-related changes in the brain exhibit regional physiological differences, including decreased hippocampal volume, cortical thinning, uncoupling of the neurovascular unit (NVU), and increased permeability of the blood brain barrier (BBB) [29]. Global changes include loss of gray matter volume and white matter volume [30]. Gray matter and white matter volume reach their peak in humans just before the age of 29 before declining, with humans reaching a peak rate of white and gray matter loss between the ages of 40 and 50 [30-31]. Changes in the volume or functionality of brain regions, uncoupling of the NVU, and increasing permeability of the BBB are the most common changes attributed to age-related neurological disorders.
The NVU and BBB create a barrier to limit exchange of exogenous materials between circulating blood and brain tissue. The NVU is composed of neurons, endothelial cells, astrocytic end feet, pericytes, microglia, and a basal lamina to act as a barrier between brain parenchyma and peripheral blood [32]. Endothelial cells and pericytes secrete the basal lamina, part of the BBB. The BBB contains tight junctions (e.g., occludins, claudins, ZO-1, ZO-2, and ZO-3) that create a physical barrier and limit exchange of chemicals between the environment and the brain [33-34]. The degree of BBB protection varies throughout the brain and some brain regions are less protected (e.g., olfactory bulb, circumventricular organs) [35-36]. Permeability of the BBB increases with age and occurs early in the development of neurological disorders, such as AD and PD [36-39]. In AD, increased permeability and dysfunction of the BBB precedes and contributes to regional deposition and impaired clearance of amyloid-β in the brain [40]. Permeability of the BBB increases following insults such as traumatic brain injuries (TBIs) and ischemic stroke [41]. Damage or deterioration of the BBB increases risk for neurotoxic outcomes by increasing accessibility for exogenous chemicals [41]. Chemicals targeting the BBB will increase permeability, similar to TBIs and the normal aging process. Increased BBB permeability may increase access of exogenous materials to the brain and exacerbate neurotoxicity. Heavy metal toxicity involving the BBB is introduced later.

At a cellular level, changes associated with brain aging include decreased neurotransmission, accumulation of chromosomal instability, induction of senescence pathways, a primed immune response in microglia, and trace element dyshomeostasis [26, 37, 42-45]. While we understand all these changes are associated with an aging
brain, the interactions between exogenous materials and the aging brain are not fully elucidated.

1.4 Persistent Environmental Pollution of Heavy Metals

The ubiquitous presence of environmental pollutants exacerbates the threat of an aging crisis. Environmental pollution is a pervasive threat to global health and there is ample evidence environmental pollution contributes to and exacerbates the severity of age-related diseases [2-3]. Environmental pollutants include compounds such as persistent organic pollutants, particulate matter, xenobiotics, and heavy metals. Heavy metals cannot be broken down and often cannot be metabolized into less toxic forms, making remediation techniques for heavy metals very complex. Studies across taxa show intermediate and long-term exposures heavy metals induce the most common age-related diseases including AD, coronary heart disease, chronic kidney disease, diabetes, and chronic obstructive pulmonary disorder [46-51]. Humans encounter heavy metals through contaminated drinking water and inhalation exposures, but heavy metals also contaminate food such as fish, plants, and baby food [52-54]. The contributions of heavy metals to age-related diseases are discussed in detail elsewhere, including neurotoxicity and the induction of age-related neurological disorders, see reviews [2-3, 55].

1.5 Heavy Metals Neurotoxicity

Heavy metals induce a variety of neurological and neurodegenerative disorders [56-60]. Heavy metals such as lead (Pb), cadmium (Cd), and mercury (Hg) cross the
BBB to exert toxic effects and contribute to neurological disorders such as AD, PD and ALS [62-63]. Additionally, aging in the brain increases permeability of the BBB and potentially results in elevated toxicant levels entering the brain. Some heavy metals even target the BBB to increase permeability of the barrier, including Pb, Hg, and arsenic (As) [64-65]. The ability of heavy metals to cross or target the BBB raises serious issues concerning permeability of this first-line defense. As permeability of the BBB increases over an individual’s lifespan, it is important to consider age in assessing neurotoxic outcomes from heavy metals.

Metals can induce distinct neurotoxic outcomes across life stages. Age-differences in Pb neurotoxicity are well-described [66]. Pb neurotoxicity during neurodevelopment induces irreversible alterations to the central nervous system, causing cognitive and motor impairment [67]. The known neurotoxic effects of Pb are most detrimental to children and include significant learning and memory impairments [61, 68]. In adults, Pb exposure is associated with olfactory impairment, cognitive deficits, and schizophrenia [69-71]. Accumulation of Pb in bones can lead to exposure later in life [72]. Various physiological changes occurring in middle-aged and geriatric individuals (e.g., pregnancy, menopause, osteoporosis) increase bone turnover and remobilize Pb from bone accumulation [73-75]. The ability of Pb to redistribute in the body complicates the interaction between heavy metals exposures and age-related diseases [76]. Age differences in neurotoxic outcomes following Cd exposure are reported as well. Cd exposure in children causes cognitive impairment and behavioral deficits [77]. Cd exposure in adults results in neuropathy, locomotor impairment, neuronal loss, and accumulation of neurofibrillary tangles and amyloid peptides [78-79]. Evidence for age-
related differences in the neurotoxicity of heavy metals such as Pb and Cd are well-documented, but we lack similar evidence for other neurotoxic heavy metals [e.g., chromium (Cr), silver (Ag), zinc (Zn)].

Mechanisms of heavy metal neurotoxicity are reported in varying detail. Pb, Cd, and Hg are the best characterized neurotoxic heavy metals and exert neurotoxic effects by inducing oxidative stress, neuroinflammation, and alterations to neurotransmission [61, 78, 80]. Evidence suggests these metals, as well as copper (Cu) and manganese (Mn), also induce mitochondrial dysfunction as part of their neurotoxic mechanism [61, 78, 80-82]. Pb is linked to AD, as it impairs clearance of amyloid peptides [46, 83]. Cd induces glutamate toxicity and increases the abundance of amyloid peptides, linking it with AD, PD, and ALS [81, 83]. Hg is linked to these same three disorders, though its mechanism involves increased amyloid peptides, loss of dopaminergic neurons, and accumulation in motor neurons, the brain stem, and the spinal cord [84]. Cu is an essential metal and requires maintenance of Cu levels in the brain. Cu is utilized in energy metabolism, neurotransmitter synthesis, and neurotransmission, but excess copper induces neurotoxic effects [85]. Excess Cu is primarily associated with Wilson’s Disease but also impairs amyloid peptide clearance and accumulates in the spinal cord, providing evidence Cu is implicated in AD and ALS [81, 86-87]. Mn accumulates in the substantia nigra, globus pallidus, and striatum to induce neurodegeneration in dopaminergic neurons and is primarily associated with PD and manganism [88].

Metals such as Pb, Cd, Hg, Mn, iron (Fe), and As are well-described as neurotoxicants in terms of mechanism and pathological conditions. Some heavy metals, such as hexavalent chromium [Cr(VI)], uranium (U), Zn, and Ag still require greater
investigation. Cr(VI) in particular requires more research given its threat to human health. Cr(VI) is ranked 17th on the Agency for Toxic Substance and Disease Registry’s (ATSDR) Substance Priority List – a ranking of chemicals deemed to pose the most significant potential threat to human health [89]. We have some knowledge of Cr(VI) neurotoxicity, discussed later, but the information is far from adequate to inform regulations or disease mechanisms. This thesis will address some of these knowledge gaps.

1.6 Knowledge Gaps in Hexavalent Chromium Neurotoxicity

Cr is a heavy metal ubiquitously present in the environment. In the Earth’s crust, Cr primarily exists in its trivalent form [Cr(III)] which is innocuous to human health. The toxic hexavalent form is produced by natural sources and industrial practices. Natural sources of Cr(VI) arise from the oxidation of Cr(III) to Cr(VI) during soil and mineral leaching, bringing Cr(VI) to groundwater [90-91]. Cr(VI) is highly desired for industrial purposes due to its hardness, bright colors, and anticorrosive properties. Cr(VI) is used in welding, production of pigments, tanneries, and production of materials such as concrete. Industrial sources of Cr(VI) pollution include burning fossil fuels, chromate dust from facilities, e-waste and recycling centers, and the dumping or improper disposal of Cr(VI) containing waste. The EPA currently lists 171 Superfund Sites contaminated with Cr(VI) [92]. Whether produced naturally or from industrial practices, human populations live in close contact with Cr(VI) and the health effects of this heavy metal require significant considerations.
Humans most often encounter Cr(VI) through inhalation (air pollution, cigarette smoke, welding fumes) or ingestion (contaminated food or ground water). Groundwater contamination is a significant concern for human exposure, as millions of Americans rely on well water directly fed by groundwater. One study found North Carolina groundwater concentrations of Cr(VI) exceeded the state advisory level (0.07 μg/L) and reached up to 22.93 μg/L Cr(VI) [93]. Cr(VI) induces negative health effects such as contact dermatitis, cancer, reproductive dysfunction, and hepatic fibrosis, but assessing the neurotoxic effects of Cr(VI) requires more detail [94-97]. While we understand Cr accumulates in the brain and induces a variety of neurological outcomes, we lack details concerning mechanisms of neurotoxicity, cell type effects, regional effects, and aging effects of Cr(VI).

I will briefly summarize Cr(VI) neurotoxicity; for a more detailed discussion, see review by Wise et al, 2022. [98]. Mechanisms of Cr(VI) toxicity are best described in peripheral tissues, though neurotoxicity studies identify key effects (e.g., neuroinflammation, decreased acetylcholinesterase). In general, Cr(VI) enters cells through sulfate or phosphate anion channels and is rapidly reduced to Cr(III) by antioxidants such as glutathione and ascorbic acid [99]. Reduction of Cr(VI) creates ephemeral species Cr(V) and Cr(IV) until stabilizing as Cr(III), while releasing reactive oxygen species (ROS) [99]. Cr(VI) exposure induces several changes to the physiology of cells, including significant oxidative stress, mitochondrial dysfunction, inflammation, epigenetic modifications, membrane damage, and DNA damage [95, 100-104]. These cellular changes have the potential to culminate into neurological disorders or neurodegenerative diseases.
Neurotoxic effects of Cr(VI) are described in human populations, animal models, and cell cultures. In adults, Cr(VI) neurotoxicity is associated with polyneuropathy, neurocognitive deficits, olfactory impairment, hearing loss, acute schizophrenia, depression, spatial memory loss, and motor neuron disease [98]. Childhood exposure to Cr(VI) is linked to autism spectrum disorder, impaired attention, and impaired neurocognitive performance [105-106]. One study linked exposure to Cr(VI) in utero to development of autism spectrum disorder in children [107]. Cr(VI) exposure in geriatrics is linked to hearing loss, vision impairment and cognitive decline [98]. It is worth noting the route of exposure to Cr(VI) can change with age. While exposure to Cr(VI) through environmental pollution occurs at all ages, adults and geriatric individuals have unique exposures such as an occupational exposures or use of medical devices (e.g., metal-on-metal joint replacements). Age-related differences in Cr(VI) exposure and Cr(VI) neurotoxic outcomes are apparent but require greater research.

We currently lack informative data for Cr regional accumulation in the brain. Human studies of non-occupationally exposed individuals show brain Cr accumulates with age, with greatest accumulation observed in the temporal cortex and pituitary gland (Figure 1.1A) [98]. One study found individuals who lived in Mexico City had greater concentrations of Cr in their brains and the olfactory bulb exhibited increased expression of genes associated with inflammation, when compared to brains of individuals who lived in rural areas without significant air pollution [108]. Cr(VI) neurotoxicity is attributed to motor neuron disease but its global effects in the brain necessitate greater research to fully demonstrate the potential threats of Cr(VI) neurotoxicity in other diseases [109]. To a degree, Cr(VI) neurotoxicity is better understood in animal models. Multiple animal
models (e.g., rat, mouse, goat, chicken, fish) reported Cr accumulation and widespread neurodegeneration. One study utilizing a goat model reported regional accumulation of Cr in the brain, with the highest concentration reported in the meninges [110]. One study using a fish model found exposure to Cr(VI)-contaminated water increased Cr concentrations in the brain; while brain Cr accumulation was the lowest of the organs studied, the brain was the only organ to not decrease Cr levels following depuration [111]. One group found Cr levels increased in the hypothalamus and pituitary gland of Wistar rats following exposure to Cr(VI), though these studies did not assess other regions (Figure 1.1B) [112-113]. These studies provide a foundation for studying Cr(VI) neurotoxicity by providing evidence Cr accumulates in the brain and bioaccumulates across the lifespan.
Figure 1.1 Summary of Regional Chromium Accumulation in Human and Rodent Brains.

Figure 1.1A depicts chromium accumulation in the human brain. Global accumulation is observed, with higher accumulation reported in the temporal cortex and pituitary gland.

Figure 1.1B depicts chromium accumulation in the rodent brain. Available data indicates accumulation in the hypothalamus and pituitary gland, but no other regions have been individually assessed. All other studies report whole brain Cr only.
There are multiple reports of Cr(VI)-induced neurological effects in rodents and chickens. These reports detail a variety of changes in neurobehavior, neuroanatomy, and cell functions. Rats exposed to Cr(VI) exhibited elevated levels of oxidative stress (lactate dehydrogenase, catalase, glutathione, superoxide dismutase, malondialdehyde, vitamin C, and non-protein thiol) and inflammatory cytokines (IL-1β, PI3K, PKB) [114-115]. These studies found Cr(VI) exposure resulted in pathologies including neurodegeneration and hypertrophy of glial cells [114-115]. Cr(VI) exposure in chickens resulted in increased oxidative stress (superoxide dismutase, glutathione, and malondialdehyde) and neurodegenerative changes to brain organization (vacuolated neurons, edema of glial cells, edema in vasculature, and loss of Purkinje cells) [116]. One study found exposure to tannery effluent, containing high Cr concentration, altered sociability and social memory in mice [117]. Other rodent studies found neuropathological changes from Cr(VI) exposure altered neurobehavior, including impaired locomotion, cognition, and social memory [115, 118]. In zebrafish, Cr(VI) exposure induced changes in brain organization (disorganization of the optic tectum), oxidative stress (glutathione, malondialdehyde, and catalase), and increased expression of inflammatory (Nrf2, Nqo1, Ho1, Ucp2, and AchE) and apoptotic genes (p53, Bax, Bcl2, Caspase 9, and Caspase 3) [119]. Animal studies provide some insight into the neurotoxic effects of chromium, though no studies have examined the neurotoxic effects of chromium in drinking water at regulatory standards; the lowest drinking water exposures currently reported in animal studies are about 700x higher. Further, additional studies are needed to elucidate mechanistic details and distinct cell type responses of Cr(VI) neurotoxicity.
Cell culture experiments demonstrate Cr(VI) reduced cell viability in primary rat anterior pituitary and immortalized human neuroblastoma (SH-SY5Y) [120-122]. These same cell culture experiments demonstrated Cr accumulated in cells, increased oxidative stress, induced apoptosis, and led to mitochondrial dysfunction in primary rat pituitary cell lines and human neuroblastoma cell lines. One study found treating cerebellar granular neurons at immature and mature stages with Cr(VI) induced mitochondrial dysfunction, oxidative stress, lipid peroxidation, and glutathione activity equally in both stages [123]. Importantly, this study found Cr(VI) cytotoxicity was more pronounced in mature neurons compared to immature neurons [123].

While Cr(VI) is not described as a brain gerontogen, studies of peripheral tissues and cell culture models demonstrate potential gerontogenic effects. One study reported welders exposed to Cr(VI) increased serum levels of Apolipoprotein J/Clusterin, a biomarker of aging [124]. In cell cultures (L0-2 hepatocytes, BEAS-2B lung fibroblasts, WI-38 embryonic fibroblasts, primary skin fibroblasts), Cr(VI) increased expression of senescence associated β-galactosidase, SASP, clusterin, senescence marker proteins 30, p53, and p21 [125-128].

Neurotoxic effects of Cr(VI) are described in humans, animals, and cell culture models. Further, there is evidence Cr(VI) induces cellular senescence, though aging effects in the brain are not known. Persisting knowledge gaps concerning Cr(VI) neurotoxicity include: precise neurotoxic mechanisms, cell type vulnerability, regional accumulation, regional vulnerability in the brain, and the potential to act as a gerontogen.
1. Heads or Tails? Assessing the Intersection of Toxicology and Aging Using a Toxic Aging Coin

Recognizing 1) geriatrics will require specific considerations in environmental regulations, and 2) the threat of gerontogens accelerating age-related diseases, emphasizes the need for a two-sided approach to bridge aging research and toxicology. Clearly, individuals at different ages may experience distinct toxic outcomes, but this is a grave oversight in environmental regulations. The recognition that chemicals can accelerate aging at a biological level presents a new challenge to understand the roles of chemicals in aging and age-related diseases. We address this two-sided challenge by employing a “toxic aging coin” approach: on the heads side we examine how age impacts toxic outcomes, while on the tails side we consider how chemicals accelerate biological aging. Together, our toxic aging coin bridges toxicology and aging to address key knowledge gaps. The present work will examine the heads side of this coin by interrogating age-related differences in neurotoxicity following Cr(VI) exposures.

Current knowledge gaps regarding Cr(VI) neurotoxicity include a lacking understanding of Cr(VI) regional effects in the brain, a lacking understanding of Cr regional accumulation in the brain, the absence of a precise mechanism for Cr(VI)-induced neurotoxicity, and a lack of understanding for the influence of age on Cr(VI) neurotoxic outcomes. To address these knowledge gaps, we exposed male and female Sprague-Dawley rats at three different ages (3, 7, and 18 months) to 0.05 mg/L or 0.1 mg/L Cr(VI) in drinking water for 90 days. Drinking water levels of 0.05 and 0.1 mg/L Cr(VI) are the current drinking water standards established by the World Health Organization (WHO) and Environmental Protection Agency (EPA), respectively. This
study investigates the neurotoxic outcomes of Cr(VI) in rats at three different life stages by examining changes in neurobehavior, regional Cr accumulation, and regional neurodegeneration. We hypothesize Cr will accumulate preferentially in the hippocampus. We further hypothesize Cr(VI) exposure will alter neurobehavior in rats (anxiety, locomotor function, hyperactivity, spatial memory) and Cr(VI) will induce neurodegeneration in brain regions associated with this behavior (cerebellum, striatum, hippocampus). We expect increased Cr accumulation, Cr(VI)-induced neurodegeneration, and changes in neurobehavior will be most severe in geriatric rats.
2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Sodium chromate (307831), potassium phosphate dibasic trihydrate (P5504-500G), and paraformaldehyde (158127-500G) were purchased from Sigma Aldrich. Sodium phosphate monobasic monohydrate (BDH9298-500G), sodium hydroxide (BDH9292-500G), and ethylene glycol (BDH1125-4LP) were purchased from VWR Chemicals BDH. Sucrose (470302-804) was purchased from Ward’s Science. Sodium chloride was purchased from Millipore Sigma (SX0420-3). Ethanol (2716) and xylenes (1601) were purchased from Decon Labs, Inc. Permount was purchased from Electron Microscopy Sciences (17986-05). Nitric acid was purchased from Fischer Chemical (A509P212). Hydrogen peroxide was purchased from J.T. Baker (5155-01). 10x DPBS without magnesium and calcium was purchased from Corning (20-031-CV). FD NeuroSilver™ Kit II was purchased from FD NeuroTechnologies, Inc. (PK301).

2.2 Animal Study

162 male and female Sprague-Dawley rats ages 3, 7, or 18 months were purchased from Envigo (Indianapolis, IN) and fed rodent diet with 10% kcal from fat (Research Diets, D12450Ji). Rats from each age and sex were divided into 3 exposure groups, for a total of 18 groups. One group for each age and sex received tap water, 0.05
mg/L Cr(VI) in tap water, or 0.1 mg/L Cr(VI) in tap water. All groups of young and middle-aged rats consisted of 8 rats. Groups of geriatric rats with no Cr(VI) exposure consisted of 9 rats. Groups of geriatric rats exposed to 0.05 mg/L or 0.1 mg/L Cr(VI) consisted of 12 rats. Additional rats were included in geriatric groups to account for attrition due to age. The animal study design is shown in Figure 2.1.

Figure 2.1 Study Model

Figure 2.1 illustrates the design of the animal study. We exposed 3-, 7-, and 18-month-old male and female rats to Cr(VI) contaminated drinking water for 90 days. Cr(VI) levels in drinking water were consistent with WHO (0.05 mg/L) and EPA (0.1 mg/L) regulations. Rats performed a grip strength assay, open field assay, elevated plus maze, and Y-Maze assay twice each during exposure. At the end of the 90-days, rats were sacrificed, and brains were collected for histology and ICP-MS analyses of metal levels.
Rat cages were changed on a weekly basis. When changing cages, rat tails were marked with a permanent marker for identification. Cr(VI) was administered as sodium chromate dissolved in water. Cr(VI) exposures were prepared weekly from 1000x stocks in tap water: 50 mg/L and 100 mg/L Cr(VI) stocks were diluted to 0.05 mg/L and 0.1 mg/L final concentrations in drinking water. Body mass and drinking water mass were measured weekly. For QA/QC, drinking water samples were collected weekly for metals analyses to validate Cr(VI) concentration and measure background metals levels. Each cage was provided two water bottles containing a combined volume of 1 L drinking water.

Various chambers for open field assay, elevated plus maze assay, and Y-maze assay were built by Dr. Jun Cai (UofL Dept. of Pediatrics). Rats performed each neurobehavior assay twice during Cr(VI) exposure but did not perform more than one neurobehavior assay each week. The schedule for neurobehavior assays is described in Table 1. One-Step Disinfectant Cleaner and Deodorizer (VHH073) from Peroxigard or 70% ethanol was used to sanitize and deodorize equipment used in between each rat.
<table>
<thead>
<tr>
<th>Week</th>
<th>Behavior Assay Performed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No behavior assessed, Cr(VI) exposure begins</td>
</tr>
<tr>
<td>1</td>
<td>Grip Strength Assay</td>
</tr>
<tr>
<td>2</td>
<td>Open Field Assay</td>
</tr>
<tr>
<td>3</td>
<td>Elevated Plus Maze</td>
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<tr>
<td>4</td>
<td>Y-Maze</td>
</tr>
<tr>
<td>5</td>
<td>3-Chamber Assay*</td>
</tr>
<tr>
<td>6</td>
<td>No behavior assessed</td>
</tr>
<tr>
<td>7</td>
<td>Grip Strength Assay</td>
</tr>
<tr>
<td>8</td>
<td>Open Field Test</td>
</tr>
<tr>
<td>9</td>
<td>Elevated Plus Maze</td>
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<tr>
<td>10</td>
<td>Y-Maze</td>
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<tr>
<td>11</td>
<td>3-Chamber Assay*</td>
</tr>
<tr>
<td>12</td>
<td>No behavior assessed</td>
</tr>
<tr>
<td>13</td>
<td>Rats sacrificed</td>
</tr>
</tbody>
</table>

Table 1.1 outlines the schedule of behavior assays, identifying which assay was performed each week. *Data is not discussed in this thesis.
2.3 Neurobehavior Assays

Neurobehavior assays were performed in the same room. The room was kept dark aside from red light used to illuminate the assay chambers. Assay chambers were placed inside a separate apparatus surrounded by four walls to limit light and sound exposure from computers, hallways, etc. Rats were assigned a day of the week (Monday, Tuesday, Wednesday, Thursday, Friday); Cr(VI) exposure was started on this day and rats performed behavior assays on this same weekday throughout the study. This ensured that rats performed no more than 1 neurobehavior assay per week and that neurobehavior assays were performed at weekly intervals from the beginning of Cr(VI) exposure. At least two rats from each group (sex, age, and Cr(VI) exposure) were assigned to each day. Rat cages were covered during transport from the animal facility to the assay room to limit anxiety. Water bottles were upturned prior to transporting rats from the animal facility to the assay room to limit leakage. Neurobehavior assays were performed in afternoons during the light period of the 12-hour light-dark cycle.

We recorded aerial views of neurobehavior assays using a 4 mm C Series Fixed Focal Length Lens camera (Edmund Optics, 33300). We recorded side views of the open field assay using a 6 mm C Series Fixed Focal Length Lens camera (Edmund Optics, 33301). Grip strength was recorded using an ALMEMO Universal Measuring Instrument (24910). Dr. Nick Mellen (UofL Dept. of Pediatrics) created software to record and analyze neurobehavior assays (open field assay, elevated plus maze, Y-Maze) using LabVIEW 2019 (National Instruments, v.190fl).
2.3.1 Grip Strength Assay

Grip strength was assessed during weeks 1 and 7 of Cr(VI) exposure. Rat forefeet were gently lowered onto a horizontal bar to grip the bar. The bar diameter was 1.016 mm and the width was 6.35 cm. Rats were held horizontally by the tail and pulled backwards at a steady rate until the rat lost grip of the horizontal bar [129]. Peak force exerted during each trial was recorded. Measurements were only recorded when rats released grip from both forefeet simultaneously. Rats refusing to grip the bar after 10 attempts were recorded as noncompliant and measurement was not recorded. Grip strength for each rat was measured in triplicate and the median values were used for analyses and comparisons across groups. Decreasing grip strength occurs with age and is an indicator of frailty in rats [129]. Following each test, instruments were cleaned and deodorized using Peroxigard.

2.3.2 Open Field Assay

Open field assay was performed during weeks 2 and 8 of Cr(VI) exposure. The open field chamber consisted of a square, open area surrounded by four walls (Figure 2.2). One wall was transparent to allow side-view camera recording. The dimensions of the open field chamber were 71 cm x 71 cm x 43 cm (LxWxH). Rats were individually placed in the center of the open field chamber and allowed to explore the chamber for 10 minutes. Behavior recordings were assessed for changes in thigmotaxis (anxiety), distance traveled (locomotor function), and rearing behavior (locomotor function) [130]. The open field chamber was cleaned with 70% ethanol between recording sessions.
Thigmotaxis and distance traveled were measured using a program created in LabVIEW 2019 by Dr. Nick Mellen. The number of rears performed by each rat was scored manually. A ‘rear’ was defined as a rat standing on its hindlegs and raising its forefeet above its center of mass; a rear was completed when the rat returned its forefeet to the floor of the chamber. Recorded trials were blinded prior to manual scoring.

Figure 2.2 Open Field Assay Schematic

Figure 2.2 shows a schematic of the open field chamber, including dimensions and general structure. Dimensions of the open field assay chamber were 71 cm x 71 cm x 43 cm (LxWxH).
2.3.3 Elevated Plus Maze

Elevated plus maze assay was performed during weeks 3 and 9 of Cr(VI) exposure. The elevated plus maze consisted of four arms connected in the shape of a ‘plus’ (Figure 2.3). Two arms were open (without walls) and two arms were enclosed on three sides by walls. Each arm was 51 cm x 15 cm (LxW). The two closed arms had 43 cm tall walls. The middle of the maze contained a 15 cm x 15 cm (LxW) square, which created distinct borders between and connected the four arms. The entire elevated plus maze was placed on a stand such that the total elevation of the rat in the maze was 74.5 cm from the ground. Rats were individually placed in the middle of the elevated plus maze chamber facing an open arm and allowed to explore the maze for 5 minutes. Behavior was analyzed to assess changes in percent time spent exploring the open arm of the maze (anxiety) [131]. The maze was cleaned with 70% ethanol in between recording sessions. Decreased time spent exploring the open arm of the maze demonstrates increased anxiety. Digital analyses of time spent exploring open arms of the elevated plus maze were performed using LabVIEW 2019.
Figure 2.3 Elevated Plus Maze Schematic

Figure 2.3 shows the schematics of the elevated plus maze, including dimensions and general structure. Dimensions of the elevated plus maze arms were 15 cm x 51 cm x 43 cm (LxWxH). Walls were only present on enclosed arms. The center of the maze contains a 15 cm x 15 cm (LxW) square.
2.3.4 Y-Maze

Y-Maze assay was performed during weeks 4 and 10 of Cr(VI) exposure. The Y-Maze consisted of three radial arms angled in the shape of a ‘Y’ fully enclosed by walls (Figure 2.4). Each arm was 51 cm x 15 cm x 43 cm (LxWxH). The arms of the Y-Maze were connected by an equilateral triangle with 15 cm sides. Rats were individually placed in the middle of the Y-maze and allowed to explore for 8 minutes. Behavior was recorded and later analyzed to assess changes in non-alternating arm entry (spatial memory) and frequency of arm entries (activity) [132]. The maze was cleaned with 70% ethanol between each recording sessions. Rat behavior was scored manually by tracking number and sequence of arm entries to determine the frequency of alternations or non-alternations. An alternation occurred when a rat explored all three arms in sequence; a non-alternation occurred when rats entered an arm more than once in a sequence of three arm entries. Rats inherently explore novel areas; thus, increased non-alternating exploration capitulates impaired spatial memory. The percent of non-alternations was calculated using the following equation:

\[
\left(\frac{\text{Number of Non-Alternations}}{\text{Total Number of Non-Alternations} + \text{Total Number of Alternations}}\right) \times 100
\]

Arm entries per minute were scored manually to assess hyperactivity. A rat was determined to have entered an arm if all limbs left the center triangle and entered an arm. Increased arm entries per minute was interpreted as increased hyperactivity.
2.4 Euthanasia and Brain Collection

Prior to sacrifice, rats were anesthetized via intraperitoneal injection of 100 mg/mL xylazine and 100 mg/mL ketamine (combined in a 1:9 ratio) at a dose of 1 μL/g bodyweight. Additional xylazine/ketamine was administered as needed for sedation. Rats were euthanized by exsanguination, using cardiac perfusion with 250-300 mL of filtered 1x DPBS (without calcium or magnesium). Rats were decapitated following perfusion. Brains were extracted by using rongeurs to remove the skull and blunted spatulas to sever the optic nerve. Brains were rinsed in cold 1x DPBS (without calcium or magnesium) and blotted to remove excess liquid. Whole brain mass was recorded. Brains were bisected.
using a Rodent Brain Matrix (ASI Instruments, RBM-4000C). One hemisphere was fixed in 4% paraformaldehyde for histology; one hemisphere was microdissected into cortex and hippocampus before being stored at -20 °C in metal-free 1.5 mL Eppendorf tubes (Nest Biotechnology, 615001) for ICP-MS analyses. Following paraformaldehyde fixation, hemispheres for histology were immersed in a 30% sucrose solution and stored at 4 °C. Hemispheres were sectioned coronally at 30 μm increments using a sliding microtome (Leica Biosystems, SM2010R) with a 16 cm Profile C Steel Knife (Leica Biosystems, 14021607100). Sections were stored in 24-well plates in a cryoprotectant solution at -20 °C until staining.

### 2.5 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

Metal levels for tissues, water, and food were measured using inductively coupled plasma-mass spectrometry (ICP-MS). Tissues amassing up to 20 mg were digested in 500 μL 70% nitric acid for 3 hours at 85 °C; tissues up to 80 mg were digested in 800 μL 70% nitric acid for 3 hours at 85 °C. The cortex was well-beyond the mass needed for ICP-MS analyses, so the cortex was bisected coronally to obtain a usable mass and the rostral portions were used for ICP-MS analyses. 100 μL of each liquid sample (e.g., blood, water) were digested in 500 μL 70% nitric acid for 3 hours at 85 °C. After acid digestion, samples were cooled to room temperature and incubated with 100 μL 3% hydrogen peroxide for 3 hours. Samples were then diluted to a final concentration of 5% nitric acid in Millipore water. Samples were inverted to mix and filtered using an Acrodisc 32 mm 0.45 μm Supor® filters (Pall Corporation, 4654) into a trace element-free 15 mL centrifuge tubes (VWR Avantor, 89049-170). Filtered digestates were stored at -20 °C
until ICP-MS analyses. ICP-MS was performed by the Metallomics Core at the University of Louisville.

For QA/QC, additional samples were analyzed to examine other potential sources of metal contamination, including: standard rat chow used prior to study enrollment, 10% kcal rat chow (Research Diets, D12450Ji), chew toy, and cob bedding. These samples were digested and prepared in the same manner as brain tissue. Liquid samples assessed included: sodium citrate (for blood samples), tap water, treatment water, 1000x stock sodium chromate, which were prepared and digested in the same manner as other liquid samples. ICP-MS limit of detection for cortical samples was 0.008 ng/mL and the limit of detection for hippocampal samples was 0.05719 ng/mL. No samples were below the detection limit.

2.6 Silver Stain

Sections were stained using FD NeuroSilver™ Kit II from FD NeuroTechnologies, Inc. according to manufacturer protocols. Stained sections were transferred to 100 mm dishes containing 1x DPBS (without calcium and magnesium) and mounted on charged microscope slides. After drying overnight, slides were cleared 3 times for 3 minutes each with xylenes. Slides were allowed to dry again before mounting with Permount and No. 1 glass coverslips. Sections were imaged at 20x and 100x magnification with an Olympus microscope (BX43F). Images were captured using a camera (Olympus Corporation, DP74) and cellSens Standard software (Olympus Corporation, v.1.18).
2.7 Statistical Analyses

The Grubb’s Test was used to identify outliers ($\alpha = 0.05$). The Shapiro-Wilk test was used to assess normality. The one-way ANOVA and multiple comparisons tests (Tukey Test) were used to assess statistical differences across Cr(VI) exposures and age groups. Student’s T-Test, or Mann-Whitney U test were used to determine whether there was evidence of differences between sexes, regardless of age or Cr(VI) exposure. The criterion for statistical significance was $p < 0.1$ for behavior assays, $p < 0.05$ for metals analyses. All analyses were conducted using GraphPad Prism 9 (v.9.5.1). Data expressed as mean ± SEM.
3. RESULTS

3.1 Neurobehavior

3.1.1 Frailty

We assessed the effects of Cr(VI) on grip strength to evaluate frailty after 7 weeks of Cr(VI) exposure. Data shown in Figure 3.1. In control males, grip strength decreased with age (young males = 429.3 ± 48.46 g; middle-aged males = 384.1 ± 41.08 g; geriatric males = 331.5 ± 33.89 g), but we did not observe an age effect in females.

After Cr(VI) exposure, young males exhibited a concentration-associated decrease in median grip strength (control = 429.3 ± 48.46 g; 0.05 mg/L = 374.9 ± 11.03 g; 0.1 mg/L = 314.6 ± 13.65 g, p < 0.1). Middle-aged males exhibited increased grip strength after 0.05 mg/L Cr(VI) exposure but decreased grip strength after 0.1 mg/L Cr(VI) exposure (control = 384.1 ± 41.08 g; 0.05 mg/L = 543.1 ± 43.61 g, p < 0.05; 0.1 mg/L = 328.6 ± 29.02 g). Differences in grip strength between middle-aged males exposed to 0.05 mg/L Cr(VI) and 0.1 mg/L Cr(VI) were statistically significant (p < 0.01). In geriatric males, we observed a concentration-associated increase in median grip strength following Cr(VI) exposure (p < 0.01) (control = 331.5 ± 33.89 g; 0.05 mg/L = 367.4 ± 27.34 g; 0.1 mg/L = 462.7 ± 21.12 g, p < 0.05).

Young females exhibited decreased grip strength following Cr(VI) exposure (control = 308.8 ± 24.68 g; 0.05 mg/L = 266.4 ± 8.91 g; 0.1 mg/L = 277.3 ± 20.67 g).
Middle-aged females exhibited a concentration-associated decrease in median grip strength (control = 415.2 ± 32.48 g; 0.05 mg/L = 377.3 ± 27.09 g; 0.1 mg/L = 315.4 ± 32.5 g, p< 0.1). Geriatric females exhibited increased grip strength following Cr(VI) exposure (control = 271.5 ± 20.64 g; 0.05 mg/L = 359.5 ± 25.02 g, p < 0.1; 0.1 mg/L = 323.1 ± 26.59 g).

When we consider sex differences in these data, males exhibited greater grip strength than females across all study groups (median male grip strength = 400.6 ± 13.6 g; median female grip strength = 326.7 ± 9.9 g, p < 0.0001). Young males and females exhibited decreased grip strength following Cr(VI) exposure. Grip strength fluctuated in middle-aged males with Cr(VI) concentration, contrasting the consistently decreased grip strength in middle-aged females. Geriatric males and females exhibited increased grip strength following Cr(VI) exposure. While it is unlikely Cr(VI) exposure reduced frailty in middle-aged males, geriatric males, and geriatric females, we suggest this increase in grip strength reflects increased anxiety.
These data show changes in grip strength after 7 weeks of Cr(VI) exposure in drinking water. At young ages, both sexes exhibited decreased grip strength following Cr(VI) exposure. Grip strength fluctuated in middle-aged males, contrasting consistently decreased grip strength in middle-aged females. Geriatric rats of both sexes exhibited increased grip strength following Cr(VI) exposure. *p < 0.05 **p < 0.01
3.1.2 Locomotor Function

We measured the number of rears and distance traveled (cm) in the open field assay after 8 weeks of Cr(VI) exposure to assess changes in locomotor function, shown in Figure 3.2.

*Open Field Rearing Count*

We assessed the number of rears performed by each rat in the open field assay as a measure of locomotor function. Data shown in Figure 3.2A. Rearing behavior was defined as a rat lifting both forefeet from the floor, such that the forefeet were above the rat’s center of gravity. A rear was considered completed when one or both forefeet returned to the floor. The number of rears was counted during the full 10-minute duration of the open field assay. Both sexes exhibited statistically significant decreased rearing behavior with age in control groups (young males = 34 ± 2; middle-aged males = 11 ± 2, p < 0.01; geriatric males = 9 ± 1, p < 0.01; young females = 54 ± 5; middle-aged females = 40 ± 3, p < 0.01; geriatric females = 15 ± 3, p < 0.01). This age-related decrease in rearing demonstrates the validity of this assay to measure age-related impairment of locomotor function. Notably, the aging effect on rearing was more apparent in male rats.

Young and middle-aged males exhibited slightly decreased rearing behavior following 0.05 mg/L Cr(VI) exposure (young control = 34 ± 1; young 0.05 mg/L Cr(VI) = 29 ± 3; young 0.1 mg/L Cr(VI) = 32 ± 3; middle-aged control = 11 ± 2; middle-aged 0.05 mg/L Cr(VI) = 8 ± 1; middle-aged 0.1 mg/L Cr(VI) = 11 ± 2). Geriatric males exhibited decreased rearing behavior after 0.1 mg/L Cr(VI) exposure (control = 9 ± 1; 0.05 mg/L Cr(VI) = 11 ± 2; 0.1 mg/L Cr(VI) = 5 ± 0). Changes in geriatric male rearing
behavior approached statistical significance following 0.1 mg/L Cr(VI) exposure (p = 0.1056 vs. control) and was statistically significant between Cr(VI)-exposed groups (0.05 mg/L vs. 0.1 mg/L, p < 0.05). These data suggest Cr(VI) impaired locomotor function in males of all age, but the effects of Cr(VI) were more pronounced in geriatric males.

Young females did not exhibit changes in rearing behavior following Cr(VI) exposure (control = 54 ± 5; 0.05 mg/L Cr(VI) = 52 ± 3; 0.1 mg/L Cr(VI) = 55 ± 3). Middle-aged females decreased rearing behavior after Cr(VI) exposure (control = 40 ± 3; 0.05 mg/L Cr(VI) = 24 ± 2, p < 0.05; 0.1 mg/L Cr(VI) = 31 ± 5). Geriatric females exhibited no change in rearing behavior following Cr(VI) exposure (control = 15 ± 3; 0.05 mg/L Cr(VI) = 15 ± 2; 0.1 mg/L Cr(VI) = 16 ± 3). These data suggest Cr(VI) only impaired locomotor function in middle-aged females.

Considering sex differences, females exhibited 2-fold greater rearing behavior across all study groups (mean rearing by males = 16.3 ± 1.4; mean rearing by females = 32.3 ± 2.2, p < 0.0001). In young animals, Cr(VI) reduced rearing behavior in males following 0.05 mg/L Cr(VI) exposure but had no effect on young females. Middle-aged females exhibited a decrease in rearing behavior following Cr(VI) exposure, while middle-aged males were largely unaffected. Geriatric females exhibited no change in rearing behavior after Cr(VI) exposure, but geriatric males exhibited a significant decrease in rearing behavior after exposure to 0.1 mg/L Cr(VI).

Open Field Distance Traveled

We measured distance traveled during the open field assay as an additional assessment of locomotor function. Data are shown in Figure 3.2B. Control males and
females exhibited an age-dependent decrease in distance traveled in the open field assay (young males = 4,538 ± 305.1 cm; middle-aged males = 3,129 ± 450 cm, p < 0.01; geriatric males = 2,893 ± 388 cm, p < 0.01; young females = 5,483 ± 273 cm; middle-aged females = 4,845 ± 352 cm, p < 0.1; geriatric females = 4,700 ± 474 cm, p < 0.01). Age-dependent decreases in distance traveled validate the use of this assay to study age-related changes in locomotor function.

Young males exhibited increased distance traveled following 0.1 mg/L Cr(VI) exposure (control = 4,538 ± 305 cm; 0.05 mg/L Cr(VI) = 4,417 ± 439 cm; 0.1 mg/L Cr(VI) = 5,121 ± 308 cm). Middle-aged males traveled less distance after 0.05 mg/L Cr(VI) exposure (control = 3,129 ± 450 cm; 0.05 mg/L Cr(VI) = 2,438 ± 333 cm; 0.1 mg/L Cr(VI) = 3,149 ± 270 cm). Similarly, geriatric males traveled slightly less distance after 0.05 mg/L Cr(VI) exposure (control = 2,893 ± 388 cm; 0.05 mg/L Cr(VI) = 2,596 ± 184 cm; 0.1 mg/L Cr(VI) = 2,809 ± 379 cm). These data suggest Cr(VI) exposure decreased distance traveled by middle-age and geriatric males exposed to 0.05 mg/L Cr(VI), while 0.1 mg/L Cr(VI) exposure increased distance traveled by young males.

Young and middle-aged females exhibited no change in distance traveled after Cr(VI) exposure (young control = 5,483 ± 273 cm; young 0.05 mg/L Cr(VI) = 5,251 ± 236 cm; young 0.1 mg/L Cr(VI) = 5,427 ± 159 cm; middle-aged control = 4,845 ± 352 cm; middle-aged 0.05 mg/L exposed = 4,611 ± 368 cm; middle-aged 0.1 mg/L Cr(VI) = 5,090 ± 550 cm). Geriatric females traveled slightly less distance after 0.05 mg/L Cr(VI) exposure but exhibited no change following 0.1 mg/L Cr(VI) exposure (control = 4,700 ± 474 cm; 0.05 mg/L exposed = 3,948 ± 218 cm; 0.1 mg/L exposed = 4,516 ± 384 cm).
These data suggest geriatric females are more susceptible to Cr(VI)-induced impairments of locomotor function, compared to young and middle-aged females.

Considering sex differences, females typically traveled greater distances than males across all study groups (mean distance traveled by males = 3383 ± 149 cm; mean distance traveled by females = 4899 ± 133 cm, p < 0.0001). Young males and females exhibited sex differences, as 0.1 mg/L Cr(VI) exposure increased distance traveled in young males, but young females were unaffected. Middle-aged males and females also exhibited sex differences, as 0.05 mg/L Cr(VI) exposure reduced distance traveled in males, but females were less affected. No differences for Cr(VI) effects on distance traveled were observed between male and female geriatric groups. These data suggest young and middle-aged males may be more susceptible to neurotoxic effects of Cr(VI) impacting locomotion than other groups.
Figure 3.2 Changes in Locomotor Function Following Cr(VI) Exposure
3.1.3 Anxiety

We assessed anxiety by measuring the amount of time spent exploring center area of the open field assay and the amount of time spent exploring the open arm of the elevated plus maze. Data are shown in Figure 3.3.

Open Field Assay

We assessed anxiety in the open field assay as thigmotaxis, measuring the time spent exploring the center area of the chamber. Thigmotaxis refers to the tendency of an animal to travel along a wall rather than an open area; elevated anxiety is associated with higher thigmotaxis [133]. Data are presented in Figure 3.3A. Center area exploration decreased with age in both male and female controls (young males = 12.3 ± 2.7%; middle-aged males = 1.6 ± 0.3%, p < 0.05; geriatric males = 0.9 ± 0.3%, p < 0.01; young females = 10.4 ± 1.4%; middle-aged females = 7.1 ± 0.7%; geriatric females = 2.8 ± 1.3%, p < 0.01).
Young, middle-aged, and geriatric males exhibited no change in center area exploration after Cr(VI) exposure (young control = 12.3 ± 2.7%; young 0.05 mg/L Cr(VI) = 14.7 ± 3.4; young 0.1 mg/L Cr(VI) = 9.8 ± 1.7%; middle-aged control = 1.6 ± 0.3%; middle-aged 0.05 mg/L Cr(VI) = 0.4 ± 0.2%; middle-aged 0.1 mg/L Cr(VI) = 1.5 ± 0.6; geriatric control = 0.9 ± 0.3%; geriatric 0.05 mg/L Cr(VI) = 2.1 ± 0.7%; geriatric 0.1 mg/L Cr(VI) = 0.9 ± 0.2%). These data suggest Cr(VI) exposure did not affect anxiety in males, assessed during the open field assay.

Young females exhibited no change in center area exploration following Cr(VI) exposure (control = 10.4 ± 1.4%; 0.05 mg/L Cr(VI) = 13.8 ± 2.5; 0.1 mg/L Cr(VI) = 10.6 ± 1.3). Middle-aged females exhibited a concentration-associated decrease in center area exploration (control = 7.1 ± 0.7%; 0.05 mg/L Cr(VI) = 6.2 ± 1.2%; 0.1 mg/L Cr(VI) = 4.9 ± 1.2%). Geriatric females exhibited no change in center area exploration (control = 2.8 ± 1.3%; 0.05 mg/L Cr(VI) = 2.3 ± 0.6%; 0.1 mg/L Cr(VI) = 2.2 ± 0.7%). These data suggest Cr(VI) increased anxiety in a concentration-associated manner in middle-aged females.

Considering sex differences, middle-aged and geriatric females exhibited more center area exploration across all study groups (male center area exploration = 5.0 ± 0.8%; female center area exploration = 6.1 ± 0.6%, p < 0.005). Young males and females exhibited similar center area exploration and similar responses to Cr(VI). Middle-aged females explored the center area for a greater amount of time than middle-aged males. In geriatric males and females, Cr(VI) had no effect on thigmotaxis.

_Elevated Plus Maze_
We assessed anxiety using elevated plus maze after 9 weeks of Cr(VI) exposure; elevated anxiety is quantified as decreased exploration of the open arm. Anxiety data from the elevated plus maze are shown in Figure 3.3B. Control males and females exhibited decreased open arm exploration with age (young males = 52.3 ± 5.8%; middle-aged males = 5.6 ± 2.3%; geriatric males = 0.0 ± 0.0%, p<0.01; young females = 41.1 ± 3.0%; middle aged females = 18.5 ± 5.0%; geriatric females = 17.7 ± 5.0%, p < 0.01). Age differences in these control groups indicate the validity of the elevated plus maze to examine age-related changes in anxiety.

Young males exhibited a concentration-associated decrease in open arm exploration (control = 52.3 ± 5.8%; 0.05 mg/L = 45.4 ± 6.3%; 0.1 mg/L = 42.6 ± 4.8%). Middle-aged males exposed to 0.1 mg/L Cr(VI) exhibited a slight reduction in open arm exploration following 0.1 mg/L Cr(VI) exposure (control = 5.6 ± 2.3%; 0.05 mg/L = 6.1 ± 2.5%; 0.1 mg/L = 2.4 ± 1.4%). Geriatric males exhibited increased open arm exploration after Cr(VI) exposure (control = 0.0 ± 0.0%; 0.05 mg/L = 6.3 ± 2.1%; 6.5 ± 2.3%). These data suggest Cr(VI) exposure may increase anxiety in young and middle-aged males in a concentration-associated manner, but Cr(VI) induced anxiolytic effects in geriatric males.

In young females, Cr(VI) induced a concentration-associated decrease in open arm exploration (control = 41.5 ± 3.0%; 0.05 mg/L 39.0 ± 3.1%; 0.1 mg/L = 37.1 ± 3.1%). Cr(VI) slightly decreased open arm exploration in middle-aged females after exposure to 0.05 mg/L Cr(VI) (control = 18.5 ± 5.0%; 0.05 mg/L = 13.9 ± 2.6%; 0.1 mg/L = 18.2 ± 6.9%). Geriatric females exhibited a concentration-associated decrease in open arm exploration (control = 17.7 ± 5.0%; 0.05 mg/L = 14.9 ± 3.6%; 0.1 mg/L = 9.7 ±
2.1\%). These data suggest Cr(VI) exposure increased anxiety in young and geriatric females in a concentration-associated manner but had a weaker effect in middle-aged rats following 0.05 mg/L Cr(VI) exposure.

Considering sex differences, females exhibited greater open arm exploration than males across all study groups (male open arm exploration = 19.1 ± 2.5\%; female open arm exploration = 22.4 ± 1.8\%, p < 0.05). Young females, overall, spent less time exploring the open arm compared to young males. However, middle-aged and geriatric females spent a greater amount of time exploring the open arm compared to middle-aged and geriatric males, respectively. Data suggest Cr(VI) may induce anxiety in young rats of both sexes, though the effects appeared stronger in young males. Middle-aged males only exhibited an effect following 0.1 mg/L Cr(VI) exposure, whereas middle-aged females only exhibited an effect following 0.05 mg/L Cr(VI) exposure. Geriatric males exhibited increased open arm exploration, while geriatric females exhibited decreased open arm exploration. We observed an interesting sex difference when looking across age groups. In females, Cr(VI) induced anxiogenic effects at all ages, whereas effects on anxiety in males changed with age. In young males, Cr(VI) induced an anxiogenic effect in both exposed groups. In middle-aged males, Cr(VI) also induced an anxiogenic effect, but the effect was weaker and only after 0.1 mg/L. In geriatric males, the effect reversed, and Cr(VI) induced an anxiolytic effect.
Figure 3.3 Changes in Anxiety Following Cr(VI) Exposure
3.1.4 Spatial Memory

Spatial memory was assessed using the Y-maze after 10 weeks of Cr(VI) exposure. We assessed the percent of non-alternations completed by each rat. Data shown in Figure 3.4. Non-alternations were defined as a rat entering the same arm more than once in any sequence of three arm entries. Control males exhibited no change in percent non-alternations between young and middle-aged groups, but geriatric males exhibited increased non-alternations (young males = 35.9 ± 4.9%; middle-aged males = 33.8 ± 6.9%; geriatric males = 53 ± 7.8%, p < 0.01). Control middle-aged and geriatric females exhibited greater frequency of non-alternations than control young females (young females = 37.6 ± 3.2%; middle-aged females = 43.4 ± 2.8%; geriatric females = 41.4 ± 4.9%, p < 0.01).

Young males exhibited no difference in non-alternations following Cr(VI) exposure (control = 35.9 ± 4.9%; 0.05 mg/L Cr(VI) = 38.3 ± 6.3%; 0.1 mg/L Cr(VI) =
33.3 ± 3.1%). Middle-aged males exhibited a concentration-associated increase in non-alternations following Cr(VI) exposure (control = 33.8 ± 5.7%; 0.05 mg/L Cr(VI) = 39.8 ± 6.9%; 0.1 mg/L Cr(VI) = 46.3 ± 3.5%). Geriatric males exhibited no substantial change in non-alternations following Cr(VI) exposure (control = 53 ± 7.8%; 0.05 mg/L exposed = 50.9 ± 4.2%; 0.1 mg/L exposed = 47.7 ± 5.2%). These data suggest middle-aged males suffered from impaired spatial memory after Cr(VI) exposure, but young and geriatric males were unaffected.

Young females exhibited no substantial change in non-alternations following Cr(VI) exposure (control = 37.3 ± 3.2%; 0.05 mg/L Cr(VI) = 39.3 ± 4.2%; 0.1 mg/L Cr(VI) = 40.6 ± 4.1%). Middle-aged females demonstrated a concentration-associated decrease in non-alternations (control = 43.4 ± 2.8%; 0.05 mg/L Cr(VI) = 36.3 ± 3.4%; 0.1 mg/L Cr(VI) = 30.3 ± 3.9%, p < 0.05). Geriatric females exhibited no change in non-alternations following Cr(VI) exposure (control = 41.4 ± 2.8%; 0.05 mg/L Cr(VI) = 42.8 ± 2.9%; 0.1 mg/L exposed = 39.5 ± 2.8%). These data suggest Cr(VI) exposure slightly impaired spatial memory of young females, but Cr(VI) exposure improved spatial memory in middle-aged females.

Considering sex differences, females exhibited significantly greater arm entries per minute than males, across all study groups (male entries per minute = 2.4 ± 0.1; female entries per minute = 3.3 ± 0.1, p < 0.0001). These data show opposite sex differences in middle-aged groups: middle-aged males exhibited increased non-alternations in a concentration-associated manner, whereas middle-aged females exhibited a concentration-associated decrease in non-alternations. These data suggest
Cr(VI) impairs spatial memory in middle-aged males but improves spatial memory in middle-aged females.

**Figure 3.4 Changes in Spatial Memory Following Cr(VI) Exposure**

This figure demonstrates Cr(VI) effects on non-alternations after 10 weeks Cr(VI). Notably, middle-aged males increased percent non-alternations in a concentration-associated manner, whereas middle-aged females exhibited a concentration-associated decrease in percent alternations. **p < 0.05 ***p < 0.01
3.1.5 Activity

We assessed the frequency of arm entries in the Y-maze after 10 weeks Cr(VI) exposure to measure of changes in activity. Data are presented in Figure 3.5. Control females exhibited an age-associated decrease in arm entries per minute, whereas control males only exhibited decreased arm entries per minute in geriatrics (young males = 2.9 ± 0.2; middle-aged males = 2.8 ± 0.1; geriatric males = 1.6 ± 0.2, p < 0.01; young females = 3.9 ± 0.1; middle-aged females = 3.4 ± 0.1; geriatric females = 2.6 ± 0.2, p < 0.01). These data indicate females became hypoactive with age and males were only hypoactive as geriatrics, validating the use of the Y-Maze to assess age-related changes in activity.

Young males exhibited a slight concentration-associated increase in activity following Cr(VI) exposure (control 2.9 ± 0.2; 0.05 mg/L Cr(VI) = 3.1 ± 0.0; 0.1 mg/L Cr(VI) = 3.3 ± 0.2). Middle-aged males decreased arm entries per minute following Cr(VI) exposure (control = 2.8 ± 0.1; 0.05 mg/L Cr(VI) = 1.8 ± 0.3, p < 0.01; 0.1 mg/L Cr(VI) = 2.2 ± 0.1). Geriatric males exhibited no change in activity following Cr(VI) exposure, and all groups were hypoactive compared to younger groups (control = 1.6 ± 0.2; 0.05 mg/L Cr(VI) = 1.9 ± 0.1; 0.1 mg/L Cr(VI) = 1.8 ± 0.1). These data suggest Cr(VI) exposure induced hyperactivity in young males and hypoactivity in middle-aged males, but geriatric males were unaffected.

Young females exhibited a slight, concentration-associated increase in arm entries per minute following Cr(VI) exposure (control = 3.9 ± 0.1; 0.05 mg/L Cr(VI) = 4.1 ± 0.2; 0.1 mg/L Cr(VI) = 4.2 ± 0.2). Middle-aged and geriatric females exhibited no change in activity following Cr(VI) exposure (middle-aged control = 3.4 ± 0.1; middle-aged 0.05 mg/L Cr(VI) = 3.2 ± 0.2; middle-aged 0.1 mg/L Cr(VI) = 3.5 ± 0.2; geriatric control =
2.6 ± 0.2; geriatric 0.05 mg/L Cr(VI) = 2.3 ± 0.1; geriatric 0.1 mg/L Cr(VI) = 2.8 ± 0.2). These data suggest Cr(VI) may have induced hyperactivity in young females but did not affect middle-aged or geriatric females.

Considering sex differences, females were generally more active than males across all study groups (male activity = 2.4 ± 0.1; female activity = 3.3 ± 0.1, p < 0.0001). Young males and females exhibited slight increases in activity following Cr(VI) exposure, suggesting Cr(VI) may contribute to hyperactivity at a young age. Middle-aged males became hypoactive following Cr(VI) exposure, while middle-aged females exhibited little change in activity following Cr(VI) exposure. Neither geriatric males or females exhibited changes in activity following Cr(VI) exposure.
Figure 3.5. Changes in Activity Following Cr(VI) Exposure

This figure shows arm entries per minute in the Y-maze. Arm entries per minute decreased with age in control rats of both sexes. Data suggest middle-aged males become hypoactive, while young males and females may become hyperactive following Cr(VI) exposure. Geriatrics were generally hypoactive and unaffected by Cr(VI). ***p < 0.01
3.2 Total Cr Measured by Inductively Coupled Plasma-Mass Spectrometry

We used inductively coupled plasma-mass spectrometry (ICP-MS) to measure total Cr levels across brain regions. Brains were microdissected to isolate the hippocampus and frontal cortex for ICP-MS analyses.

Cortex

Cortical Cr levels are shown in Figure 3.6. Control males exhibited slightly increased cortical Cr with age (young males = 8.9 ± 7.7 ng/g; middle-aged males = 12.1 ± 2.3 ng/g; geriatric males = 14.6 ± 2.7 ng/g). Control females exhibited no change in cortical Cr levels across age groups (young females = 14.6 ± 2.9 ng/g; middle-aged females = 16.8 ± 5.1 ng/g; geriatric females = 14.7 ± 3.0 ng/g).

Males did not exhibit changes in cortical Cr levels in young (control = 8.9 ± 2.7 ng/g; 0.05 mg/L Cr(VI) = 14.9 ± 4.3 ng/g; 0.1 mg/L Cr(VI) = 9.1 ± 1.7 ng/g), middle-aged (control = 12.1 ± 2.3 ng/g; 0.05 mg/L Cr(VI) = 19.6 ± 6.2 ng/g; 0.1 mg/L Cr(VI) = 15.8 ± 2.4 ng/g), or geriatric groups (control = 14.6 ± 2.7 ng/g; 0.05 mg/L Cr(VI) = 14.6 ± 3.0 ng/g; 0.1 mg/L Cr(VI) = 10.9 ± 1.5 ng/g).

Young females exhibited decreased cortical Cr levels following 0.1 mg/L Cr(VI) exposure (control = 14.6 ± 2.9 ng/g; 0.05 mg/L Cr(VI) = 17.8 ± 4.7 ng/g; 0.1 mg/L Cr(VI) = 2.9 ± 0.5 ng/g). Changes in cortical Cr levels were statistically significant between 0.05 mg/L and 0.1 mg/L Cr(VI) exposed groups (p < 0.05). Middle-aged (control = 12.5 ± 2.9 ng/g; 0.05 mg/L Cr(VI) = 15.6 ± 2.9 ng/g; 0.1 mg/L Cr(VI) = 14.5 ± 3.1 ng/g) and geriatric females (control = 14.7 ± 3.0 ng/g; 0.05 mg/L Cr(VI) = 16.8 ± 3.0 ng/g).
2.89 ng/g; 0.1 mg/L Cr(VI) = 15.5 ± 3.1 ng/g) exhibited no changes in cortical Cr levels following Cr(VI) exposure.

The only relevant sex difference noted is that young females exhibited decreased cortical Cr levels, while Cr levels in young males did not change following Cr(VI) exposure.

Figure 3.6 Cortical Cr Accumulation Following 90-Day Cr(VI) Drinking Water Exposure

Young females exhibited lower cortical Cr levels following exposure to 0.1 mg/L Cr(VI) for 90 days, otherwise there were no differences across study groups.
Hippocampus

Hippocampal Cr levels are shown in Figure 3.7. Cr hippocampal levels increased with age in control males (young males = 21.5 ± 2.7 ng/g; middle-aged males = 26.1 ± 4.7 ng/g; geriatric males = 35.9 ± 4.8 ng/g, p < 0.1). Control females exhibited lower hippocampal Cr levels in middle-aged and geriatric rats compared to young rats (young females = 39.9 ± 10.1 ng/g; middle-aged females = 21.4 ± 4.7 ng/g; geriatric females = 32.8 ± 4.6 ng/g).

Males did not exhibit changes in hippocampal Cr levels in young (control = 21.5 ± 2.7 ng/g; 0.05 mg/L Cr(VI) = 21.8 ± 3.3 ng/g; 0.1 mg/L Cr(VI) = 20.0 ± 3.9 ng/g), middle-aged (control = 26.1 ± 4.7 ng/g; 0.05 mg/L Cr(VI) = 22.3 ± 4.5 ng/g; 0.1 mg/L Cr(VI) = 20.0 ± 4.1 ng/g), or geriatric groups (control = 35.9 ± 4.8 ng/g; 0.05 mg/L Cr(VI) = 51.1 ± 8.0 ng/g; 0.1 mg/L Cr(VI) = 38.3 ± 6.5 ng/g). These data suggest Cr does not accumulate in the male hippocampus.

Young females exhibited slightly higher hippocampal Cr in young females after 0.1 mg/L Cr(VI) exposure (control = 39.4 ± 10.1 ng/g; 0.05 mg/L Cr(VI) = 31.9 ± 5.3 ng/g; 0.1 mg/L Cr(VI) = 47.0 ± 11.5 ng/g). Middle-aged females exhibited a concentration-associated increase in hippocampal Cr levels (control = 21.4 ± 4.7 ng/g; 0.05 mg/L Cr(VI) = 32.3 ± 6.8 ng/g; 0.1 mg/L Cr(VI) = 34.9 ± 4.7 ng/g). Geriatric females exhibited the most drastic concentration-associated increase in hippocampal Cr levels of all the study groups, exhibiting 3x and 4x higher Cr in Cr(VI) exposed groups compared to geriatric female controls (control = 32.8 ± 4.7 ng/g; 0.05 mg/L Cr(VI) = 103.7 ± 16.9, p < 0.01; 0.1 mg/L Cr(VI) = 137.5 ± 16.2 ng/g, p < 0.01). These data
suggest hippocampal levels of Cr in females increased in a concentration-associated manner and was more likely to accumulate in hippocampus in older animals.

We observed some key sex differences for Cr accumulation in hippocampus. Females exhibited greater hippocampal Cr levels than males across all study groups (male hippocampal Cr = 31.27 ng/g; female hippocampal Cr = 51.45 ng/g, p < 0.01). Hippocampal Cr levels were higher in young and geriatric females when compared to age-matched males. Young females exhibited higher hippocampal Cr levels following 0.1 mg/L Cr(VI) exposure, but hippocampal Cr levels did not change in males following Cr(VI) exposure. Further, middle-aged females exhibited a concentration-associated increase in hippocampal Cr levels, while hippocampal Cr levels in middle-aged males were unaffected. Geriatrics of both sexes exhibited higher hippocampal Cr following Cr(VI) exposure, but hippocampal Cr levels in geriatric females are 2-4x higher than hippocampal Cr levels in geriatric males.
3.3 Neurodegeneration: Silver Stain

Silver stain was used to identify brain regions with Cr(VI)-induced neurodegeneration. We focused analyses on brain regions associated with the
neurobehaviors interrogated in our study. Figures demonstrating silver stain are composed of images captured at 200x and 1000x magnification.

*Cerebellum*

We examined neurodegeneration in the cerebellum to determine if neurodegeneration in these regions contributed to Cr(VI)-induced effects on locomotor function. We examined simple lobule B of the cerebellum, a region related to coordinated, voluntary movement and proprioception in rats. Data from females are shown in **Figure 3.8** (200x magnification). Regions of interest are shown at greater magnification (1000x) in **Figure 3.9**. Age-dependent neurodegeneration occurred in the cortex, white matter, and molecular layers of this region in both sexes.

Young and middle-aged females exhibited increased neurodegeneration in the cerebellar cortex and white matter following Cr(VI) exposure. Control geriatric females exhibited neurodegeneration in these regions and neurodegeneration was not exacerbated following Cr(VI) exposure.

Young males exhibited increased neurodegeneration in the cerebellar white matter following 0.1 mg/L Cr(VI) exposure. Control middle-aged and geriatric males exhibited neurodegeneration in the cerebellar cortex and white matter, which was not exacerbated following Cr(VI) exposure.

Concerning sex differences, females exhibited increased susceptibility to age- and Cr(VI)-induced neurodegeneration in simple lobule B of the cerebellum.
Figure 3.8 Neurodegeneration in the Female Cerebellum Following 90-Day Cr(VI) Drinking Water Exposure (200x Magnification)

This figure shows silver stain in simple lobule B of the cerebellum, 200x magnification. Females exhibited Cr(VI)-induced neurodegeneration in the cortex and white matter of the cerebellum. Boxes indicate regions in the cerebellar cortex shown at 1000x magnification in Figure 3.9. CTX = Cortex; ML = Molecular Layer; WM = White Matter. Scale Bar = 50 μm.
Figure 3.9 Neurodegeneration in the Female Cerebellum Following 90-Day Cr(VI) Drinking Water Exposure (1000x Magnification)

This figure shows silver stain in the cerebellar cortex of simple lobule B of the cerebellum, 1000x magnification. Females exhibited Cr(VI)-induced neurodegeneration in the cerebellar cortex. Images correspond to the appropriate boxes shown in Figure 3.8. Scale Bar = 10 μm.
Dorsal Caudate Putamen

We examined the dorsal caudate putamen to determine if changes in locomotor function or activity may be related to neurodegeneration. Data from males are shown in Figure 3.10 (200x magnification). Regions of interest are shown at greater magnification (1000x) in Figure 3.11. Neurodegeneration increased with age in control males. In females, control geriatrics exhibited increased neurodegeneration compared to younger ages, but there was no noticeable difference in neurodegeneration between young and middle-aged control females. Notably, neurodegeneration was most prominent in the white matter of the dorsal caudate putamen.

Young males exhibited neurodegeneration in the dorsal caudate putamen following Cr(VI) exposure. Young males exhibited neurodegeneration in the gray matter of the dorsal caudate putamen following 0.05 mg/L Cr(VI) exposure, but exposure to 0.1 mg/L Cr(VI) induced greater neurodegeneration in the white matter of the young male caudate putamen. Control middle-aged males exhibited some neurodegeneration in the white matter, which was exacerbated following Cr(VI) exposure. When considering the gray matter, middle-aged males exhibited increased neurodegeneration compared to control middle-aged males. Geriatric males did not exhibit an increase in neurodegeneration following Cr(VI) exposure.

Young females did not exhibit Cr(VI)-induced neurodegeneration in the dorsal caudate putamen. Middle-aged females exhibited slightly increased neurodegeneration following Cr(VI) exposure. Geriatric females did not exhibit increased neurodegeneration following Cr(VI) exposure.
Sex differences are apparent, as young males exhibited Cr(VI)-induced neurodegeneration in the dorsal caudate putamen, but young females did not. Middle-aged males were more impacted by Cr(VI)-induced neurodegeneration, compared to middle-aged females. Cr(VI) did not exacerbate neurodegeneration in geriatric animals of either sex. In both sexes, neurodegeneration appears most prominent in white matter tracts of the dorsal caudate putamen, rather than gray matter areas.
Figure 3.10 Neurodegeneration in the Male Caudate Putamen (Dorsal) Following 90-Day Cr(VI) Drinking Water Exposure (200x Magnification)

This figure shows changes in neurodegeneration of the male dorsal caudate putamen following Cr(VI) exposure, 200x magnification. Young and middle-aged males exhibited Cr(VI)-induced neurodegeneration in the white matter of the dorsal caudate putamen, though Cr(VI)-induced neurodegeneration was not increased in geriatric males following Cr(VI) exposure. Boxes indicate regions of interest shown at 1000x in Figure 3.11. GM = Gray Matter; WM = White Matter (Note: only one WM tract is labeled in each image, though more are present). Scale Bar = 50 μm.
Figure 3.11 Neurodegeneration in the Male Caudate Putamen (Dorsal) Following 90-Day Cr(VI) Drinking Water Exposure (1000x Magnification)

This figure shows changes in neurodegeneration of the male dorsal caudate putamen following Cr(VI) exposure, 1000x magnification. Young and middle-aged males exhibited Cr(VI)-induced neurodegeneration in the white matter of the dorsal caudate putamen, though Cr(VI)-induced neurodegeneration was not increased in geriatric males following Cr(VI) exposure. Images correspond to boxes in images shown at 200x magnification in Figure 3.10. GM = Gray Matter; WM = White Matter (Note: only one WM tract is labeled in each image, though more are present). Scale Bar = 10 μm.
Dentate Gyrus and CA3 of the Dorsal Hippocampus

We assessed the dorsal hippocampus to determine if spatial memory impairment may be linked to neurodegeneration in this region. Data from females are shown in Figure 3.10A and Figure 3.11A. Control young males exhibited the greatest amount of neurodegeneration in the dorsal hippocampus, compared to control middle-aged and geriatric males. Control middle-aged females exhibited more neurodegeneration compared to control young females; however, control geriatric females exhibited less neurodegeneration than both control young and middle-aged females. We propose age-related priming of microglia has enhanced clearance of degenerating neurons in control middle-aged and geriatric males, as well as in control geriatric females.

Young females exhibited increased neurodegeneration in the dentate gyrus and CA3 following Cr(VI) exposure. Middle-aged females exhibited increased neurodegeneration in the dentate gyrus and CA3 region following Cr(VI) exposure. Geriatric females exhibited increased neurodegeneration in the dentate gyrus and CA3 following Cr(VI) exposure.

Young males did not exhibit increased neurodegeneration in the dentate gyrus following Cr(VI) exposure. Middle-aged males exhibited increased neurodegeneration in the dentate gyrus following Cr(VI) exposure. Geriatric males exhibited increased neurodegeneration in the dorsal hippocampus and CA3 following Cr(VI) exposure.

Considering sex differences, young females exhibited increased neurodegeneration in the dentate gyrus following a 0.05 mg/L Cr(VI) exposure relative to exposure-matched young males; however, neither sex exhibited increased
neurodegeneration following 0.1 mg/L Cr(VI) exposure. Middle-aged males and females exhibited increased neurodegeneration in the dentate gyrus and CA3 region following Cr(VI) exposure; however, middle-aged females exhibited more severe neurodegeneration in the dentate gyrus and CA3 region of the hippocampus than middle-aged males (further observations described below with Figure 10.B). Cr(VI) induced neurodegeneration was consistent between geriatric males and females following 0.05 mg/L Cr(VI) exposure. Interestingly, 0.1 mg/L Cr(VI) exposure induced neurodegeneration in the male dentate gyrus, but Cr(VI)-induced neurodegeneration was not present in the geriatric female dorsal hippocampus following 0.1 mg/L Cr(VI) exposure.

Given our results on the Y-maze for spatial memory showing opposite Cr(VI) effects in middle-aged groups (Figure 3.5), we compared differences in hippocampal neurodegeneration in these groups (Figure 10.B). More substantial neurodegeneration in the dentate gyrus and CA3 of middle-aged females, compare to middle-aged males, suggests the development of side preferences may have skewed data concerning spatial memory obtained using the Y-Maze.
Figure 3.12. Neurodegeneration in the Dorsal Hippocampus (Dentate Gyrus and CA3) Following 90-Day Drinking Water Cr(VI) Exposure (200x magnification).
Figure 3.12 (description) These figures demonstrate Cr(VI)-induced neurodegeneration in the dentate gyrus and CA3 region of the hippocampus, 200x magnification. (A) Cr(VI)-induces significant neurodegeneration in the dentate gyrus and CA3 of female rats at all ages. (B) Middle-aged female rats exhibited more severe neurodegeneration in the dentate gyrus and CA3 region compared to middle-aged males. Boxes in these images indicate the region of interest shown in the corresponding image at 1000x in Figure 3.13. DG = Dentate Gyrus, CA3 = CA3 Region. Scale Bar = 50 μm.
Figure 3.13. Neurodegeneration in the Female Hippocampus Following 90-Day Cr(VI) Drinking Water Exposure (1000x magnification)
Figure 3.13 (description) These figures demonstrate Cr(VI)-induced neurodegeneration in the dentate gyrus of the hippocampus, 1000x magnification. (A) Cr(VI)-induces significant neurodegeneration in the dentate gyrus of female rats at all ages. (B) Middle-aged female rats exhibited more severe neurodegeneration in the dentate gyrus and CA3 region compared to middle-aged males. Images correspond to boxes in images shown at 200x magnification in Figure 3.11. DG = Dentate Gyrus, CA3 = CA3 Region. Scale Bar = 10 μm.
4. DISCUSSION

4.1 Project Summary

This thesis interrogated the neurotoxic effects of long-term exposure to low concentrations of Cr(VI) in drinking water in rats at three different ages. This project utilized a toxic aging coin approach, specifically employing the heads side of the toxic aging coin to examine how age impacts neurotoxic outcomes. Primary goals of this project examined regional Cr accumulation in the brain, Cr(VI)-induced behavior changes, and Cr(VI)-induced neurodegeneration. Our data strongly suggest Cr(VI) exposure induced neurotoxic effects in animals across all three ages.

4.2 Cr Brain Accumulation

This is the first study to examine Cr brain accumulation in the context of age- and sex-differences. Our data demonstrate hippocampal Cr accumulation occurred in a concentration-associated manner, only in middle-aged and geriatric females. Cr(VI)-exposed geriatric females exhibited the greatest hippocampal Cr levels, with 3- or 4-fold higher levels than age-matched controls (control geriatric female = 32.8 ± 4.7 ng/g; 0.05 mg/L Cr(VI) geriatric female = 103.7 ± 16.9; 0.1 mg/L Cr(VI) geriatric female = 137.5 ± 16.2 ng/g). The only change observed in cortical Cr levels was decreased cortical Cr in young females exposed to 0.1 mg/L Cr(VI), suggesting region-specific accumulation of Cr.
To put our results in context, Table 4.1 summarizes reported Cr brain accumulation following drinking water exposures in animal studies. Literature regarding Cr accumulation in the brain is inconsistent and mostly reported as whole brain Cr levels, with data regarding region-specific accumulation largely absent. Cr accumulation is not yet reported in the hippocampus, though several studies have examined Cr accumulation in other regions or in the whole brain. Nudler et al. (2009) exposed adult male Wistar rats to 100 ppm Cr(VI) in drinking water for 30 days and reported hypothalamic accumulation of 26 ng/g Cr and anterior pituitary gland accumulation of 274 ng/g Cr [113]. This study utilized a Cr(VI) concentration 1000x greater than ours but reported a hypothalamic Cr level only slightly higher than the hippocampal Cr accumulation in our middle-aged males exposed to 0.1 mg/L Cr(VI) (our study = 19.98 ng/g). The pituitary gland exhibits increased BBB permeability for hormone production and release, providing a possible explanation for substantially increased Cr accumulation in the pituitary gland. Suljević et al. (2021) reported a 20-day exposure to 1.2 or 2.4 mg/L Cr(VI) in drinking water significantly increased whole brain Cr accumulation in 5-month-old male Japanese quails (10,580 ng/g and 10,620 ng/g, respectively; Note: Cr levels in control brains of this study were below detectable limits) [134]. It is important to note these studies only utilized young or middle-aged males of each species. Our data suggest females and geriatrics likely accumulate higher brain Cr, evident by increased hippocampal Cr levels in these animals. Collectively, these data demonstrate whole brain Cr accumulation does not accurately reflect the impact of Cr in the brain, as the regions of the brain are highly specialized. Understanding the regional effects and accumulation of Cr is critical for determining how neurotoxic outcomes are directly or indirectly
related to Cr(VI). Results obtained using our model demonstrate the limitations of assessing Cr brain accumulation using a single age or sex and highlight the importance of these characteristics in studying chemical-induced neurotoxicity.

Control animals in our study exhibited hippocampal Cr concentrations ranging from 8.43 ng/g Cr to 96.62 ng/g Cr and cortical Cr levels in our control animals ranged from 2.71 ng/g Cr to 42.77 ng/g Cr. These data suggest the hippocampus is prone to greater Cr accumulation than the cortex. Cr levels in control groups of other studies varied greatly. Suljević et al. (2021) reported Cr levels measured by UV-Vis spectroscopy in control quail brains were below detectable limits. Nudler et al. (2009) reported control Cr levels measured by AAS were 7 ng/g in the hypothalamus and 25 ng/g in the pituitary gland of rats. This lack of consistency in control animal brains may be due to differences in model species or detection methods. AAS and ICP-MS have detection limits significantly lower than UV-Vis Spectroscopy, allowing for very low levels to be detected. ICP-MS offers detection of more elements than AAS, making ICP-MS a more efficient method for determining multiple metal concentrations; however, maintaining consistency in reported values between studies and ICP-MS spectrometers is difficult. Our study utilized ICP-MS.

Nudler et al. (2009) utilized used significantly greater amounts of Cr(VI) in their exposures and exposed rats for a much shorter time in their study, but they reported hypothalamic levels similar to control hippocampi in our study. The highest hippocampal Cr level (233.8 ng/g Cr) in our study was observed in a geriatric female exposed to 0.05 mg/L Cr(VI) for 90 days. This Cr level is similar to pituitary Cr levels found in rats exposed to 100 ppm Cr(VI) for 30 days by Nudler et al. (2009), but the hippocampal
level is much greater than the exposed hypothalamus in this study. Altogether, evidence from our study and the literature demonstrate Cr will accumulate in the hippocampus and pituitary but not in hypothalamus or cortex.
<table>
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<th>Research Model</th>
<th>Cr(VI) Exposure (Length of Exposure)</th>
<th>Regions Examined</th>
<th>Mean Cr Level (Cr Level Range)</th>
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<td>Anterior Pituitary Gland</td>
<td>274 ng/g (N.R.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Month-Old Male Japanese Quail</td>
<td>1.2 μg/mL (20 days)</td>
<td>Whole Brain</td>
<td>10,580 ng/g (N.R.)</td>
<td>UV-Vis Spectroscopy</td>
<td>[134]</td>
</tr>
<tr>
<td></td>
<td>Concentration</td>
<td>Tissue</td>
<td>Method</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------------------------</td>
<td>--------------</td>
<td>---------</td>
<td>-----------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>Female Swiss Mice</td>
<td>2.4 μg/mL</td>
<td>Whole Brain</td>
<td>AAS</td>
<td>496 ng/g* (N.R.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(20 Days)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>25 mg/kg orally in drinking water</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>(3 Days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Month-Old Male Sprague-Dawley Rats</td>
<td>0.05 mg/L</td>
<td>Cortex</td>
<td>ICP-MS</td>
<td>14.85 ng/g (3.84 ng/g - 38.09 ng/g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(90 Days)</td>
<td></td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.092 ng/g (1.679 ng/g - 14.71 ng/g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.725 ng/g (1.74 ng/g - 14.64 ng/g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 mg/L</td>
<td>Cortex</td>
<td>ICP-MS</td>
<td>18.71 ng/g (15.19 ng/g – 24 ng/g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(90 Days)</td>
<td></td>
<td>This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16.3 ng/g (11.2 ng/g - 19.85 ng/g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hippocampus</td>
<td>ICP-MS</td>
<td>17.8 ng/g (2.442 ng/g - 41.57 ng/g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05 mg/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(90 Days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 mg/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(90 Days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age Group</td>
<td>Dose (mg/L)</td>
<td>Time (Days)</td>
<td>Tissue</td>
<td>Concentration (ng/g)</td>
<td>Method</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-----------------</td>
<td>------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>3-Month-Old Female</td>
<td>0.1</td>
<td>90</td>
<td>Hippocampus</td>
<td>9.321 (1.415 - 30.28)</td>
<td>ICP-MS</td>
</tr>
<tr>
<td>Sprague-Dawley Rats</td>
<td>0.05</td>
<td>90</td>
<td></td>
<td>31.87 (14.67 - 58.62)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>90</td>
<td></td>
<td>46.97 (14.05 - 95.05)</td>
<td></td>
</tr>
<tr>
<td>7-Month-Old Male</td>
<td>0.05</td>
<td>90</td>
<td>Cortex</td>
<td>19.56 (5.56 - 55.63)</td>
<td>ICP-MS</td>
</tr>
<tr>
<td>Sprague-Dawley Rats</td>
<td>0.1</td>
<td>90</td>
<td></td>
<td>15.8 (5.88 - 26.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>90</td>
<td>Hippocampus</td>
<td>22.34 (5.35 - 39.28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>90</td>
<td></td>
<td>19.98 (8.79 - 38.3)</td>
<td></td>
</tr>
<tr>
<td>Age Group</td>
<td>Treatment</td>
<td>Tissue</td>
<td>Concentration</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>------------</td>
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<td>----------------</td>
<td></td>
</tr>
<tr>
<td>7-Month-Old Female</td>
<td>0.05 mg/L</td>
<td>Cortex</td>
<td>15.62 ng/g</td>
<td>5.54 ng/g - 25.36 ng/g</td>
<td></td>
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<tr>
<td></td>
<td>(90 Days)</td>
<td></td>
<td>14.48 ng/g</td>
<td>3.65 ng/g - 27.43 ng/g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 mg/L</td>
<td>Cortex</td>
<td>32.28 ng/g</td>
<td>17.84 ng/g - 69.92 ng/g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(90 Days)</td>
<td></td>
<td>34.94 ng/g</td>
<td>21.99 ng/g - 53.68 ng/g</td>
<td></td>
</tr>
<tr>
<td>7-Month-Old Female</td>
<td>0.05 mg/L</td>
<td>Hippocampus</td>
<td>51.09 ng/g</td>
<td>17.56 ng/g - 91.39 ng/g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(90 Days)</td>
<td></td>
<td>12.3 ng/g</td>
<td>4.67 ng/g - 22.83 ng/g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 mg/L</td>
<td>Hippocampus</td>
<td>10.88 ng/g</td>
<td>5.44 ng/g - 18.57 ng/g</td>
<td></td>
</tr>
<tr>
<td>18-Month-Old Male</td>
<td>0.05 mg/L</td>
<td>Cortex</td>
<td>10.88 ng/g</td>
<td>5.44 ng/g - 18.57 ng/g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(90 Days)</td>
<td></td>
<td>51.09 ng/g</td>
<td>17.56 ng/g - 91.39 ng/g</td>
<td></td>
</tr>
</tbody>
</table>

ICP-MS: This study
<table>
<thead>
<tr>
<th>18-Month-Old Female Sprague-Dawley Rats</th>
<th>0.05 mg/L (90 Days)</th>
<th>Cortex</th>
<th>14.34 ng/g (5.89 ng/g - 24.66 ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-Month-Old Female Sprague-Dawley Rats</td>
<td>0.1 mg/L (90 Days)</td>
<td>Cortex</td>
<td>15.46 ng/g (5.93 ng/g - 35.88 ng/g)</td>
</tr>
<tr>
<td>18-Month-Old Female Sprague-Dawley Rats</td>
<td>0.05 mg/L (90 Days)</td>
<td>Hippocampus</td>
<td>103.7 ng/g (47.29 ng/g - 233.8 ng/g)</td>
</tr>
<tr>
<td>18-Month-Old Female Sprague-Dawley Rats</td>
<td>0.1 mg/L (90 Days)</td>
<td>Hippocampus</td>
<td>137.5 ng/g (86.54 ng/g - 228.3 ng/g)</td>
</tr>
</tbody>
</table>

| 0.1 mg/L (90 Days) | 38.3 ng/g (14.2 ng/g - 80.77 ng/g) |

Table 4.1 summarizes literature where Cr levels are reported in the brain following drinking water exposure to Cr(VI). AAS = Atomic Absorption Spectrometry, ICP-MS = Inductively Coupled Plasma-Mass Spectrometry. N.R. = Not Reported. *Raw values for whole brain homogenate Cr levels not reported. Value reported as a 60% increase Cr levels of control brain homogenate.
4.3 Cr(VI)-Induced Changes in Behavior

We assessed a variety of behaviors using this model (Figure 2.1) to demonstrate Cr(VI) neurotoxicity. We assessed behaviors using the grip strength assay, elevated plus maze, open field assay, and Y-Maze. We used results from these assays to interrogate the effects of Cr(VI) on neurobehaviors such as frailty, anxiety, locomotor function, activity, and spatial memory. Data gathered from control animals of each age in these assays are consistent with aging effects reported in the literature and validate the use of these assays to study age-related changes in grip strength, anxiety, and locomotor function [129, 136-148]. Behavior data presented in this thesis demonstrated several age- and sex-specific changes in behavior following Cr(VI) exposure.

Regarding age-differences, we observed decreased grip strength in young animals following Cr(VI) exposure, whereas geriatric animals increased grip strength. We suggested that increased grip strength in geriatric animals was a result of increased anxiety rather than improvement of their frailty state, but our anxiety data did not support this hypothesis. In males, we observed age-specific differences in open arm exploration during the elevated plus maze: young males exhibited less open arm exploration following Cr(VI) exposure, whereas geriatric males exhibited increased open arm exploration. We observed two key age-differences in the Y-Maze. First, middle-aged females performed fewer non-alternations following Cr(VI) exposure, which was inconsistent with other female age groups and may indicate a neuro-hormonal effect by Cr(VI). Further, we observed patterns towards concentration-associated hyperactivity in young animals of both sexes, while activity was unchanged or decreased in other age groups.
Regarding sex-differences, middle-aged males exhibited significantly increased grip strength following 0.05 mg/L Cr(VI) exposure, while females exhibited slightly decreased grip strength following exposure to the same concentration of Cr(VI). In the elevated plus maze, geriatric males exhibited increased open arm exploration following Cr(VI) exposure, while geriatric females exhibited a concentration-associated decrease in open arm exploration. The clearest sex difference in this study was observed in the Y-Maze, where middle-aged males exhibited a concentration-associated increase in non-alternations while middle-aged females exhibited a concentration-associated decrease in non-alternations. These data strongly suggest Cr(VI)-induced neurotoxicity is impacted by sex and age.

Locomotor function is of particular concern in the context of Cr(VI) exposure, as environmental exposure to Cr(VI) is linked to motor neuron disease in humans [109]. We measured rearing behavior during the open field assay to assess locomotor function and observed Cr(VI) decreased rearing behavior, with the largest reduction observed in middle-aged females (control = 40 ± 3; 0.05 mg/L = 24 ± 2, p < 0.05; 0.1 mg/L = 31 ± 5) (Figure 3.2A). We also measured distance traveled in the open field assay and observed Cr(VI) exposure decreased distance traveled in most groups, but not in young (2-month-old) rats. We observed the largest change in distance traveled in middle-aged males (control = 3,129 ± 450 cm; 0.05 mg/L Cr(VI) = 2,438 ± 333 cm; 0.1 mg/L Cr(VI) = 3,149 ± 270 cm). Singh and Chowdhuri (2016) observed fruit flies exposed to 20 μg/mL Cr(VI) as larvae exhibited decreased performance in climbing and jumping assays, suggesting locomotor impairment in adult flies following a juvenile exposure [139]. Hegazy et al. (2021) reported 2-month intranasal exposure to 0.125-, 0.25-, or 0.5 mg/kg/day potassium
dichromate significantly reduced balancing time on the Rotarod test in male Wistar rats after 2-month exposures compared to controls (approximately 25%, 35%, and 35% reduction, respectively), an assessment of locomotor function [115]. Salama et al. (2016) reported reduced locomotor activity in the grid floor activity cage assay follow exposure to 0.5 (42% decrease), 1 (59% decrease), and 2 (77% decrease) mg/kg Cr(VI) via intraperitoneal injection, when compared to controls receiving saline [118]. These studies demonstrated Cr(VI) impairs locomotor function, but none examined the influence of age or sex.

Changes in locomotor function or locomotor behavior are also linked to AD. One study reported decreased rearing may be indicative of neurodegeneration in the hippocampus of transgenic mice expressing three familial mutations linked to AD (3xTg mice) [140]. That study reported decreased rearing behavior may be a prodromal indicator of AD in 3xTg mouse models when compared to non-transgenic mice (2-month-old non-transgenic = 7.3 ± 2.1; 4-month-old non-transgenic = 5.9 ± 1.3; 6-month-old non-transgenic = 3.2 ± 7.4; 2-month-old 3xTg = 2.5 ± 0.6; 4-month-old 3xTg = 3.2 ± 1.2; 6-month-old 3xTg = 2.5 ± 0.8). In this context, the behavioral changes and hippocampal neurodegeneration observed in our study are suggestive of an AD-like effect.

Regarding locomotor activity, we observed a concentration-associated increase in activity of young rats, which may reflect an association for Cr(VI) exposure in children and ASD-like outcomes. Modi et al. (2018) found Shank2 knockout rats exhibited increased rearing behavior and distance traveled during the open field assay [141]. The Shank2 knockout is a rat model for ASD and induces dysregulation of striatal function by
increasing expression of a glutamate receptor (mGluR1) in the striatum. Another model of juvenile neurological deficits, the spontaneously hypertensive rat (SHR), is an established model for ADHD and exhibits increased activity in the open field assay. Pathology of the SHR model includes decreased volume of the caudate putamen [142]. Ishido et al. (2011) reported hyperactivity in juvenile rats following Bisphenol-A-induced neurodegeneration in the substantia nigra and ventral tegmental area, implicating neurodegeneration in these regions with locomotor changes observed in our young rats [143]. We observed increased activity in young males and females following Cr(VI) exposure, a behavioral change consistent with the ASD and ADHD rodent models previously described. Whereas this was observed in both sexes in the Y-Maze, we only observed a pattern towards increased distance traveled in the open field assay in young males. This supports a hypothesis for Cr(VI) as a risk factor for ASD, as prevalence of ASD is four times higher in boys than girls [144]. Males diagnosed with ASD present with increased external behaviors (e.g., hyperactivity) compared to females, also consistent with our study [144]. Importantly, Cr(VI) has been linked to ASD and impaired childhood attention development [4, 105-106].

We observed impaired spatial memory following Cr(VI) exposure, a result not yet reported in animal studies. An absence of studies assessing the effects of Cr(VI) on spatial memory is critical, especially as metal-on-metal hip replacement failure induces short-term memory deficits and disorientation in place [23]. This same study reported toxic levels of Cr and Co in the blood of individuals suffering from these neurological deficits, indicating these neurological deficits may be a result of heavy metal exposure [23]. One study reported the effects of Cr(VI) on social memory in mice [117]. Estrela et
al. (2017) exposed 2-3-month-old female Swiss mice to 500 mL tannery effluent as an environmental exposure for 2 hours per day over 20 consecutive days. Social memory was assessed by observing the frequency and amount of time spent exploring an unfamiliar “intruder” mouse during an initial introduction and during a retest 15 minutes later. Their results demonstrated mice exposed to the tannery effluent exhibited no changes in anogenital exploration or aggressive behavior between the initial introduction and the retest, indicating impairment of social memory as test mice did not recognize the intruder mouse during the retest. Importantly, the authors demonstrated olfaction was not impaired (using the buried food test) and locomotion was not impaired (observed during social recognition test), indicating olfactory dysfunction and locomotor impairment did not skew anogenital or body exploration in the social recognition test. This study also demonstrated that mice treated with Vitamin C before or after tannery effluent exposure did not exhibit impairments in social memory, compared to controls. This last point is critical for Cr(VI), as vitamin C reduces Cr(VI) to Cr(III), which is considered nontoxic. While the study includes some notable differences from our study (e.g., environmental exposure to all chemicals in tannery effluent, testing social memory rather than spatial memory, mouse model rather than rat model), results from this study are relevant to this thesis. Spatial and social memory are both dependent on the hippocampus, where we observed substantial Cr accumulation and neurodegeneration.

4.4 Cr(VI)-Induced Neurodegeneration

Silver stain demonstrated age- and Cr(VI)-induced neurodegeneration in multiple regions relevant to behaviors assessed in this thesis. This assay served as a qualitative
assessment to determine where Cr(VI) induced neurodegeneration in the brain to guide future investigations for biochemical and mechanistic endpoints. Silver stain is reported here for the cerebellum, dorsal caudate putamen, and dorsal hippocampus.

Our results show neurodegeneration increased in the cerebellum. We observed silver-stained neurons in the granular layer of the cerebellum at all ages, but we observed Cr(VI)-induced neurodegeneration in the white matter and cerebellar cortex only in young animals of both sexes and middle-aged females (Figures 3.8 and 3.9). These regions are instrumental in communicating with the cerebrum to elicit coordinated, voluntary movements. Changes in locomotor function during behaviors assays may be related to neurodegeneration in the cerebellum. One study found exposing chickens to potassium dichromate in drinking water led to disorganization in the cerebrum and cerebellum, including significant damage to Purkinje cells of the cerebellum [116]. While silver stain in our study was unable to detect damage specific to the Purkinje cells, we showed Cr(VI) damage in the cerebellum. Hao et al. (2017) observed edema of Purkinje cells, glial cells, and granule cells in the cerebellum following Cr(VI) exposure. Edema and damage to Purkinje cells following Cr(VI) exposure has been reported in other studies as well [114, 145]. Fahmy (2017) observed a decrease in Purkinje cells in the cerebellum and in increase in astrocytes following Cr(VI) exposure in guinea pigs. That study also reported Cr(VI) increased expression of caspase-3 in the Purkinje cells, granule cells, and cells of the cerebellar cortex. In future studies, we will examine biochemical and molecular changes within specific cell types, including the Purkinje cells of the cerebellum.
Positive silver staining in the dorsal caudate putamen increased with age and Cr(VI) exposure in males, particularly in white matter tracts (Figures 3.10 and 3.11). Cr(VI)-induced neurodegeneration in the caudate putamen was more severe in middle-aged and geriatric rats compared to young rats, though neurodegeneration was still noticeable in young males. Changes in functionality of the caudate putamen are related to neurological disorders found in individuals at multiple life stages. Reports indicate dysfunction in the caudate putamen may contribute to neurodevelopmental disorders (e.g., ASD, ADHD) and neurodegenerative diseases (e.g., PD, AD) [146-149].

Silver stain of the dorsal hippocampus revealed significant amounts of neurodegeneration across ages and Cr(VI) exposures (Figures 3.12 and 3.13). Cr(VI) induced the most severe neurodegeneration in the dorsal hippocampus in middle-aged females. Notably, there were significant sex differences observed in middle-aged rats. Middle-aged males and females both exhibited substantial neurodegeneration following Cr(VI) exposure, though females were more severely impacted. Evidence suggests that severe neurodegeneration in the dorsal hippocampus may induce side preference [150-151]. Developing a side preference results in animals turning in only one direction in the Y-Maze, rather than relying on spatial memory for navigation. This reduces the number of non-alternations performed, regardless of spatial memory function. Results from silver staining in middle-aged rats demonstrated severe hippocampal neurodegeneration, reflecting a likelihood they developed a side preference in the Y-Maze.
4.5 Application of Research

In summary, this study addressed knowledge gaps for Cr(VI)-induced neurotoxicity by identifying age differences, sex differences, region-specific Cr accumulation, changes in behavior, and evidence of neurodegeneration following long-term exposure to Cr(VI) at low concentrations (0.05 or 0.1 mg/L). Importantly, this exposure regimen was only 90 days and behavioral changes occurred as early as 7-10 weeks (49-70 days) after Cr(VI) exposure began.

Currently, there are no regulations for Cr(VI) neurotoxicity, and the vulnerability of geriatrics is not typically considered in toxicological studies. No other groups have reported low-level Cr(VI) neurotoxic effects or distinct neurotoxic outcomes across ages. Our data emphasize the need for a more in-depth assessment for Cr(VI) levels in drinking water and emphasize the need for regulations to consider Cr(VI) neurotoxicity and susceptibility across ages.

The neurotoxic effects of Cr(VI) have direct impacts on the health of human populations and may pose significant threats to geriatric populations. The unique geriatric exposome includes exposures to Cr(VI) and other heavy metals from environmental pollution, prosthetic implants, use of medical devices, polypharmacy, and remobilization of metals within the body. The geriatric exposome threatens to exacerbate the aging crisis by increasing the prevalence and severity of age-related diseases.

The results of this study addressed knowledge gaps in aging and toxicology, providing evidence Cr(VI)-induced neurotoxicity and age-specific outcomes require greater consideration. Understanding age-specific outcomes in toxicology becomes
critical in the context of the ongoing aging crisis. Successful application of the toxic aging coin approach in this study builds confidence in applying this concept to future studies. This study specifically interrogated the heads side of the toxic aging coin and identified areas where age impacted neurotoxicity. Continued investigation of the heads side of the toxic aging coin will consider further targets of Cr(VI) in the brain (other brain regions, cell types, biochemical processes, etc.) and determine how the effects of Cr(VI) in the brain are impacted by age. Future considerations of the tails side of the toxic aging coin will assess Cr(VI) effects on various aging hallmarks and interrogate a gerontogenic mechanism.
5. CONCLUSION

We conclude low concentrations of Cr(VI) in drinking water induced distinct neurotoxicity in male and female rats across three ages. This thesis demonstrated the neurotoxic effects of Cr(VI) can manifest as behavioral changes after a long-term exposure to low concentrations of Cr(VI), indicating Cr(VI) induced a variety of neurotoxic effects. Our data demonstrate Cr(VI) exposure altered frailty, locomotor function, activity, and spatial memory; however, the degree and direction of change in these neurobehaviors depended on age and sex. We observed age-, sex-, and region-specific changes in brain Cr accumulation. Cr accumulated in the geriatric male, middle-aged female, and geriatric female hippocampus, but did not accumulate in the cortex of any study groups. Notably, females exhibited age- and concentration-associated increases in hippocampal Cr levels. Geriatric females accumulated Cr levels substantially higher than those found in their male counterparts. Histologically, Cr(VI) exposure induced neurodegeneration in the cerebellum, dorsal caudate putamen, and dorsal hippocampus, with the greatest effect observed in middle-aged female dorsal hippocampus.

This thesis addressed multiple knowledge gaps in Cr(VI) neurotoxicity regarding age-, sex-, and region-specific effects. We identified region-specific accumulation in the hippocampus not yet reported in the literature. We demonstrated the effects of Cr(VI) (accumulation, pathology, behavior) are dependent on age and sex, a critical observation as most rodent Cr(VI) neurotoxicity studies only examined adult males. Identification of
age-dependent changes in Cr(VI) neurotoxicity validated the *heads* side of the ‘toxic aging coin’ approach. Behavior data from control animals demonstrated an age-associated effect, validating the use of this model and approach to assess gerontogenic effects and providing credence for the *tails* side of the toxic aging coin. Both sides of the toxic aging coin are crucial in bridging toxicology and aging to address the ongoing aging crisis.

The prevalence of age-related diseases will continue to rise alongside a growing geriatric population and increasing average lifespan. Environmental pollution can induce and exacerbate age-related diseases; as geriatrics are more susceptible to environmental pollutants, this compounds the ramifications of the aging crisis and threatens societal health. For these reasons, we strongly encourage the use of a toxic aging coin approach in toxicology and aging research to address the health, societal, and economic impacts of the aging crisis.
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2022  
Professional Development Award, Health and Environmental Sciences Institute Genetic Toxicology Technical Committee (HESI GTTC)

2022  
Student and New Investigator Travel Award, Environmental Mutagenesis and Genomics Society (EMGS)

2022  
Graduate Student Council Research Grant

2021 – 2023  
Pre-Doctoral Fellowship, Integrated Programs in Biomedical Sciences, University of Louisville

2021  
First Overall Oral Presentation, Louisiana State University Discover Day

2021  
Randall Outstanding Undergraduate Researcher, University of Alabama

2020  
President’s Award, The University of West Alabama Research Conference

2019  
First Place Undergraduate Research Poster, Alabama Water Institute Research Symposium

2017 – 2021  
Presidential Scholarship, University of Alabama

**Professional Memberships & Societies:**

2023-Present  
Society of Toxicology

2023-Present  
Ohio Valley Society of Toxicology
**Continuing Education Coursework:**

2023  Toxicology Excellence for Risk Assessment Dose Response Assessment Bootcamp, Cincinnati, Ohio, September 25-29, 2023

**Meetings Attended:**

2023  Ohio Valley Society of Toxicology Summer Trainee Meeting, Online  
       June 12, 2023

2023  Society of Toxicology Annual Meeting, Nashville, Tennessee  
       March 19 – 23, 2023

2022  Ohio Valley Society of Toxicology Annual Meeting, Louisville, Kentucky  
       October 14, 2022

2022  Research!Louisville, University of Louisville, Louisville, Kentucky  
       September 19 – 23, 2022

2022  Ohio Valley Society of Toxicology Summer Trainee Meeting, Online  
       July 29, 2022

2022  Environmental Mutagenesis and Genomics Society International Conference on Environmental Mutagens, Ottawa, Canada  
       August 27 – September 1, 2022

2021  Louisiana State University Discover Day, Online  
       April 21, 2021

2021  Undergraduate Research & Creative Activity Conferences, University of Alabama, Tuscaloosa, Alabama  
       March 31, 2021

2021  University of West Alabama Undergraduate Research Conference, Online  
       March 9, 2021

2020  Undergraduate Research & Creative Activity Conferences, University of Alabama, Online  
       February 28, 2020
Peer-Reviewed Publications:


Abstracts:

Extramural Abstracts


Exposure Contributing to Parkinson’s Disease. Presented at the Ohio Valley Society of Toxicology Summer Trainee Meeting, July 2023.


Intramural Abstracts


4. Vivee, S., Lackey, K., O’Shields, B., Cullop, P., Reed, L., and Chaudhuri, A. Spectracide, an Environmental Pollutant, Causes Parkinsonian Symptoms in Fly

**Teaching:**

2021  Teaching Assistant: Microbiology and Man, University of Alabama
2021  Teaching Assistant: Introduction to Biological Research, University of Alabama
2020  Teaching Assistant: Introduction to Biological Research, University of Alabama
2020  Teaching Assistant: Principles of Biology I Laboratory, University of Alabama
2019  Teaching Assistant: Principles of Biology I Laboratory, University of Alabama

**Students Trained:**

*Undergraduate Students*

2023 – Present  Liam Buchanan (University of Louisville)
2023 – Present  Spencer Roof (University of Louisville)

*High School Students*

2022 – 2022  Maitri Patel (Manual High School)

**Service:**

2023  Judge: Neuroscience Special Category Award, Louisville Regional Science & Engineering Fair
2022  Mentor: Louisville Science Pathways
2022  Judge: Middle School Division, Louisville Regional Science & Engineering Fair