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Curcumin supplementation in the rhesus monkey: effects on cognitive decline and neuroinflammation

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CURCUMIN SUPPLEMENTATION IN THE RHESUS MONKEY: EFFECTS ON COGNITIVE DECLINE AND NEUROINFLAMMATION

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ABSTRACT

Human and non-human primates (NHP) undergo age-related cognitive decline beginning as early as middle-age, even in the absence of an underlying pathology or disease. Growing evidence indicates that an increase in white mater pathology related to rising chronic levels of inflammation may be key contributors to age related cognitive decline. Curcumin (CUR), the active ingredient in turmeric, is a polyphenol nutraceutical with potent anti-inflammatory and antioxidative effects. Several ongoing research studies are underway to explore this potential anti-aging compound. For the first time in a rhesus monkey model of aging, we studied the effects of CUR supplementation on cognition and inflammation. Baseline MRI, blood, CSF and cognitive data were collected for all monkeys. Monkeys were fed daily doses of 500mg of CUR or a vehicle control over 18-months during which three rounds of a battery of cognitive testing was performed along with regular collection of blood, CSF and MRI. Following completion of this testing and specific to this thesis, monkeys were further tested on object discrimination, object and spatial reversal tasks. No significant differences were observed between groups in object discrimination task performance. CUR treatment improved performance on object reversal testing, with treated monkeys making fewer perseverative type errors. At the completion of behavioral testing,

serum samples from two-year post treatment onset and brain tissue were harvested for post-mortem analysis of markers of inflammation. The density and morphology of microglia, the resident immune cells of the brain, were examined using immunohistochemistry on serial coronal sections through frontal cortical gray (A46, A25) and while matter (FWM, CC, and CngB) regions that are implicated in cognitive aging. We demonstrated that CUR treatment did not significantly alter the density of presumably immune-activated microglia expressing the MHC class II marker LN3. However, treatment did affect morphological features of microglia specifically within the while matter. Within the white matter, CUR treatment was associated with a significant increase in microglial ramification, evidenced by greater process length, number of nodes and convex-hull area and volume. Increased microglial ramification suggests greater likelihood of microglial surveillance within the white matter associated with CUR treatment. No significant group differences however were observed in the select serum cytokine levels quantified using multiplex ELISA, or in inflammatory gene expression in brain tissue measured with qRT-PCR. While our findings show the benefit of CUR supplementation on cognitive performance and its effects on microglial morphology, further study is needed to understand the precise changes that CUR supplementation may have on inflammation.

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CHAPTER ONE:

INTRODUCTION

Rhesus Monkey Model

The United Nations Department of Economic and Social Affairs projects that by 2050, globally, there will 1.5 billion individuals aged 65 and older, a near doubling of the 2019 aged population. As aging is a risk factor for most diseases and increases the chances of comorbidity, a dramatic rise in the cost of care for the elderly can be expected (Divo et al., 2014). Even in the absence of disease, aging alone is sufficient to negatively impact cognitive function (Harada et al., 2013). Understanding the effects that aging has on cognition is necessary to form a clear backdrop of changes which disease states may then exacerbate. Therefore, models in which the effects of aging on cognition, uncomplicated by underlying disease or neuropathy, can be studied are extremely valuable.

The rhesus monkey (Macaca mulatta), an old-world primate, is an ideal model to study human aging. Rhesus monkeys have a life span of roughly 35 years and age at a rate 3:1 in comparison to humans (Tigges et al., 1988). Rhesus monkeys have a gryencephalic brain with many similar structures and organization to the human brain. Furthermore, in contrast to rodents, rhesus monkeys have a greater white to grey matter ratio that more closely resembles the human brain. Additionally, monkeys can be tested with a wide variety of cognitive assessments that have direct parallels to human cognitive testing (Hara et al., 2012, Herndon et al., 1997, Moore et al., 2003, Moore et al., 2006). Similarly, to humans, rhesus monkeys develop age related cognitive decline in short-term memory, working memory and executive function, beginning in midlife (Albert, 1984; Chodosh et

al., 2002; Drag and Bieliauskas; 2010; Hara et al., 2012; Kwon et al., 2016; León et al., 2016; Light 1991; Simen et al., 2011; Zeamer et al., 2012; Zeamer et al., 2011, Park and Reuter-Lorenz, 2009; Moore et al., 2003, 2006). Unlike humans, rhesus monkeys do not typically develop Alzheimer's Disease like pathology with age. This allows for study the effects of aging on cognition, absent confounding factors such as neurodegeneration typically observed in diseased brains. Lastly, while human brain tissue is extremely valuable, it often exhibits cellular deterioration due to delays in processing the brains post mortem. The brains of rhesus monkeys however, can be harvested in a controlled setting preserving the tissue in an optimal manner for post mortem experiments. For these reasons the rhesus monkey is ideal organism to model and study age related cognitive decline.

Cognitive Changes in Aging

Aging alone is sufficient to negatively impact cognition, with roughly 30% of individuals above the age of 65 experiencing severe cognitive deficits (Harada et al., 2013). In particular the hippocampus (HPC) and prefrontal cortex (PFC) are two regions that are highly susceptible to aging effects. Subsequently, memory and executive function are two domains that have been demonstrated to be particularly vulnerable to the aging process. Working memory and executive function are among the first domains to exhibit age related deficits. (Cahn-Weiner et al., 2000, Fristoe et al., 1997, Lai et al., 1995, Moore et al., 2003, 2006; Moss et al., 1997, Rabbitt and Lowe 2000; Souchay et al., 2000). Deficits in abstraction, set shifting, working memory and rule learning, have been observed as early as middle age in the rhesus monkey (Albert, 1984; Chodosh et al., 2002; Drag and Bieliauskas; 2010; Hara et al., 2012; Kwon et al., 2016; León et al., 2016; Light 1991;

Simen et al., 2011; Park and Reuter-Lorenz, 2009; Moore et al., 2003, 2006; Zeamer et al., 2012; Zeamer et al., 2011). In our laboratory, we have demonstrated that both middle-aged and aged monkeys are impaired on our Category Set Shifting Task (CSST) and the Delayed Recognition Span Task (DRST) that assess working memory (Moore et al., 2003, Moore et al., 2006). In contrast, impairments on the Delayed Non-Match to Sample (DNMS) that assesses recognition memory is mainly limited to the most elderly monkeys (Herndon et al., 1997, Killiany et al., 2000, Moss et al., 1988). Other tasks such as object discrimination and pattern separation, which engage the temporal lobe have also been demonstrated to decline in elderly monkeys (Burke et al., 2011). These findings and others provide support that the earliest detectable changes in cognition due to age occur first in tasks mediated by the prefrontal cortices, while advanced age reveals impairments in temporal lobe dependent tasks (Arshad et al., 2016; Borella et al., 2014; Funahasi et al, 2017; Johnson et al., 2016; Kwon et al., 2016; McEwen and Morison 2013; Moore et al., 2005; Raz et al., 1997; Toepper et al., 2014). While the mechanisms of age-related cognitive decline are not yet fully understood, growing evidence points to disruption of myelin as being a heavily contributing factor.

Myelin and Mechanisms Underlying Cognitive Decline

Brody in 1955 demonstrated that aging resulted in a dramatic loss of neurons. Following the application of stereological counting techniques, it has become clear since then, that aging does not typically result in a net loss of neurons in either human and non-human primates (NHP) (Peters et al., 1994, Morrison and Hof 1997). Rather than an overt loss of neurons, conclusive evidence demonstrates that aging results in disruption of the

myelin sheaths (Bowley et al., 2010; Peters, 2002; Peters and Kemper, 2012; Peters and Sethares, 2002).

MRI studies have demonstrated age related decreases in white matter volume and in myelinated nerve fiber length in both humans and NHPs (Albert 1993, Sandell and Peters 2001, Sandell and Peters 2002, Wisco et al., 2008). Additionally, the loss of white matter volume and myelinated fibers has been observed to occur in frontal white matter regions like the PFC, but less so in posterior structures like the occipital lobe (Brickman et al.,, 2006; Fjell and Walhovd, 2010; Guttmann et al.,, 1998; Marner et al.,, 2003, Head, 2004; O'Sullivan et al., 2001; Salat et al., 2005; Sullivan et al., 2006; Yoon et al., 2008). In addition to the loss of myelinated fibers, aging results in accumulation of myelin damage and degeneration of myelin sheaths in the frontal white matter underlying the lateral PFC area A46 and in white matter tracks responsible for interhemispheric communication such as the corpus callosum, and anterior commissure. Further, these myelin deficits are correlated with cognitive decline (Feldman and Peters 1998, Peters and Salthares 2002, Sandell and Peters 2003). It is plausible that the damage and loss of myelinated fibers in these white matter regions is therefore altering transmission capability between brain regions leading to cognitive impairment.

This loss of myelinated fibers is likely resulting from in part changes to both oligodendrocytes precursor cells (OPCs) and oligodendrocytes. As myelin debris can inhibit the differentiation and proliferation of oligodendrocyte progenitor cells (OPCs), the repair and replacement of damaged myelin is likely impaired with age (Kottter et al., 2006). Aging has also been shown to decrease proliferation and impair functioning of

oligodendrocytes, and is believed to be related to a rise in chronic low levels of inflammation (Juurlink et al., 1998).

Role of Microglia in Inflammation and Aging

As stated previously, aging results in a loss of myelinated fibers and significnt myelin damage, the causes of which are not fully understood but may be related to a rise in chronic low levels of inflammation.

Inflammation and immune activation are normal responses. In aging however, there is a rise in persistent low levels of inflammation (Franceschi et al., 2000). This change is likely a consequence of decreases in intrinsic antioxidant and anti-inflammatory capability, as well as a shift towards increased and prolonged immune response (Ye and Johnson 2001). Unlike acute inflammation which can lead to repair and clearing of debris, chronic or prolonged inflammation can result in damage to surrounding tissue.

Microglia are the resident macrophages of the brain, derived from yolk sac cells during development and can self-renew within the central nervous system (Alliot et al., 1999). Microglia mediate the immune response, at baseline scanning the environment. In reaction endogenous or exogenous stimuli microglia can initiate breakdown, clearing debris and can recruit additional microglia to aid in such efforts. This activity is reflected in microglial morphology (Karperien et al., 2013). In their surveilling state, microglia are characterized by a ramified morphology with a small dense soma with multiple long thin processes radiating outward. Following ‘activation’, microglia retract their processes altering their morphology to be either hypertrophic, having few and short processes, or ameboid, having an enlarged soma with no processes. In this state microglia are primed for

pro-inflammatory activities including the generation of reactive oxygen species (ROS), proinflammatory cytokine secretion and phagocytosis of debris (Nimmerjahn et al., 2005, Bohatschek et al 2001, Lee et al 2008). Following resolution of activation, microglia are able to return to their surveillance state and corresponding morphological features (Hiero-Bujalance et al., 2018, Miller et al., 2019).

Within aging, multiple changes are noted to occur to microglia. Aged microglia appear to have an impaired ability to phagocytose debris (Floden and Combs 2011, Natrajan et al., 2015, Safaiyan et al.,, 2016). Additionally, aged microglia are likely to be primed towards a proinflammatory phenotype with an exaggerated response resulting in increased proinflammatory cytokine and ROS secretion (Sierra et al., 2007, Perry et al., 2014, Boehmer et al., 2004). Finally, aged microglia were also found to have increased expression of major histocompatibility complex antigens (Conde and Streit 2006, Sheng et al., 1998).

The shift in microglial activity with age likely contributes to the disruption of the white matter, cognitive decline and increase in neuro-inflammation. A key function of microglia is to remove damaged myelin, the presence of which impairs remyelination efforts and OPC differentiation (Kotter et al., 2006). As age related myelin damage builds up, microglia are recruited to damaged areas to clear the debris but are unable to effectively do so, leading to a persistent and inefficient immune response. Evidence in support of this theory includes the increase in activated microglia within the white but not grey matter of aged monkeys (Shobin et al., 2017). The rise of chronic inflammation and oxidative stress in age likely contributes to myelin pathology and thus are potential targets for therapeutics

to reduce aging related myelin damage and cognitive decline (De la Fuente and Miquel, 2009; Poliani et al., 2015; Rawji et al 2016; Ruckh et al., 2012)

Curcumin Metabolism and Effects on Neuroinflammation

CUR a yellow pigment, is a polyphenol and primary active ingredient found in the rhizome turmeric. Turmeric has been used traditionally as a spice but also a dye and medicine by Asian cultures. More recent application and study of CUR medicinal properties have revealed that CUR has potent anti-inflammatory, anti-oxidative, anti-neoplastic and neuroprotective effects. CUR has been reported to act on many cellular pathways regulating inflammatory and cell division processes, notable targets of CUR include transcription factors, nuclear factor kappa-light-chain enhance of activated b cells (Nf-kB), protein kinase B, mammalian target of rapamycin, interleukin 6 and transforming growth factor b (Gupta et al., 2012, Nahar et al., 2015).

CUR has a poor bioavailability, the restricting factors include a poor solubility, inefficient GI absorption, rapid metabolism and excretion (Choudhury et al., 2015, Gupta et al., 2012). CUR is primarily metabolized in the gut and liver. Metabolism occurs through reduction and conjugation, with the metabolites having poor bioactivity in comparison to CUR, for complete review see Schneider et al., 2015. Briefly, the primary metabolites following reduction within the gut are di-, tetra-, hexa-, and octahyrocurcumin. Further metabolism of CUR by alcohol dehydrogenase within the liver leads to tetra-, and hexahydrocurcumin, which are the highest concentration detected metabolites formed. When absorbed, CUR and metabolites are found conjugated with glucuronic acid and sulfates in the plasma, forming di-, tetrahydrocurcumin glucronoside or mixed sulfate. Oral

CUR supplementation has been found reach peak serum concentration ranging from 1-2 hours after administration. Due to CUR poor bioavailability numerous strategies have been developed to increase absorption including supplementation with added piperine or lecithin, encasement in hydrophilic nanoparticles, micellar systems and solid lipid particle formulations. Though all such strategies have demonstrated improved CUR absorption, comparison across studies is limited by high variability in CUR dosage administered across studies. A brief description of each method of enhancing bioavailability follows for review see Stohs et al., 2020. Piperine, is an alkaloid of black pepper (Piper nigrum) prevents glucuronidation of CUR. Lecithin is a phospholipid phytochemical that has increases GI absorption of CUR due to its amphipathic phospholipid structure. Hydrophilic nanoparticles allow for colloidal dispersion of CUR and enhance GI absorption. Lipidated and micellar methods, such as the solid lipid curcumin particle used in the current study, have be observed to improve bioavailability due to a combination of enhanced absorption through the GI system, reduction of metabolism and breakdown of CUR. Furthermore, solid lipid CUR particle formulations have been demonstrated to increase blood brain permeability of intact CUR.

Despite having a poor bioavailability, several studies demonstrate the benefits of CUR supplementation in various models including cell culture, rodent, human and non-human primates. While CUR has demonstrated ability to dampen inflammatory response in general, several studies demonstrate that CUR can affect neuroinflammatory processes. Cell culture experiments with rodent BV2 microglia stimulated with either lipoteichoic acid or lipopolysaccharide (LPS) application, show attenuated proinflammatory response

in the presence of CUR. Specifically inflammatory mediators and cytokines such as tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2), Nitric Oxide (NO), cyclooxygenase-2 (COX-2), interleukin (IL)-1β, IL-6, map kinase (MapK) were reduced in CUR treated cells (Jin et al 2007, Yu et al., 2018). Additionally, Yu et al., 2018 show that CUR induces anti-inflammatory processes in their model including increasing heme oxygenase and nuclear factor erythroid 2 (NRF2) expression.

In addition to cell culture experiments benefits CUR supplementation has been observed with *in vivo* models. Kim et al., 2008 showed in adult mice, CUR supplementation enhanced neurogenesis, likely by inhibition of the mitogen activated protein kinase pathway. Nam et al 2014 also show that CUR can enhance neurogenesis by increasing brain derived neurotrophic factor (BDNF) in the subgranular zone of the dentate gyrus while also demonstrating that supplementation results in improved water maze performance, decreasing escape latency, in mice. Yu et al., 2012 show that CUR can dampen nitric oxide synthase (NOS) production following hypoxic ischemic damage in a rodent model of brain injury. Human studies also show multiple benefits resulting from CUR supplementation. Cox et al., 2015 show that acute supplementation (1-3 hours after treatment) with CUR yields improvements on sustained attention and working memory tasks in aged individuals, while chronic supplementation (4 weeks) also resulted in improvements to working memory, alertness and contentedness. Our laboratory has demonstrated that in middle age rhesus monkeys, CUR supplementation can improve performance on the Delayed Recognition Span Task-Spatial (DRSTsp), and motor function (Moore et al., 2017, Moore et al., 2019). While is it important to note that CUR

supplementation can improve cognitive function, another key aspect of supplementation is that it may prevent or slow aging related cognitive decline. Rainey-Smith et al., 2016 show that control subjects decline in performance on repeat testing of the Montreal Cognitive Assessment in comparison to subjects receiving CUR over a 6-month time span. Because of its potent anti-inflammatory activity CUR has been touted a pleiotropic therapeutic for a wide array of diseases including cancer and neurological disorders. While there is a rich body of evidence demonstrating many pathways known to be affected by CUR, and many modalities in which supplementation is beneficial, it remains unclear how CUR improves cognition.

Summary and Hypothesis

Age related increases in inflammation and oxidative stress may be associated with increased white matter damage and thus contribute to the cognitive decline observed in aging. The purpose of this study was to investigate whether CUR supplementation can improve cognition in middle age monkeys by mitigation of age-related increases in inflammation and oxidative stress. Specific to this study, monkeys were tested on an object discrimination task as well as object and spatial reversal learning tasks. As these tasks are known to be mediated by the temporal and frontal lobes, performance on these tasks by monkeys treated with CUR may confer insight into potential regional specificity of CUR supplementation. While no significant effect was observed on object discrimination, reversal testing showed promising results following supplementation. In order to understand changes related to inflammatory processes in CUR treated monkeys that may have resulted in improved object reversal task performance, microglia density and

morphology were assessed using immunohistochemistry. Additionally, blood and CSF samples taken from the monkeys at various times points across treatment were assayed for markers of inflammation using enzyme linked immunosorbent assays (ELISA), while the serum was tested for protective or dampening effects on LPS stimulation of rat microglia cells *in vitro*. Finally, brain gene expression for several inflammatory markers was measured using qRT-PCR. These experiments together tested the hypothesis that daily dietary CUR supplementation improves cognition and that this improvement results from decreases in inflammation either systemically or within the brain.

CHAPTER TWO:

CURCUMIN SUPPLEMENTATION EFFECTS ON OBJECT DISCRIMINATION AND REVERSAL LEARNING

Portions of this chapter under review for publication and has been reproduced here with minor changes and permission from all authors and is submitted to Behavioral Neuroscience.

Introduction

Age-dependent cognitive decline occurs both in humans and non-human primates (Cahn-Weiner et al., 2000; Fristoe et al., 1997; Lai et al., 1995; Moore et al., 2003, 2006; Moss et al., 1997; Rabbitt and Lowe 2000; Souchay et al., 2000). These cognitive changes occur as early as middle age, predominately in the domains of short-term memory, working memory and executive function (Albert, 1984; Chodosh et al., 2002; Drag and Bieliauskas; 2010; Hara et al., 2012; Kwon et al., 2016; León et al., 2016; Light 1991; Simen et al., 2011; Park and Reuter-Lorenz, 2009; Moore et al., 2003, 2006; Zeamer et al., 2012; Zeamer et al., 2011). Though age associated cognitive decline was originally thought to be due to loss of neurons from the cerebral mantle (Brody 1955), evidence has accumulated to show that there is no significant loss of neurons with age. In contrast, significant disruptions of the myelin sheaths have been observed with aging (Peters et al, 1998). These pathologic changes occur predominantly in the frontal lobe and include cytoplasmic ballooning, degeneration of myelin sheaths, as well as impaired and reduced remyelination. Further, this myelin pathology is correlated with diminished cognitive performance in both

human and non-human primates (Bowley et al., 2010; Drag and Bieliauskas 2010; Guttmann et al., 1998; Makris et al., 2007; Peters and Sethares 2002; Wisco et al., 2008).

It is also clear that inflammation and oxidative stress increase with age, are cofactors of many diseases, they likely contribute to myelin pathology and are potential targets for therapeutics to reduce aging related myelin dysfunction (De la Fuente and Miquel, 2009; Poliani et al., 2015; Rawji et al 2016; Ruckh et al., 2012). Curcumin (CUR), a naturally occurring polyphenol derived from the rhizomes of Curcuma Longa, has been shown to be a potent antioxidant and anti-inflammatory nutraceutical (Queen & Tollefsbol 2010; Salminen et al., 2008; Sikora et al., 2010a, Sikora et al., 2010b). Accordingly, CUR has received increasing attention as a potential anti-aging therapeutic that may slow or reverse inflammaging and cognitive decline. Numerous studies have demonstrated that administration of CUR improves cognitive and motor performance in rodents and humans alike. (Cox et al., 2015; Nam et al., 2014; Rainey-Smith et al., 2016; Salvioli et al., 2007; Sikora et al., 2010a; Sikora et al., 2010b). More recently our laboratory has demonstrated beneficial effects of CUR supplementation in the middle-aged non-human primate on a task of spatial working memory, but not one of object recognition memory (Moore et al., 2018; 2019). These findings are of particular interest as several studies have demonstrated that the domains of working memory and executive function, of which both are mediated in large part by the pre-frontal cortices are among the first to exhibit and most sensitive to age-related cognitive decline (Arshad et al., 2016; Borella et al., 2014; Funahasi et al, 2017; Johnson et al., 2016; Kwon et al., 2016; McEwen and Morison 2013; Moore et al., 2005; Raz et al., 1997; Toepper et al., 2014; Writ and Hyman 2017). To this end, one might

hypothesize that CUR supplementation would ameliorate deficits in executive function in the aged monkey (Moore et al, 2009; 2017; Bartus et al 1979; Herndon et al., 1997; Moss et al., 1988).

Having found a dissociation of CUR supplementation affecting frontal but not temporal lobe function in our previous experiments, in the present study, we assessed the effects of long-term CUR administration on object discrimination and two forms of a reversal learning task, object and spatial to further our understanding of this effect. Object discrimination has been shown to be reliant on the perirhinal cortex, particularly when a discrimination cannot be made based on a single feature and impaired in age (Burke et al., 2011) Performance on reversal tasks is mediated in part by the prefrontal cortex as it requires subjects to alter their behavioral response following changes in reward contingencies, and has also been demonstrated to be reliant on HPC function (Lai et al., 1995, Jones and Mishkin 1972; Mahut 1971; 1972 Pohl 1971). Numerous studies have demonstrated that reversal learning, is impaired in aged monkeys relative to young monkeys, with aged monkeys making more perseverative errors (Gray et al., 2017; Munger et al., 2017; Lai et al., 1995; Rapp 1990; Bartus et al., 1979). Therefore, in our longitudinal study of the efficacy of oral administration of CUR, we asked the question if CUR supplementation would reduce age-related impairments of performance on object discrimination and object and spatial reversal learning tasks given that both tasks show a susceptibility to age related cognitive decline, while being heavily reliant on processing in varying brain regions.

Methods

Subjects

Seven middle aged male and female rhesus monkeys (Macaca mulatta) were tested on object discrimination, while eight middle aged male and female rhesus monkeys were tested on object and spatial reversals in this study (Table 1). The monkeys ranged in age from 12-21 years of age, equivalent to approximately 36-63 human years of age (Tigges et al., 1988). All monkeys were obtained from either private vendors or National Primate Research facilities. All monkeys arrived with complete health records and underwent a full medical examination including magnetic resonance imaging to rule out occult brain pathology. All monkeys were healthy at the start of the study. Monkeys were individually housed in cages, within visual and auditory range of the other monkeys of the colony, located in rooms at the Animal Science Center (ASC) at Boston University School of Medicine. As several studies are ongoing within our laboratory, and new cohorts of monkeys are introduced regularly, the decision, to individually house monkeys, was made to minimize disruption to the consistency and quality of behavioral testing while also lessening disturbances that may prolong overall study duration. Furthermore, we aimed to reduce the risk of injury that can occur with pair housing of rhesus macaques. Lastly, individualized housing also allowed for greater control of the food intake and enrichment that each monkey received. Monkey rooms were kept at a 12-hour light-dark cycle. The daily diet included Purina Monkey Chow (Purina Mills Inc, St. Louis, MO) with 12-20 biscuits per day (by weight), fresh fruit and vegetables given after completion of daily testing and following treatment administration. During testing monkeys received small food rewards including fruit and candy. Monkeys had free access to water in their home

cage and enrichment in the form of toys and food treats, that were changed regularly, located either directly inside or attached to their cages. The Boston University ASC is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All procedures were performed following the guidelines set by the National Institutes of Health and the Institute of Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals (2011). This study was approved by the Boston University Institutional Animal Care and Use Committee (IACUC).

Prior to starting CUR supplementation, all monkeys were cognitively tested to establish a baseline cognitive performance and were then randomly assigned into two groups, receiving either CUR or VEH treatments. It should be noted that for the reversal experiments the CUR treatment group was significantly older than the VEH ($t(6)=2.77$, $p=0.03$, no group differences were observed in the initial acquisition of any phase of testing. Testers were blind to the treatment condition of the monkeys. All monkeys received CUR or VEH daily (including weekends) for 3 years prior to testing and dosing continued during testing. CUR dosage was 500mg per day. The CUR and vehicle control (dextran) were provided courtesy of Verdure Sciences (Noblesville, IN). Their formulation is an optimized natural CUR for enhanced bioavailability, to increase its absorption within the gastrointestinal system and permeability of the blood brain barrier. Both CUR and VEH were mixed with either 150ml of yogurt or Prima-Burger TM (BioServ, Flemington, NJ). Treatments were given immediately following testing and monkeys were observed to verify that the treatment was consumed. If a monkey did not immediately eat the treatment, (e.g. discarding or dropping it into the waste pan of their cage), a second treatment was

given at a later time. Monkeys ate the treatments (both the VEH and CUR) on more than 98% of the study days.

Cognitive Testing

Apparatus

All testing was conducted in a Wisconsin General Testing Apparatus, in a darkened room with white noise played on overhead speakers. Monkeys used in this study had already completed a battery of cognitive and motor tests (Moore et al., 2017; 2018) in this apparatus and were thus acclimatized to the testing environment. Briefly, the monkeys and tester were separated by a double set of windows, one fully opaque (between monkey and testing board) the other semi-opaque (between tester and testing board) allowing for one way viewing of the monkey by the tester. A stimulus tray of 3 equally linear spaced wells was located between the two doors. Monkeys had been previously trained to displace grey plaques to uncover the wells and retrieve a food reward (small pieces of candy, fruit or nuts). For reversal testing only the left and right wells were utilized. The opaque door was raised to initiate and terminate trials, while the semi-transparent door remained lowered during each trial allowing the tester to observe the monkey's behavior.

Object Discrimination

For the Object Discrimination Task (OD), two objects covering the right and left wells are simultaneously presented to the animals. The locations of the objects switch each trial in a pseudo-random order and are balanced for both left/right sides. The animal is tasked with displacing one of the objects to uncover the well underneath. A trial begins when the door is raised by the experimenter and terminates following the displacement of

an object and once the door is lowered by the experimenter. One object within a pair is always rewarded and the other is never rewarded. A single object pair is used for a total of 30 trials/day, with a 15 second interval between each trial, until criterion is reached. Criterion is met when within a single day's testing the rewarded object is selected for 90% of the trials. If criterion is met on the first day of testing a second day of repeat testing is done to rule out the possibility of reaching criterion by chance. Once criterion for an object was met following a 48-hour delay recall of the discrimination was performed for a total of 30 trials.

Testing begins with object pairs with least amount of feature overlap and progresses to object pairs with higher degrees of feature overlap. Object pairs were created using Legos, and feature overlap was calculated by dividing the 'pips' in common between the pair by the total number of pips within in a single object. Pairs consisting of 60, 70, 80, 90 percentages of feature overlap are used. Legos used in each object pair are the same across all pairs, the spatial orientation however is particular to a single pair.

Reversal Learning Tasks

Two unique pairs of dissimilar objects were used for object reversals and two identical black plaques were used for spatial reversals (figure 6). For both tasks, each trial consisted of the simultaneous presentation of the rewarded and unrewarded stimulus over the lateral wells. Thirty trials per day were administered with an inter-trial interval of 15 seconds. Initial learning criterion for the acquisition phase was defined as selection of the rewarded stimulus for 90% (27/30 trials) of trials within a single session of testing. For each task, initial acquisition of the rewarded stimulus was established and then four

reversals were administered. The object Reversal Learning Task was administered twice followed by the spatial Reversal Learning Task which was only administered once due to budgetary constraints.

For both object and spatial reversals, the day after the initial learning criterion was achieved, testing was conducted with the same rewarded stimulus as the day prior. If the monkey selected the rewarded stimulus for 90% of the first 20 trials (18/20 trials), meeting the reversal criterion, the rewarded and unrewarded stimuli were switched on the 21st trial with testing continuing for an additional 20 trials for a total of 40 trials on the reversal day. If the monkeys did not select the rewarded stimulus for 90% of the first 20 trials, testing continued for 30 trials per day until the reversal criterion was met. The next day, following a reversal, testing continued with the 'new' rewarded stimulus for 30 trials/day until the reversal criterion was met (90% correct of the first 20 trials in a single day of testing) triggering another switch between the rewarded and unrewarded stimuli. This pattern of 40 trial reversal days, and 30 trial testing days was performed for a total of 4 reversals of the rewarded and unrewarded stimuli.

Object Reversal Learning Task

For object reversals, the location of the rewarded object was pseudo-randomized and balanced for both the right and left wells to prevent side-based selection bias. One object from a pair of objects was selected at random to be the rewarded stimulus, this was randomized for each monkey. The test began with an initial acquisition phase and once criterion was reached on the initial acquisition phase (< 3 errors in 30 trials), the 1st reversal was administered the following day. Testing continued until reversal criterion was again

reached and then the 2nd reversal was administered (the initially rewarded object was once again rewarded). This pattern continued for 2 additional reversals. Once criterion was reached on the last reversal, the entire test began again with a new pair of objects until a total of 4 reversals were accomplished. Following completion of both object reversals tasks, spatial reversal testing was administered.

Spatial Reversal Learning Task

For spatial reversals, both the left and right wells were simultaneously covered with identical plaques and either the left or right well was pseudo-randomly selected, for each monkey, to be rewarded for the initial acquisition. Once the monkey reached criterion on the initial acquisition, testing on the 1st reversal began the following day. When criterion was met, another reversal was administered (the previous rewarded well now became the unrewarded well and the previously unrewarded well now became the rewarded well) beginning on the 21st trial. Testing continued until reversal criterion was reached and then the 2nd reversal was administered (the initially rewarded side was once again rewarded). This pattern continued for 2 additional reversals.

Data Analysis

For object discrimination, the trials taken to reach criterion, and the total correct trials during recall were analyzed using 2-way ANOVA, with trials being the dependent measure, independent factors included treatment group and percentage of feature overlap. Pairwise comparisons with Bonferroni corrections were used for post hoc analysis when appropriate. For both the object and spatial reversal learning tasks, the number of trials and errors to reach criterion for the initial acquisition and for each reversal were calculated.

For reversal learning tasks to further assess patterns of learning on these tasks, a learning stage analysis was performed for each phase of testing, excluding trials in which a reversal criterion was met. This staging analysis was done by defining error types into specific stages. A similar analysis was performed previously (Lai et al., 1995), in which they demonstrate that a staging analysis of blocks of ten trials is more sensitive at detecting slight differences between young and old monkeys. Briefly, based on the number of errors made in each block of ten trials, each block of 10 was characterized as Stage I, II or III based on the following criterion: Stage I was defined as 7-10 errors, Stage II was defined as 4-6 errors, and Sage III was defined as 0-3 errors. Stage I errors are indicative of a failure to reverse established stimulus reward contingencies and are analogous to making preservative responses. Stage II errors are characterized as being near chance levels of selection of either presented stimuli, while Stage III errors demonstrate a shift in selection of toward the new positive stimulus. 1-way between subjects ANOVA was conducted to compare the effect of treatment on the initial acquisition trial and errors. Comparison of the two groups in trials, errors and each type of stage error were analyzed for each reversal using repeated measures ANOVA grouped by treatment with Greenhouse-Geisser corrections when appropriate. T tests, adjusting p values for the multiple comparisons using the Holm-Sidak correction method where appropriate.

Results

Object Discrimination

Trials to Criterion – All Object Pairs

The total trials made to reach criterion were compared for each group at each level of feature overlap (Table 2.2). As shown in Figure 2.1 no significant group differences were observed in discrimination performance [$F(1,20) = 0.091$, $p=0.766$)]. A significant difference however was found due to similarity in feature overlap [$F(3,20) = 5.560$, $p=0.006$)]. Pairwise comparison with Bonferroni correction show this effect is significant between the 60 and 90% as well as 70 and 90% feature overlap pairs ($p < 0.029$, $p < 0.005$, respectively). Showing that objects with higher similarity are harder to tell apart and require more trials to form a discrimination.

Correct Trials During Recall – All Object Pairs

The correct trials made during recall testing were compared for each group at each level of feature overlap (Table 2.3). As shown in Figure 2.1 no significant group differences were observed in recall once a discrimination was achieved [$F(1,20) = 0.047$, $p=0.831$)]. A significant difference however was found due to feature overlap [$F(3,20) = 3.202$, $p=0.045$)]. Pairwise comparison with Bonferroni correction show this effect is significant between the 60 and 90% feature overlap pairs ($p < 0.038$). This suggests that recall of a discrimination is diminished when objects share a high level of similarity.

Object Reversal Learning Task

Initial Acquisition – 1st and 2nd Object Pairs

The total trials and errors to criterion during the acquisition phase (prior to beginning reversals), for the 1st and 2nd object pairs, were separately analyzed using one-way between subjects ANOVA comparing the CUR and VEH groups. As shown in Table 2.4, no significant group differences were observed during the acquisition phase for either

object pair (Object Pair 1 Trials: [$F(1,6) = 0.11$, $p=0.75$)], Errors: [$F(1,6) = 0.01$, $p=0.92$)], Object Pair 2 Trials: [$F(1,6) = 0.36$, $p=0.57$)], Errors: [$F(1,6) = 0.49$, $p=0.51$)].

Reversal Performance – 1st and 2nd Object Pairs

For each of the two object pairs, the total trials and errors to criterion during each reversal were separately analyzed using a repeated measures ANOVA, with reversal as the within subjects factor, and treatment set as the between subjects factor. For the 1st object pair, there was no significant effect of reversal or treatment on either trials or errors (Table 2.5, Figure 2.2). (Reversal: Trials: Mauchly's Test: [$X^2(5) = 18.40$, $p = 0.003$, Greenhouse-Geisser corrected: [$F(1.32,7.70) = 1.14$, $p = 0.34$)], Errors: [$F(3,18) = 1.396$, $p = 0.27$); Treatment: Trials: [$F(1,6) = 0.20$, $p = 0.67$)], Errors: [$F(3,18) = 1.6$, $p = 0.54$)]).

For the 2nd object pair, there was no significant effect of reversal or treatment on trials to criterion (Table 2.5, Figure 2.3). (Reversal: Trials: [$F(3,18) = 1.5$, $p = 0.24$)]; Treatment: Trials: [$F(1,6) = 3.13$, $p = 0.13$)]). However, there was a significant effect of reversal [$F(3,18) = 4.74$, $p = 0.01$] but not treatment [$F(1,6) = 4.60$, $p = 0.08$)] on the totals errors to criterion. Pairwise comparison with Bonferroni correction showed that fewer errors were made by both groups during the 3rd reversal compared to the 1st ($p < 0.006$).

Though not significant, an interesting pattern emerges in task performance between the two groups of monkeys. Starting with the third reversal of the first object pair, monkeys treated with CUR on average were able to reach reversal criterion having made fewer than 50 errors during a reversal. This average error rate of approximately 50 errors/reversal was

importantly maintained during all subsequent object and spatial reversals. In contrast monkeys treated with VEH did not reach this sub 50 errors/reversal rate until the third reversal of the second object pair, a total of 4 reversals after the CUR treatment group. Additionally, unlike the CUR treated monkeys this error rate was not maintained by the VEH monkeys during subsequent reversal rounds. This performance level is lost in the immediate reversal after having been achieved and is not reestablished by VEH monkeys until the 2nd round of spatial reversals, 2 reversals later (Tables 2.5-2.7). Do to the small sample size a clear determination cannot be made, however subjectively, the 50 errors/reversal appears to be a ceiling, that CUR treatment group reaches first and maintains once it was achieved unlike the VEH monkeys. As it is likely that both groups of monkeys are in the earliest stages of cognitive decline, any significant improvement in task performance following treatment may be difficult to detect as significant decline in either group has yet to occur.

Staging Analysis – 1st and 2nd Object Pairs

For both object pairs, each type of stage error (I, II, III) was analyzed using a repeated measures ANOVA, with reversal as the within subjects factor and treatment as the between subjects factor (Figure 2.5). No significant effects of either reversal or treatment were observed for the 1st object pair (Reversal Stage I: Mauchly's Test: [$X^2(5) = 15.63$, $p = 0.009$, Greenhouse-Geisser corrected: [$F(1.92,11.53) = 1.14$, $p = 0.46$)], Stage II: [$F(3,18) = 1.40$, $p = 0.28$)], Stage III: [$F(3,18) = 1.4$, $p = 0.28$)]. Treatment on Stage I: [$F(3,18) = 1.93$, $p = 0.21$)], Stage II: [$F(3,18) = 0.16$, $p = 0.70$)], Stage III: [$F(3,18) = 0.16$ $p = 0.70$)]).

In contrast, for the 2nd object pair, significant differences were found in the total Stage I errors as the main effects of both reversal [$F(3,18) = 6.26$, $p=.0.004$)] and treatment, [$F(1,6) = 8.31$, $p=0.03$)], were significant. Further, a significant interaction between reversal and treatment was also observed [$F(3,18) = 4.54$, $p=0.02$)]. To explore this interaction, an analysis of simple effects followed by pairwise comparisons with Bonferroni corrections was performed. This revealed that within the VEH group, VEH monkeys made fewer Stage I errors on the 3rd reversal compared to the 1st reversal (mean difference: 5, $p < 0.02$) and 2nd reversal (mean difference: 2.5, $p < 0.05$). A similar effect of reversal within group was not observed for the CUR monkeys.

To explore the effect of treatment on Stage I errors, an analysis of simple effects followed by pairwise comparisons with Bonferroni corrections was conducted. This revealed that CUR monkeys made fewer Stage I errors on the 1st (mean difference: 3.75, $p < 0.02$) and the 4th reversal (mean difference: 2.75, $p< 0.03$) in comparison to VEH monkeys.

No significant effects of either reversal or treatment were found for either Stage II (Reversal [$F(3,18) = 1.06$, $p = 0.39$)], Treatment [$F(3,18) = 2.01$, $p = 0.20$)] or Stage III errors (Reversal [$F(3,18) = 1.17$, $p = 0.35$)], Treatment [$F(3,16) = 2.0$, $p = 0.28$)]).

Spatial Reversal Learning Task

Initial Acquisition

The total trials and errors to criterion during the acquisition phase (prior to beginning reversals) were separately analyzed using one-way ANOVA comparing the CUR and VEH groups. As shown in Table 2.4, no significant group differences were

observed during the acquisition phase of spatial reversals (Trials: [$F(1,6) = 0.243$, $p = 0.640$)], Errors: [$F(1,6) = 0.125$, $p = 0.736$)]).

Reversal Performance - Spatial

The total trials and errors to criterion during the spatial reversals were separately analyzed using a repeated measures ANOVA, with reversal as the within subjects factor, and treatment as the between subjects factor. There were no significant effects observed for either reversal or treatment (Figure 2.4 and Table 2.7) (Reversal: Trials: [$F(3,18) = 0.50$, $p = 0.61$)], Errors: [$X^2(5) = 18.16$, $p = 0.003$, Greenhouse-Geisser corrected: [$F(1.49,8.92) = 1.70$, $p = 0.20$)]; Treatment: Trials: [$F(1,6) = 0.20$, $p = 0.67$)], Errors: [$F(3,18) = 0.45$, $p = 0.53$)]).

Staging Analysis - Spatial

Each type of stage error (I, II, III) was analyzed using a repeated measures ANOVA, with both reversal and treatment as between subjects factors (Figure 2.5). No significant effects were found resulting from either reversal or treatment on any type of Stage Error (Reversal: Stage I: [$F(3,18) = 1.68$, $p = 0.21$)], Stage II: [$F(3,18) = 1.91$, $p = 0.34$)], Stage III: [$F(3,18) = 0.61$, $p = 0.62$)]; Treatment: Stage I: [$F(3,18) = .0.71$, $p = 0.43$)], Stage II: [$F(3,18) = 1.1$, $p = 0.34$)], Stage III: [$F(3,18) = 0.40$ $p = 0.55$)]).

Discussion

Summary of Results

The results of the current study show that daily oral CUR supplementation did not affect object discrimination performance nor recall 48 hours after criterion was met. Both treatment groups required more trials to reach criterion at high levels of feature overlap.

Both groups also had similar but poorer recall of discrimination when objects were more similar. CUR supplementation did enhance performance on object but not spatial reversal learning. Specifically, during the 2nd object pair testing the CUR treated group made fewer Stage I errors than the VEH treatment group during the 1st and 4th reversal of the 2nd object pair (Figures 1.5). These stage I errors are indicative of a failure to switch the established reward stimulus and by making fewer such errors the data suggest that CUR supplementation reduces the likelihood of perseverative responding. Within the 2nd object pair, VEH treated monkeys show improvement relative to themselves, making fewer Stage I errors on the 3rd reversal compared to the 1st and 2nd, this effect however is not maintained on following reversals. Overall, these findings demonstrate that CUR supplementation can improve performance on object reversal learning and suggest that supplementation improves frontal-cortical functioning in the middle-aged rhesus macaque.

Object Discrimination in Age

Unlike the age-related decline in working memory and executive function, declines of object memory and recognition memory is not typical of early senescent monkeys (Killiany et al., 2000). Deficits in object discrimination have been observed in age and following damage to the perirhinal cortex or when the objects are similar and cannot be distinguished by a single feature, (Bartko et al., 2007, Bussey, Saksida, & Murray, 2002, Barense et al., 2005, Burke et al., 2010, Burke et al., 2011). Here we found no effect of CUR treatment on either discrimination or recall. Our laboratory also previously reported that CUR supplementation did not improve performance on the DNMS (Moore 2017).

These data lend further support that CUR supplementation is likely not affecting the function of the temporal lobe in middle aged monkeys.

Cortical Regions of Executive Function and Reversal Learning

Dissimilar to recognition memory, working memory and executive function engage the prefrontal cortices, and exhibit age related decline that first manifests near midlife (Albert, 1984; Albert and Wolfe 1990 Chodosh et al., 2002; Drag and Bieliauskas; 2010; Fisk and Sharpe 2004; Hara et al., 2012; Kwon et al., 2016; Lai et al., 1995; León et al., 2016; Light 1991; Moore et al., 2003, 2006; Rapp 1990; Simen et al., 2011; Wecker et al., 2000; Voytko 1999; Zeamer et al., 2012; Zeamer et al., 2011, Park and Reuter-Lorenz, 2009). Specifically, in the rhesus monkey, it has been demonstrated that aging results in deficits in abstraction, set shifting, working memory and rule learning. In our laboratory, we have demonstrated that both middle-aged and aged monkeys are impaired on our Category Set Shifting Task (CSST) and the Delayed Recognition Span Task (DRST) both of which assess PFC function (Moore et al., 2003, 2006). Specifically, middle-age and aged monkeys require more trials, while also making more errors in learning these tasks compared to young controls. Furthermore, middle and aged monkeys have a greater tendency to perseverate in their response patterns than young monkeys. This pattern of impairment has also been observed by other groups. For example, Bartus et al., 1979 and Rapp 1990 have both demonstrated that middle-aged and aged monkeys have impairments in shifting response patterns, establishing new response patterns, demonstrated preservative response patterns and finally were more susceptible to interference on Reversal Learning Tasks. More recently Gray et al., 2017 and Munger et al., 2017 reported

that aged monkeys and marmosets were impaired in attentional updating, response shifting and committed more perseverative errors than young monkeys on reversal tasks.

Our previously published data from our CUR supplemented cohort demonstrated that supplementation improved performance on the Delayed Recognition Span Task spatial (DRSTsp), but had no significant on the DNMS (Moore et al., 2017). These results led us to hypothesize that given the age group of our monkeys (mean age 17 years), which are likely in the earliest stages of cognitive decline, we would best observe the effects of CUR supplementation on tasks necessitating frontal-cortical activation.

Reversal learning tasks have been used extensively across species in a multitude of modalities including object, spatial, auditory and olfactory reversal paradigms to assess cognitive flexibility, perseveration and rule learning. The specific cortical region most involved in mediating performance on reversal learning tasks however appears to depend on the modality of stimuli used in testing. Lesions within the orbital frontal cortex (OFC) have been shown to cause impairments in reversal tasks using visual and auditory stimuli, while medial prefrontal cortex (mPFC) lesions result in reversal learning impairments when using spatially orientated stimuli (Meunier et al., 1991; Shaw et al., 2013; Young and Shapiro 2009). Jones and Mishkin 1972 show that lesioning of either the OFC or a lesion encompassing the temporal pole and amygdala (TPA) resulted in impairments in both object and spatial reversals, while HPC lesions resulted in only in spatial reversal impairments. Importantly they showed that lesions to the OFC resulted in greater perseverative or stage I errors during object reversals than either TPA or FHH. Further Jones and Mishkin showed a double dissociation on performance during object reversals

where OFC lesioned monkeys made greater Stage I errors, while TPA lesioned monkeys made greater Stage II errors, the FHH group did not differ from controls at any stage. Mahut 1971(a, b) which reversed the order of testing between spatial and object reversals in comparison to Jones and Mishkin 1972 yielded similar results despite the difference in testing order. Lai et al., 1995 report that aged non-human primates make more errors on spatial but not object reversals in comparison to young monkeys. Furthermore, Lai et al., 1995 report that the aged monkeys made increased Stage I errors on both spatial and object reversal tasks in comparison to young monkeys. From these studies it is clear that reversal tasks engage multiple brain regions, however aging in monkeys appears to cause similar deficits to lesioning the OFC, such that aged monkeys exhibit greater perseverative responding than young monkeys during object reversals. While it is difficult to pinpoint a singular region as definitively benefitting from CUR supplementation, the lessening of perseverative errors in object reversals, our previously published data on improved DRSTsp performance (Moore et al., 2017), and the age of the tested cohort who would be in the earliest stages of cognitive decline, combined would suggest that CUR supplementation may be improving frontal cortical function.

Aging and Inflammation

The PFC is associated with age-related myelin pathology and decreased white matter volume (Wisco et al., 2008; Grady 1998; Makris et al., 2007; Peters et al., 1994; Raz et al., 1997; West 1996). In addition, there is a loss of myelin integrity as measured by decreased fractional anisotropy in PFC white matter (Makris et al., 2007). This age-related increase in myelin damage can have downstream effects as myelin debris can inhibit

oligodendrocyte differentiation and remyelination (Kotter et al., 2006). Finally, there is evidence of a rise in age related inflammation within the frontal white matter demonstrated by increased phagocytic and ameboid microglia, (Shobin et al., 2017). Together, these findings suggest an age-associated vulnerability of the PFC to alterations in the myelin sheath. It is hypothesized that changes in white matter and myelin are associated with age-related immunosenescence that contributes to a chronic neuroinflammation (Di Benedetto et al., 2017; Ownby 2010). In support of this idea, is evidence of an age-related increase in microglial proinflammatory activation (Shobin et al., 2017). Further support for this notion comes from data showing increased circulating proinflammatory cytokines that negatively impact the CNS, specifically myelin and white matter (Cornejo and von Bernhardi 2016; Robillard et al., 2016; Safaiyan et al., 2016; Xie et al., 2013).

Aging is also associated with a decrease in intrinsic antioxidant and anti-inflammatory capability, with a shift towards proinflammatory immune response (Ye and Johnson 2001, London et al., 2013). Microglia, the macrophages of the brain, have been demonstrated to have a loss in phagocytic capability, a priming toward pro-inflammatory activation, and an increased density within the frontal white matter with age (Plowden et al., 2004; Safaiyan et al., 2016; Shobin et al., 2017). The age-related increases in white matter pathology, inflammation and immune activation suggests that a cycle of inflammation induced myelin damage leads to a persistent and inefficient immune response. This inflammaging effect can then further exacerbate myelin pathology and may drive the cognitive decline observed in aging. Finally, changes in myelin ultrastructure, increased microglial activation, and increased markers of inflammation are all strongly

correlated with age-related cognitive decline and therefore are potential targets for anti-inflammatory compounds, such as CUR (Simen et al., 2011). Many studies have demonstrated that CUR is a potent anti-inflammatory agent (Lee et al., 2007; Nahar et al., 2015; Parada et al., 2015; Tegenge et al., 2014; Yang et al., 2014) observed to inhibit of the activity the NF-κB transcription factor, a key regulator of the inflammatory process which can promote the production of many pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Jin et al., 2007; Kure et al., 2017; Lee et al., 2007). Together, these actions of CUR may have downstream effects that result in improved performance on our Reversal Learning Task by monkeys receiving long-term daily administration of CUR. Future studies, examining brain tissue and CSF from these monkeys, will help to elucidate the potential effects on inflammation that CUR treatment has in these monkeys.

Study Limitations

Human clinical trials of CUR treatment with aged individuals, show that control subjects exhibit noticeable decline in performance on the Montreal Cognitive Assessment in comparison to those who received CUR (Rainey-Smith et al., 2016). These data suggest that while CUR is not improving performance, it is perhaps delaying or mitigating aging effects on cognition. A key limitation of this study, in addition to the small group size, is that perhaps the cohort of monkeys tested have not developed significant cognitive impairment such that a similar mitigation of decline by CUR treatment can be easily observed. Further conspicuous improvements following supplementation may be partially masked by the lack of pronounced decline in cognitive ability in the cohort tested. For this reason, we believe that a thorough understanding of CUR treatment effects would be more

readily assessed in longitudinal studies with treatment beginning in middle age and continuing through to advanced old age (15 to 25 years+). A longitudinal study as described, may provide evidence that CUR has a beneficial role in delaying age-related cognitive decline. Further studies in which CUR treatment begins an older cohort of monkeys, which likely will have a poorer baseline cognitive ability, would address if CUR treatment can reverse age related cognitive decline. This current study only tested a subset monkeys from the larger study as several animals in the larger study had already been euthanized when the spatial and object reversals were added to the testing paradigm. These more "frontal" based tasks were added for the 2nd cohort based on the findings that curcumin improved performance on the DRSTsp task, another task of PFC function. While this did provide valuable insight in the impact of curcumin on PFC function, a second round of reversal testing may have provided further evidence supporting prior results from our laboratory and others that CUR supplementation can improve performance with repeated testing.

A second limitation to this study was that while both groups can be considered middle aged it should be noted that the CUR treatment group was significantly older than our VEH group. This did not impact the initial acquisition of any of the tasks and as age is a known to negatively impact reversal learning, it is plausible that any effect of age on task performance would act as a disadvantage to the CUR treatment group. That the CUR treated group made fewer Stage I then younger VEH controls provides even further evidence of the beneficial effects of curcumin.

Conclusions and Future Directions

The present study and previous investigations by our laboratory have demonstrated that long-term daily administration of CUR to middle-aged rhesus monkeys enhances performance on tasks of spatial working memory and motor function (Moore et al., 2017; 2018). Here we show that CUR treated monkeys make fewer perseverative type errors in contrast to controls. We did find that control monkeys only show improvements relative to themselves. The CUR treatment group quickly reaches and maintains a ceiling of errors made during reversals, in contrast the VEH treatment group does not, and because the CUR group are making fewer errors a significant improvement may be difficult to detect. We found no significant effect of CUR treatment on object discrimination nor recall testing performance. The improvement in performance by CUR treated monkeys on object and in DRSTsp, with no improvements on spatial reversals, object discrimination, recall and DNMS, taken together support the hypothesis that CUR supplementation is likely affecting the PFC but not the temporal lobe in our model. However, the precise role of CUR in delaying and/or improving age-associated cognitive and motor decline remains unclear. While the exact neurobiological mechanism remains a mystery, the demonstration that CUR supplementation has a beneficial role on cognition in humans and monkeys offers a nutritional approach to reducing age-related cognitive decline.

Study Subjects

Group	Monkey	Sex	Age	OD	Reversal
Control (n=4)	AM352	M	12	X	X
	AM303	M	15	X	
	AM350c	F	16		X
	AM340	F	17	X	X
	AM347	F	17		X
	AM311c	M	20	X	
Mean				16	15
SD				3.4	2.4
Treated (n=4)	AM344c	F	16		X
	AM349c	F	19		X
	AM309c	M	20	X	
	AM308c	M	20	X	
	AM312c	M	21		X
	AM310c	M	21	X	X
Mean				20	19
SD				0.6	2.4

Table 2.1 | Behavioral Study Subjects: Monkeys used in each study listed in order by age. Task Participation denoted by X.

Total Trials to Criterion for Object Discrimination

Group	Monkey	60% Trials	70% Trials	80% Trials	90% Trials
Control (n=4)	AM340	749	387	1317	1499
	AM303	240	360	360	690
	AM311c	450	120	2160	780
	AM352	849	180	570	3355
Mean		572	262	1102	1581
SD		279	132	816	1237
Treated (n=4)	AM310c	758	295	210	2246
	AM308c	539	270	500	2010
	AM309c	450	390	533	840
Mean		582	318	620	1699
SD		159	63	355	753

Table 2.2 | Object Discrimination Performance: Total trials taken to reach criterion for each object.

Total Correct Trials on Recall Testing for Object Discrimination

Group	Monkey	60% Trials	70% Trials	80% Trials	90% Trials
Control (n=4)	AM340	25	28	28	22
	AM303	22	24	24	25
	AM311c	30	27	22	22
	AM352	27	28	23	22
Mean		26	27	24	23
SD		3.4	1.9	2.6	1.5
Treated (n=4)	AM310c	25	23	25	21
	AM308c	27	26	26	22
	AM309c	29	24	23	26
Mean		27	24	25	23
SD		2	1.5	1.5	2.6

Table 2.3 | Recall Trials: Total correct trials made on recall testing 48 hours after criterion was met for each object pair.

Total Trials and Errors to Criterion for Initial Learning for each Reversal Task

Group	Monkey	Object Pair 1 Trials	Object Pair 1 Errors	Object Pair 2 Trials	Object Pair 2 Errors	Spatial Trials	Spatial Errors
Control (n=4)	AM352	102	18	164	41	124	26
	AM347c	141	69	202	62	30	3
	AM350c	56	4	53	10	97	23
	AM340	76	14	96	24	73	17
Mean		94	27	129	34	81	17
SD		37	29	67	22	40	10
Treated (n=4)	AM344c	150	49	105	29	63	7
	AM349c	75	15	51	9	91	29
	AM312c	144	36	146	34	50	10
	AM310c	48	12	120	30	77	13
Mean		104	28	106	26	70	15
SD		51	18	40	11	18	10

Table 2.4 | Reversal Initial Acquisition: Total trials and errors made during initial acquisition for each reversal task.

Performance on Object Pair 1 Reversal Testing

Group	Monkey	Rev 1 Trials	Rev 1 Errors	Rev 2 Trials	Rev 2 Errors	Rev 3 Trials	Rev 3 Errors	Rev 4 Trials	Rev 4 Errors
Control	AM352	179	79	140	70	290	160	200	68
(n=4)	AM347c	350	144	410	192	260	99	320	150
	AM350c	110	59	50	28	110	35	80	42
	AM340	80	55	50	34	80	24	80	37
Mean		180	84	163	81	185	80	170	74
SD		121	41	170	76	105	63	115	52
Treated	AM344c	566	259	80	36	50	21	110	38
(n=4)	AM349c	140	60	140	65	80	44	110	39
	AM312c	140	60	50	32	140	50	140	57
	AM310c	80	40	230	102	50	25	80	22
Mean		232	107	125	59	80	35	110	39
SD		223	102	79	32	42	14	24	14

Table 2.5 | Object Pair 1 Reversal Performance: Total trials and errors made for the 1st object pair.

Performance on Object Pair 2 Reversal Testing

Group	Monkey	Rev 1 Trials	Rev 1 Errors	Rev 2 Trials	Rev 2 Errors	Rev 3 Trials	Rev 3* Errors	Rev 4 Trials	Rev 4 Errors
Control	AM352	140	68	50	32	80	33	230	102
(n=4)	AM347c	260	128	230	102	260	82	200	110
	AM350c	140	65	140	65	80	41	110	46
	AM340	170	86	80	102	110	35	140	54
Mean		176	86	123	59	133	48	170	78
SD		57	29	79	32	86	23	55	33
Treated	AM344c	80	20	20	11	46	11	20	4
(n=4)	AM349c	140	44	110	47	170	51	110	51
	AM312c	80	35	80	24	80	28	80	35
	AM310c	110	60	110	47	92	45	110	43
Mean		103	40	80	32	97	34	80	32
SD		29	17	42	19	52	18	42	20

Table 2.6 | Object Pair 2 Reversal Performance: Total trials and errors taken during each reversal for the 2nd object pair. Repeated measures ANOVA revealed an overall effect of reversal but not treatment [$F(3,18) = 4.735$, $p=.0.013$], pairwise comparisons with Bonferroni corrections show that fewer errors were made during the 3rd reversal compared to the 1st ($p < 0.006$).

Performance on Spatial Reversal Testing

Group	Monkey	Rev 1 Trials	Rev 1 Errors	Rev 2 Trials	Rev 2 Errors	Rev 3 Trials	Rev 3 Errors	Rev 4 Trials	Rev 4 Errors
Control	AM352	170	120	80	33	170	77	140	57
(n=4)	AM347c	170	72	200	47	200	66	130	52
	AM350c	80	37	80	20	80	25	80	17
	AM340	110	36	80	18	20	8	70	17
Mean		133	66	110	30	118	44	105	36
SD		45	40	60	13	83	33	35	22
Treated	AM344c	74	20	20	9	260	119	170	71
(n=4)	AM349c	110	48	110	42	50	20	140	29
	AM312c	80	26	80	20	80	28	80	25
	AM310c	110	41	50	16	50	13	80	15
Mean		94	34	65	22	110	45	118	35
SD		19	13	39	14	101	50	45	25

Table 2.7 | Spatial Reversal Performance: Total trials and errors taken during each reversal for Spatial Reversal testing.

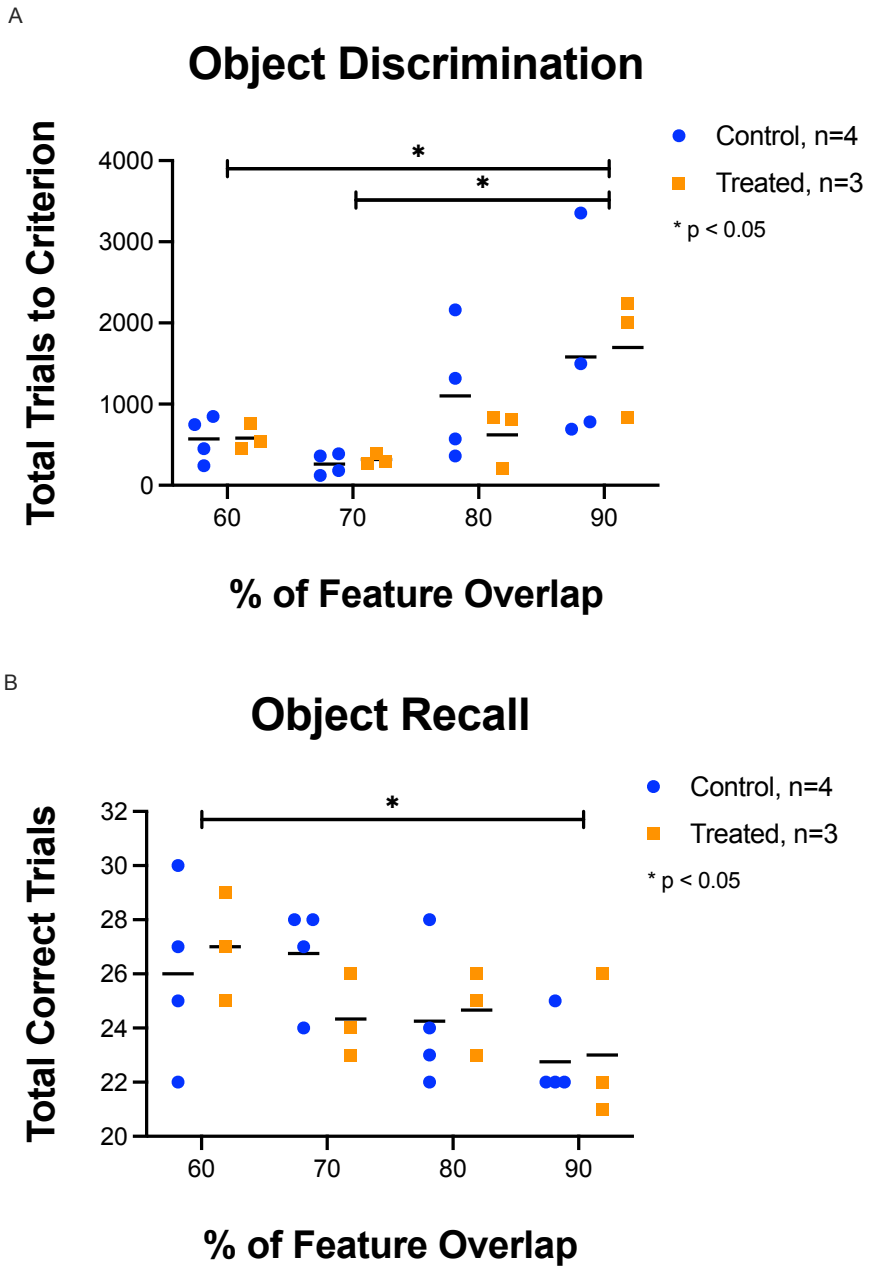

Figure 2.1 | Object Discrimination Performance A: Total trials to reach criterion for each object pair. No significant group differences were found, however a significant effect due to feature overlap was observed with 90% object pair requiring more trials to reach

criterion than either the 60 or 70% pairs overlap [$F(3,20) = 5.560$, $p=0.006$], ($p < 0.029$, $p < 0.005$, respectively. B: Total correct trials during recall for each object pair. No significant group differences were found, however recall at the 60% object pair was greater than the 90% pair [$F(3,20) = 3.202$, $p=0.045$)], $p<0.038$.

Object Pair 1 Reversal Errors

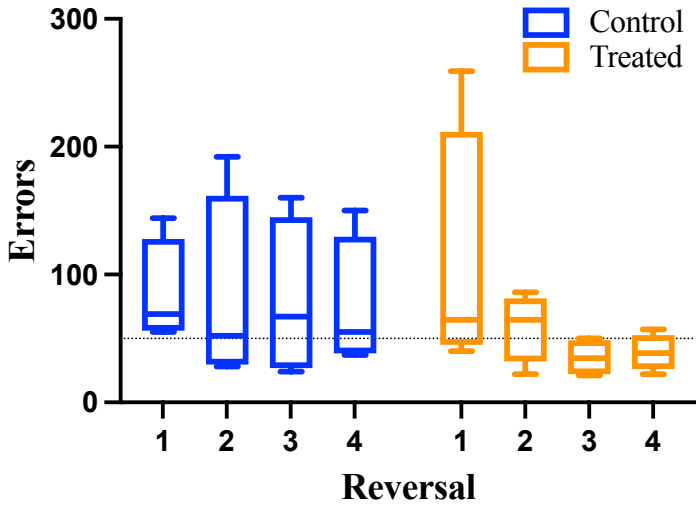

Figure 2.2 | Object Pair 1 Reversal Errors: Errors made during 1st object pair reversal testing. Monkeys treated with vehicle control are in blue and monkeys treated with CUR are in orange. A line is drawn through the 50-error mark for visual clarity. No significant group differences were observed in performance.

Object Pair 2 Reversal Errors

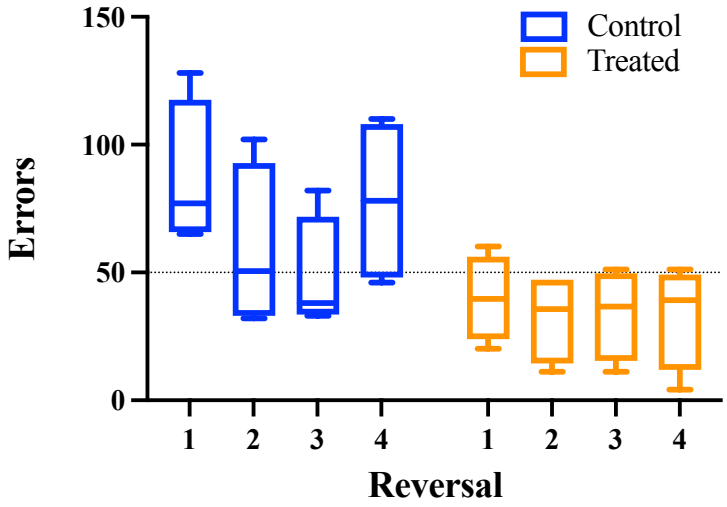

Figure 2.3 | Object Pair 2 Reversal Errors: Errors made during 2ndt object pair reversal testing. Monkeys treated with vehicle control are in blue and monkeys treated with CUR are in orange. A line is drawn through the 50-error mark for visual clarity. Repeated measures ANOVA found no significant effect of treatment, however an overall significant difference was found due to reversal, where less errors were made during the third reversal in comparison to the first (reversal [$F(3,18) = 4.735$, $p=.0.012$)], R1 v R3 $p < 0.006$).

Spatial Reversal Errors

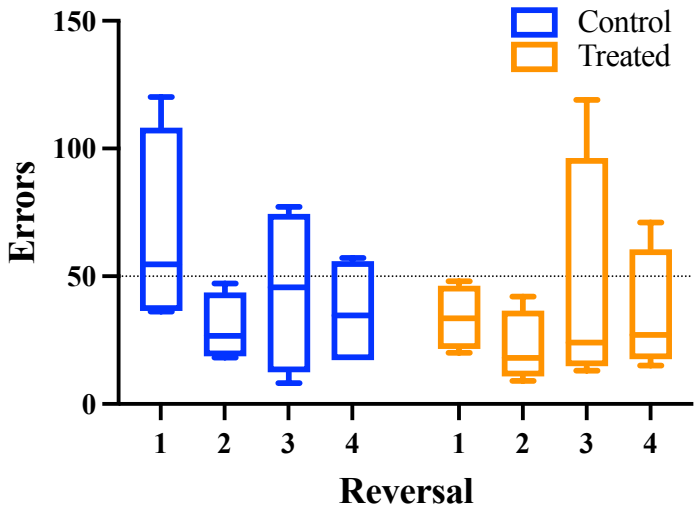

Figure 2.4 | Spatial Reversal Errors: Errors made during spatial reversal testing. Monkeys treated with vehicle control are in blue and monkeys treated with CUR are in orange. A line is drawn through the 50-error mark for visual clarity. No significant group differences were observed in errors made during other reversals.

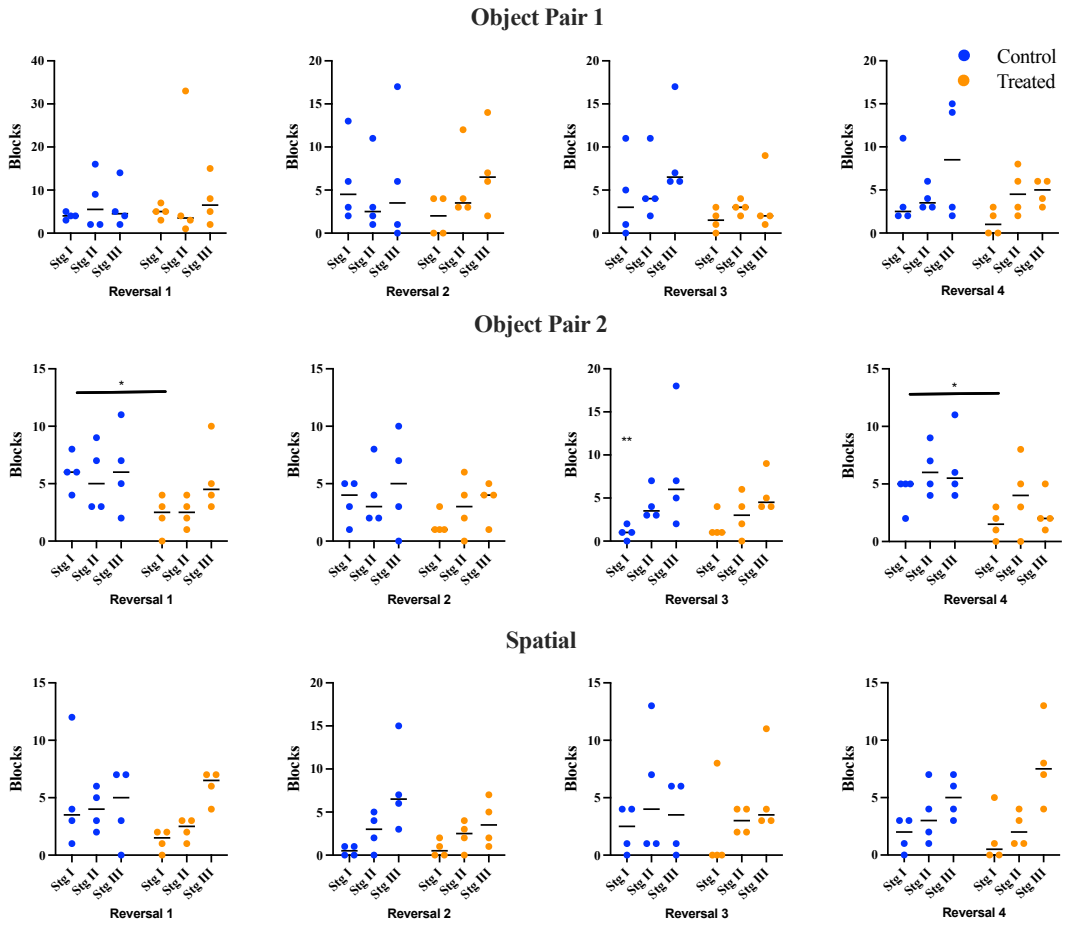

Figure 2.5 | Staging Errors: Total blocks of trials spent in each stage of error per reversal per subject. Top Row: Object Pair 1 Reversals. Middle Row: Object Pair 2 Reversals, * denotes that treated monkeys made significantly fewer Stage I errors during the 1st (mean difference: 3.75, $p < 0.019$) and on the 4th reversal (mean difference: 2.75, $p < 0.032$) reversal in comparison to VEH treated monkeys. ** Denotes within group, control monkeys made fewer Stage I errors during the 3rd reversal compared to their performance on the 1st (mean difference: 5, $p < 0.016$) and 2nd reversals (mean difference: 2.5, $p < 0.047$).

Bottom Row: Spatial Reversal, no other significant group differences were observed in the Object Pair 1 or Spatial Reversal testing.

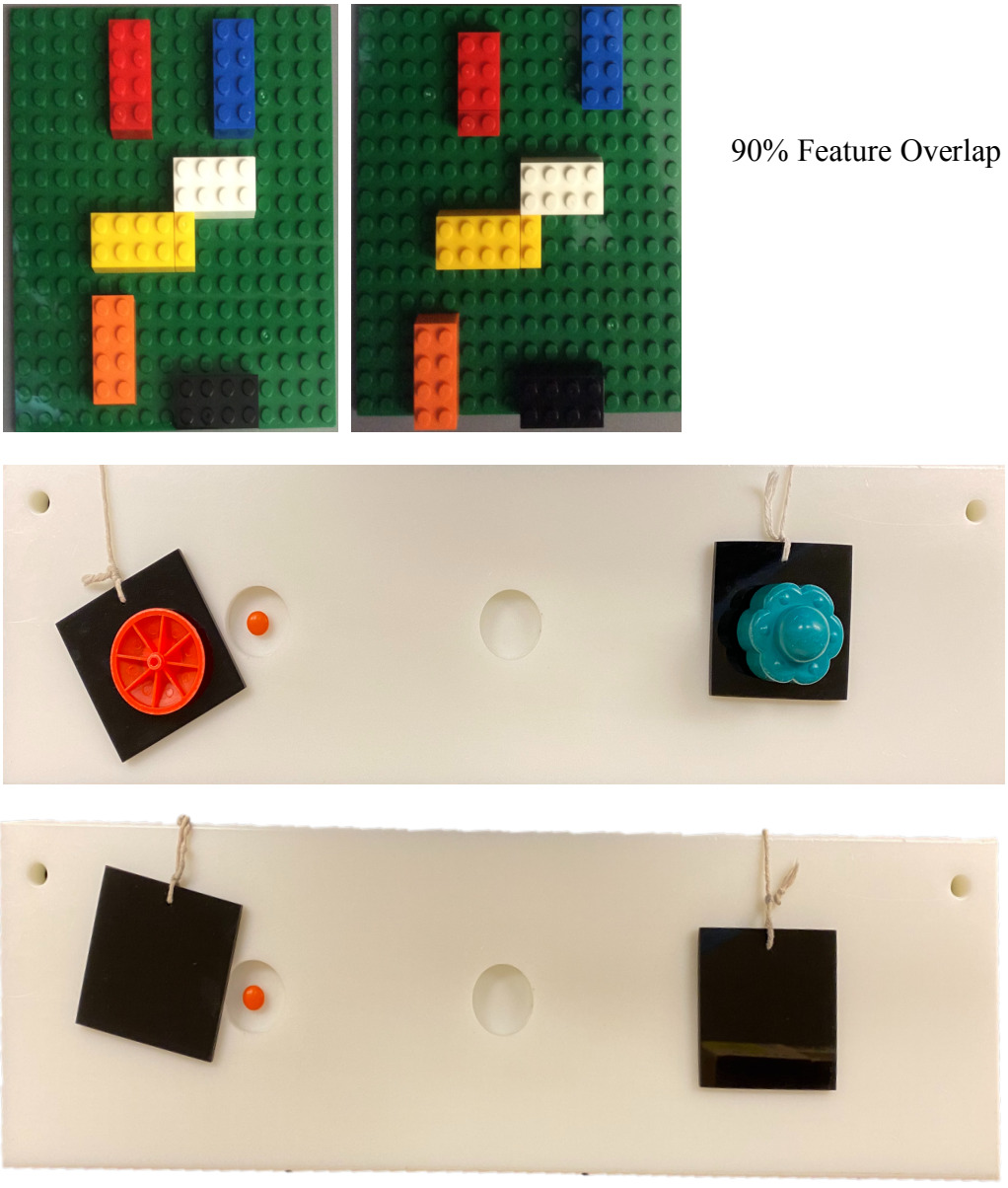

Figure 2.6 | Sample Objects and Plaques

Representative Sample of the Lego Object Plaques and Testing Board Used for the Object Reversal Learning Task

CHAPTER THREE:

CURCUMIN SUPPLEMENTATION EFFECTS ON MICROGLIA DENSITY, ANTIGENICITY AND MORPHOLOGY

Introduction

Microglia are the macrophages of the brain and are a principal component of the immune response within the central nervous system (Alliot et al 1999). Ongoing study of microglia reveal diverse and multifold functionality, ranging from synaptic pruning, secretion of either pro- or anti-inflammatory cytokines and chemokines, as well as secretion of trophic factors that are necessary for cellular growth and maintenance (Stevens et al., 2007, Ginhoux et al 2010, Schafer et al., 2012). Other key functions of microglia relate to their ability to mount an immune response to injury or insult including, phagocytosis of debris, forming glial barriers, and antigen presentation. There is growing evidence that microglia play a significant role in the neuroinflammatory processes of multiple diseases but additional evidence indicates a role of microglia in aging and age-related cognitive decline (Streit et al., 2004, Shobin et al., 2017, De la Fuente and Miquel, 2009; Poliani et al., 2015; Rawji et al 2016; Ruckh et al., 2012).

In aging there is an accumulation of damage to myelin sheaths within frontal cortex that correlated to cognitive decline (Bowley et al., 2010, Peter et al 2009, Feldman and Peters 1998, Peters and Salthares 2002, Sandell and Peters 2003). Growing evidence indicates a dysfunction in both myelin repair by oligodendrocytes as well as clearance of myelin debris by microglia which occurs with age (Kotter et al., 2006, Floden and Combs 2011, Natarajan et al., 2015, Safaiyan et al.,, 2016). Microglia demonstrate impaired

phagocytic activity with age, and a priming to pro-inflammatory response (Perry et al., 1993, Steit and Sparks 1997, Sheffield and Berman 1998, Holtman et al., 2015). Microglia morphology often is suggestive of its activity, such that ramified microglia are thought to be in a surveilling state, ameboid microglia actively phagocytosing debris, and hypertrophic microglia representing transitional states between ramified and ameboid morphologies (Karperien et al 2013, Nimmerjahn et al., 2005, Bohatschek et al 2001, Lee et al 2008). Shobin et al., 2017 show there is an accumulation of ameboid microglia within the white matter of aged monkeys and this is further correlated to cognitive decline. These data suggest that in the aged brain there is a persistent and inefficient response of microglial activation that can further potentiate damage to the myelin sheaths.

CUR supplementation has been demonstrated to dampen proinflammatory response of microglia, as well as altering microglial morphology (Ullah et al., 2020). The results from in vitro and rodent models demonstrate an attenuation of proinflammatory response by mitigating NF-kb production, as well as increasing process length and decreasing soma size, and decreasing in density following CUR supplementation (Karlstetter et al., 2011, Naeimi et al., 2018, Ullah et al 2020).

The current study tested the effects of daily long-term oral CUR supplementation on microglial density within the white matter and microglial morphology in both grey and white matter regions of rhesus monkeys. Immunohistochemical (IHC) labeling of the LN3 antibody, which identifies HLD-DR major histocompatibility complex class II receptor and stereology were used to measure microglia density within the white matter. IHC using LN3 antibodies in combination with antibodies against a purinergic receptor (P2Yr12) and a pan

microglial marker ionized calcium binding adapter molecule (Iba1), was conducted to allow for complete 3-dimensional reconstruction of microglia using Neurolucida Software. The goal of these experiments was to examine whether CUR supplementation dampened neuroinflammation reflected by changes in microglia density and morphology.

Methods

Subjects

Tissue from 22 male and female rhesus macaques aged 6-26 (18-78 human equivalency, Tigges et al., 1998) were selected for use in the following experiments. For the measurements of microglial density tissue from young and old monkeys were included in the analysis as controls to reaffirm age related increases in microglia density. Tissue samples were obtained from monkeys that received daily CUR supplementation (500mg/day) over a 3-year period. The CUR was a lipidated formulation to increase bioavailability, provided by Verdure Sciences (Noblesville, IN). CUR or VEH treatments were mixed with either 150ml of yogurt or Prima-Burger TM (BioServ, Flemington, NJ). Monkeys were observed to ensure that they consumed the treatment, if they did not consume the first treatment, a second treatment was given later in the day. Treatments for both groups were observed to be eaten on 98% of days.

Perfusion and Fixation of the Brain

Briefly, following an induction of ketamine (10mg/kg IM), blood was collected from either left/right femoral vein after which monkeys were deeply anesthetized with sodium pentobarbital (25mg/kg IV to effect). Once fully anesthetized, monkeys were placed in a stereotactic head holder in a prone position and cerebral spinal fluid (CSF) was

collected from the cisterna magna. The monkey's head was tilted ventrally exposing the atlanto-occipital junction and a 23-gauge needle was used to access the cisterna magna to withdraw 2-3cc of CSF. For perfusion the monkeys were euthanized by exsanguination during trans-cardial perfusion of the brain, first with Krebs-Heinsleit buffer (4°C, pH7.4, 3-6L), followed by 4% paraformaldehyde (37°C, pH7.4, 8L). During perfusion with Krebs-Heinsleit buffer, following clearance of the vasculature fresh tissue samples were collected prior to the fixation with paraformaldehyde. This fresh tissue was flash frozen and stored at -80°C. Following fixation, the brain was then removed and allowed to fixate further in 4% paraformaldehyde overnight. The brain was then transferred to a cryo-protectant solution (20% glycerol in 2% DMSO) to prevent freezing artifact. The brains were then flash frozen using isopentane at -75°C and then stored at -80°C prior to cutting.

Brain Cutting and Tissue Collection

The brains were cut using a frozen microtome into one 60µm series (24 vials) and eight 30µm series (96 vials). Half the 60µm tissue was mounted on slides and stained with thionin. The other half were divided, 6 vials were frozen at -80°C, tissue stored in a 15%glycerol phosphate buffer solution. The six remaining 60µm vials were kept at -20C, in a 30% ethylene glycol in 0.05M phosphate buffer solution (Medalla and Barbas 2009). All 30µm vials were stored at -80°C, tissue stored in a 15%glycerol phosphate buffer solution with the exception of one series which was stored in a 15% glycerol 1%paraformaldehyde phosphate buffer solution.

Immunohistochemistry

Brightfield Immunohistochemistry for Density Analysis

30μm coronal sections were used in the microglia density experiments. Tissue was first thawed and allowed to warm to room temperature. 6-10 serial sections per animal were selected and stained to assess microglia density. Section intervals were ~1200μm. Sections were then washed in 0.05M Tris-Buffered Saline (TBS, pH 7.5), 3X10min per wash (used in all wash steps). To improve antibody binding and break fixation related cross-linkages, antigen retrieval was done in a microwave tissue processor (Pelco Biowave) for 5min at 550W and 40°C in a solution of 10mM sodium citrate buffer. Following another wash cycle, tissue was incubated in a 3% hydrogen peroxide solution to quench endogenous peroxidases. Tissue was washed again then incubated in a blocking solution containing 10% SuperBlock (Life Technologies) 0.2% triton X in 0.05TBS for 1 hours to prevent non-specific antibody binding. Tissue was then placed into incubation buffer containing 0.1MPB 2% normal donkey serum 0.3% triton X, and primary antibody mouse anti-LN3 IgG2b 1:500 (MP Biomedicals, Santa Ana, CA) for 48 hours at 4°C. Following primary incubation and repeat washing, tissue was then placed into a second incubation buffer now containing secondary antibody: biotinylated goat anti-mouse 1:600 (Vector Laboratories). Tissue was washed again, then placed into a solution of avidin-biotin complex (Vectastain, ABC Kit, Vector Laboratories) for 1 hour. Tissue was washed then exposed to a chromagen solution (0.5mM 3-3 diaminobenzadine, Sigma Aldrich) for 5min. After a final rinse tissue was mounted onto gelatin coated slides and dried for 72 hours. Slides were dehydrated using solutions of increasing alcohol content (0, 50, 70, 90, 100% 1 min each). Slides were cleared using xylenes (2 x 5min). Slides were then coverslipped with Permount (Fischer Scientific). Slides were then blind coded prior to counting.

Fluorescent Immunohistochemistry for Reconstruction

60μm sections were used in the microglia reconstruction experiments. Tissue was first thawed and allowed to warm to room temperature. 1-2 tissue sections containing Brodmann Areas 46 and 25 were identified and used. Sections were then washed in 0.01M Phosphate Buffered Saline (PBS, pH 7.5), 3X10min per wash (used in all wash cycles unless noted otherwise). Unreacted aldehydes were then blocked by incubating the tissue 50mM Glycine in 0.01PBS for 2hours. To improve antibody binding, antigen retrieval was done in a water bath at 65C for 20min in a solution of 10mM sodium citrate buffer. Following another wash cycle tissue was incubated in a second blocking solution containing 5% bovine serum albumin 5% normal donkey serum 0.2% triton X in 0.01PBS for 2 hours to further prevent non-specific antibody binding. Tissue was incubated in primary antibodies diluted in incubation buffer containing 0.1MPB 0.2% BSAc (Aurion) 1% normal donkey serum 0.1% triton X for 12 hours at 4°C. All primary antibodies were diluted in the incubation buffer as follows: mouse anti-LN3 IgG2b 1:50 (MP Biomedicals, Santa Ana, CA), rabbit anti-P2YR12 1:250 (Wako), guinea pig anti-VGlut2 1:1000 (Synaptic Systems), goat anti-Iba1 IgG 1:1000 (Abcam). To improve antibody penetration tissue was microwaved using a Pelco Biowave for 10 minutes at 35°C for 150 watts a total 5 times. Following primary incubation and repeat washing, tissue was then placed into a second incubation buffer now containing secondary antibodies: Alexa Fluor donkey anti-mouse 405 1:200, donkey anti-rabbit biotinylated 1:200, Alexa Fluor donkey anti-guinea pig 488 1:200 and Alexa Fluor donkey anti-goat 647 1:200 for 12 hours at 4°C with microwave sessions as described above. Following secondary incubation and repeat

washes, tissue was then placed in a third incubation buffer containing streptavidin 568 1:200 for 12 hours at 4°C. Following this final incubation tissue was then washed and placed into a solution of 1mM cupric sulfate for 10 minutes to reduce auto fluorescence. Tissue was washed a final time, mounted onto slides and coverslipped using Prolong Anti-Fade solution, and blind coded for imaging.

Imaging of Tissue

Stereologic Counting and Brightfield Microscopy to Quantify LN3 Density

LN3 positive microglia were counted using a Nikon E600 light microscope, a motorized stage and StereoInvestigator software (MBF Bioscience). LN3 positive cells were estimated using the optical fractionator method (West et al., 1991). 5-7 serial sections (spaced ~1200um apart) were counted for each monkey. The regions of interest (ROI) were identified using a 2X objective, while actual counts were made using a 60X oil objective. A counting frame of 60 X 60 X 5 μm^3 was used with a dissector top and bottom guard of 1μm from the tissue surface. The soma was used as the counting object. Microglia were counted based on morphological criteria of Shobin et al., 2017. Briefly, the morphology of hypertrophic 1 microglia were defined as having rod like somas, hypertrophic 2 microglia had enlarged rounded somas, with both subtypes having short dense processes extending out from the soma. Ramified microglia were defined as compact cell bodies with long heavily branched processes. Ameboid microglia, were defined as having enlarged rounded somas with little to no processes extending outward. Microglia were counted in 3 white matter ROI. The cingulum bundle (CngB), frontal white matter (FWM) and the corpus callosum (CC). The CngB was defined as white matter bounded by the cingulum gyrus

dorsally and the corpus callosum ventrally. The CC was the region of white matter between the midline the anterior cingulate gyrus and the lateral ventricle. The FWM was defined medially as lateral to the corpus callosum and the putamen, and lateral to the CngB. The lateral boundary of the FWM followed the cortex excluding the grey matter. The Cavalieri Estimator was used to estimate volumes, and the coefficient of errors (CE) was calculated as described by Gunderson et al., 1999. For some monkeys the CE was close but not <0.1, as such here we report cell density in cubic millimeters to compare relative amounts of cells between subjects.

Confocal Imaging and 3D Reconstructions of Microglia

24 hours after mounting, slides were blinded and coded prior to imaging. Tissue was imaged using a Leica SPE confocal microscope. Regions of interest were found using a 10X objective, once defined 3-5 sites within the region were imaged using a 63X oil immersion lens for all samples. ROI included grey matter: Layers 2/3 of Area 46 and Area 25, white matter: CngB and CC. Imaging settings include: resolution 1024 x 1024 with a zoom factor of 1.5, pixel size 113.79 113.70 μm, z-step 0.35μm. Images were processed using 3D deconvolution using AutoQuant X3 (Media Cybernetics). Following deconvolution microglia were reconstructed in 3d using user guided tracing methods available in Neurolucida 360 (MBF Bioscience). Microglia were selected for reconstruction and analysis, provided that the full soma and processes were imaged. These images were also analyzed for LN3 expression.

Analysis

Density

LN3 Density data were analyzed using a three-way ANOVA. Density measurements were used as dependent variables, age, treatment and region of interest were treated as dependent variables. Post hoc testing was done by multiple comparisons with Tukey correction. For age, monkeys were divided into three groups 1-for younger monkeys: <11 years, 2-early middle aged:11-20 years, 3-late middle aged-old: >20 years. For morphological subtype comparisons between treated and age matched controls ANOVA was performed with density as the dependent variable, subtype, treatment and regions of interest were independent variables.

LN3 Expression

Images were batched processed to measure degree of LN3 expression within the cingulum bundle and corpus callosum. Using the 'Coloc 2' plugin for ImageJ (NIH), colocalized images of LN3 and P2YR12/IBA1 signals were generated. These colocalized images were then analyzed using the 'Particle Analysis' feature of ImageJ, following auto-thresholding using Otsu Dark thresholding method. The data were analyzed using an ANOVA with Average Percent Area being the dependent measure and treatment and ROIs being the independent factors.

3D-Reconstructions

Following reconstructions, cells were analyzed using Neurolucida Explorer (MBF Biosciences). Within this software cells were examined using prebuilt analysis methods including branched structure, process length, convex hull 2D area, 3D, volume and Sholl Analysis. For the Sholl analysis, the center point was defined as the soma and ring radius was set increase by 2um increments. Data output from explorer was then organized,

grouping cells by grey and white matter into a total of two groups. The data were then normalized using Euclidean normalization within MATLAB to allow for multivariate analysis. Independent variables included: Soma Surface Area, Process Length, Total Intersections, Average Diameter, Quantity, Nodes, Ends, Area, Branching Index, Convex Hull 2D Area, Convex Hull 3D Perimeter, Convex Hull 3D Volume and Convex Hull 3D Perimeter. Grey and white matter were analyzed separately.

Results

Microglial Density

A significant effect of age was observed, such that the oldest group (20+ years) had a greater density of LN3+ cells than our young monkeys (<11 years), replicating similar results found in our laboratory (Figure 3.1, [$F(1,2) = 7.63$, $p=0.001$], Tukey Post hoc $p = 0.007$) (Shobin et al., 2017). However, in middle aged monkeys, there was no significant effect of CUR on microglial density in comparison to age matched VEH controls. Further there was no effect of CUR on density of microglia when assessing by morphological subtype (Figure 3.2). For both CUR and VEH monkeys Hypertrophic 1 and 2 cells were the predominate subtypes of cells counted ([$F(3,132) = 73.05$, $p=0.001$], Tukey Post hoc $p < 0.001$, for all comparisons).

LN3 Expression

Particle analysis was run on the colocalized signal between LN3 and P2YR12/IBA1 to measure the amount of LN3 microglia were expressing. No significant effect was observed due treatment on the percent area label of the colocalized signal in either the CngB nor the CC (Figure 3.3).

3D Reconstructions

A total of 143 cells were reconstructed from the grey matter of VEH (70 cells - 49: A46, 21: A25) and CUR (73 cells - 43: A46, 30: A25) monkeys. Within the white matter a total of 31 cells were reconstructed from VEH (19 cells – 13: CC, 6: CngB), and CUR (12 cells – 7: CC, 5: CngB) monkeys. Within the white matter, significant differences were found between treatment groups with regards to almost all microglial morphological features measure except soma surface area, average process diameter and quantity of processes (Table 3.1). The microglia of CUR treated monkeys exhibited significantly greater length complexity (number of intersections, nodes, ends and branching index) and span (convex hull area and volume) in comparison to those of control monkeys ($p < 0.01$ for all comparisons) This shows that the white matter microglia of CUR monkeys, which are more branched, complex, have longer processes and larger spans that are more evocative of a ramified morphology than VEH controls (Figure 3.4). For the grey matter microglia, significant differences were only observed in soma size and process length, such that CUR microglia had smaller somas and less processes.

Discussion

Summary of Results

The goal of this study was to investigate whether if CUR can reduce neuroinflammation as reflected by microglia density and morphology measures. Microglia were quantified using unbiased stereological techniques in three white matter regions the CngB, CC and FWM in age matched CUR and VEH monkeys, as well as in a young (<11 years old) and aged control subjects (>20 years old). There was no effect of CUR on LN3+

microglia total density and density by morphological subtype in any of the ROIs. However, we did find, consistent with other reports, an increase levels of LN3+ cells in aged monkeys compared to young monkeys (Shobin et al., 2017).

The degree of LN3 expression by microglia was also measured using particle analysis of the colocalized signal of LN3 and P2YR12/IBA1. No significant effect of CUR was found on the percent area label of this signal. Microglia were reconstructed in 3D, in A46, A25, the CngB and CC, to examine if CUR resulted in more subtle morphological changes that cannot be detected with unbiased stereology. Cells from A46 and A25 were combined in a single grey matter group, while CngB and CC were a combined into a single white group for analysis. Significant differences were found for microglia primarily within the white but also to a lesser extent within the grey matter group. These morphological changes detected in the microglia of CUR treated monkeys of smaller somas, longer, more branched processes that would suggest a shift toward a ramified morphology more so than controls.

Microglia Classification and Aging Related Effects

Traditionally microglia have been divided into 2 categories: M1 associated with more proinflammatory activation, and M2 being more anti-inflammatory in nature (Cherry et al 2014). This classification of microglia stems from differing responses to interferon gamma (IFN-γ, pro-inflammatory cytokine response) or interlukin4 stimulation (IIL-4, anti-inflammatory cytokine response) (Martinez and Gordon 2014). Within this framework of M1/M2 activation states, the morphology of microglia has also been used to infer function (Karperien et al., 2013). Three major morphological subtypes have been identified: ramified, hypertrophic and ameboid. Ramified microglia are defined as having

small somas with having long thin processes, and represent a surveillance or resting state (Nimmerjahn et al., 2005). Hypertrophic microglia have enlarged somas with few short and dense processes, have been thought to be activated and potentially inflammatory, while ameboid microglia (large rounded somas with little to no processes) are associated with phagocytosis and proinflammatory activation (Bohatschek et al 2001, Lee et al 2008). Aging has been found to increase microglial 'priming,' such that they have increased expression of inflammatory markers like MHC class II (Perry et al., 1993, Steit and Sparks 1997, Sheffield and Berman 1998, Holtman et al., 2015). Further aging has also been demonstrated to result in increases in microglial density, specifically, increases in hypertrophic and ameboid microglia (Shobin et al., 2017). Additionally, Shobin et al., 2017 found the increases in hypertrophic and ameboid cells were correlated to cognitive impairment. These age-related increases in what appears to be proinflammatory microglia may therefore be driving and contributing to the myelin damage typically observed in frontal white matter regions. Mitigation of this proinflammatory microglial response in age may therefore be a mechanism to dampen age related cognitive decline.

Curcumin and Microglia

In vitro experiments show that CUR treatment can dampen pro-inflammatory microglial activation (Karlstetter et al., 2011, Shi et al., 2015). In a rodent model of chronic inflammation (GFAP-IL-6) CUR was found to decrease total numbers of IBA+ cells within the hippocampus and the cerebellum (Ullah et al., 2020). Further, Ullah et al., 2020 show that CUR increased the dendritic length and number of nodes compared to their GFAP-IL-6 mouse.

In the present study, we found no significant effects of CUR on microglial density, when counting against LN3. Neither did we find a significant change in LN3 expression as a result of CUR. Similarly, to Ullah et al., 2020 we did find significant effects of CUR on morphological features, such that microglia from CUR treated monkeys exhibit greater ramification than microglia from VEH monkeys. Despite the differences in morphological features, we found that CUR does not alter the level of activation or the degree of microglial priming as represented by LN3 expression that normal aging causes. There are other defined microglial phenotypes that we have not examined in this study that may however be affected by CUR treatment. This includes triggering receptors on myeloid cells (TREM2) or microglia neurogenerative phenotype (MGnD) microglia, which have been associated with AD (Krasemann et al., 2019). An examination of these microglial phenotypes may reveal an effect of CUR not observed simply by degree of LN3 expression. However, because we have observed a cognitive benefit resulting from CUR, this would suggest that improvement in cognitive function may not be related to changes in microglial density nor morphology (Chapter 2 and Moore et. al 2017). Alternatively, it is possible that our method of microglia assessment is too limited, and examination of microglial gene expression may reveal further effects of CUR supplementation. Indeed, the canonical definition of M1/M2 phenotype, study of morphology and or density alone has been suggested to be insufficient at accurately capturing diversity of microglia and microglial functioning (Ransohoff 2016, Hammond et. al 2019). A future study which accounts for the microglial signatures proposed by Hammond et al., 2019 could provide more in-depth analysis of the what effects CUR supplementation has on microglia in the rhesus monkey.

Conclusions

Increased inflammation and oxidative stress may be contributing to age-associated myelin damage and potentiating age-related cognitive decline. Microglia, key mediators of inflammation within the brain increase in density and activation. Research suggests that microglial phagocytosis is impaired with age leading to an ineffective clearance of debris. Further as microglia are primed to proinflammatory activation, a sustained but ineffective inflammatory response may be further exacerbating myelin damage and cognitive decline. In the present study we show that a daily dosage of 500mg of CUR does not alter microglial density or degree of antigen presentation. However, CUR alters the morphology of white matter microglia to suggest that they are more ramified and surveilling and less likely to be pro-inflammatory. Further assessment of microglial gene expression is needed to derive conclusive evidence of a dampening of proinflammatory microglial response.

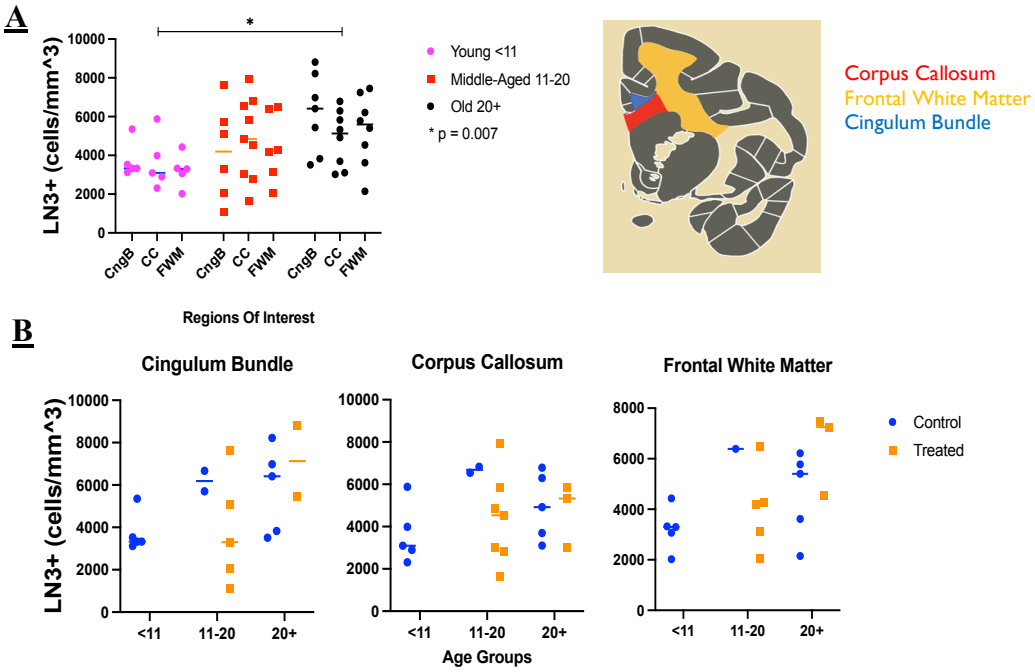

Figure 3.1 | LN3 Density Measurements: A) Left: Density of LN3+ cells sorted by age. No significant effect was observed for either treatment or region of interest. A significant effect of age group was observed [F(2,45) = 7.63, p=0.001)]. Multiple comparisons with Tukey correction show that the youngest age group has less LN3+ cells compared to the oldest age group (p = 0.007). A) Right: Cartoon representation of ROI, adapted from Scalable Brain Atlas (Bakker et al., 2015). B) LN3+ density in middle aged CUR and VEH treated monkeys for each ROI.

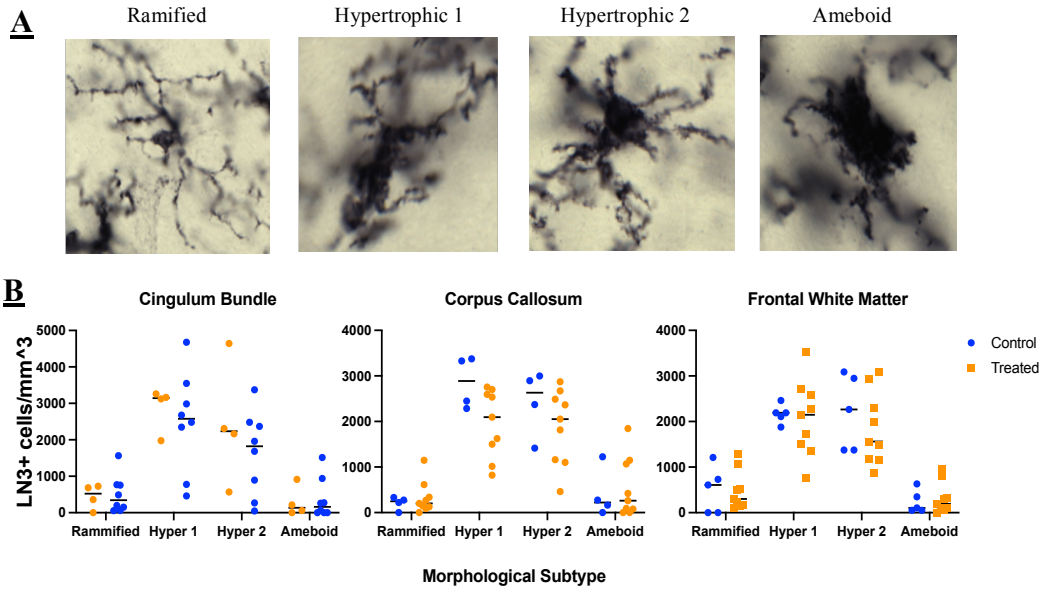

Figure 3.2 | LN3 Density by Morphology: A) Representative images of each morphological subtype. Ramified: small soma with long thin processes, Hypertrophic 1: rod like soma with short dense processes, Hypertrophic 2: rounded soma with short dense processes, Ameboid: enlarged soma with few to no processes. B): Density of cells for each ROI for each morphological subtype. No significant of treatment or ROI was observed. A significant effect of morphology was observed, such that both Hypertrophic 1 and 2 cells were the highest density of cell type measured compared to either ramified or ameboid cells ([$F(3,132) = 73.05$, $p=0.001$, Tukey multiple comparison $p < 0.001$ for all comparisons).

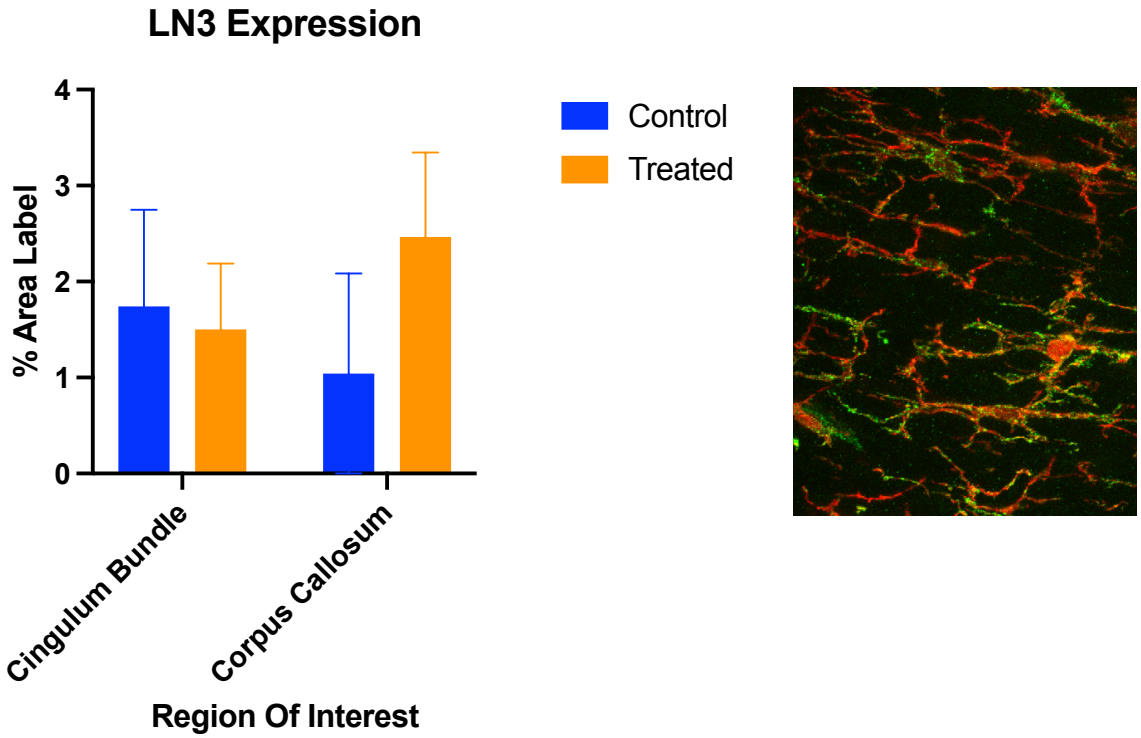

Figure 3.3 | LN3 Expression: Left: Percent Area label of colocalized signal for LN3 and P2yr12/Iba1. No significant effect of treatment was observed in either ROI. Right: Representative image of fluorescence signal, Red: P2YR12/IBa1, Green: LN3, Yellow are areas of colocalization.

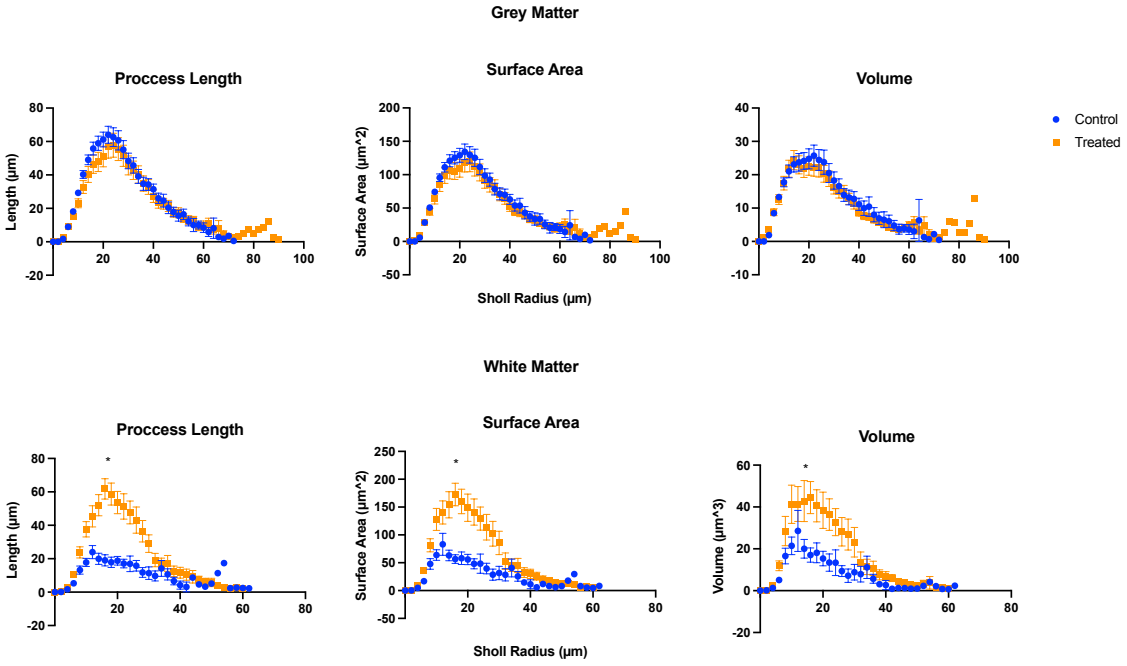

Table 3.4 | Sholl Analysis: Morphological features by Sholl radius. Grey matter includes cells from A46 and A25 (143 cells total). White matter includes cells from cingulum bundle and corpus callosum (31 cells total). Significant effects of treatment were found for both grey but predominantly white matter, summarized in Table 3.2. As a whole white matter microglia of CUR treated monkeys exhibit longer more complex branching than those of VEH monkeys.

Reconstruction Averages

	CUR		VEH	
Morphological Feature	**Grey Matter (A46/25)**	**White Matter (CngB/CC)**	**Grey Matter (A46/25)**	**White Matter (CngB/CC)**
Soma Surface Area	283.967578	462.12175	427.091182	564.432316
Total Length	736.863068	651.558083	916.812291	345.318842
Total Intersections	210.219178	254.163083	263.454545	108.315789
Average Diameter	1.25439966	0.81726952	0.62667627	0.74044216
Quantity	4	5.25	4.70909091	5.84210526
Nodes	60.6849315	38.75	70.0545455	20.5263158
Ends	69.3013699	48.1666667	81.5454545	28.7368421
Area	1585.14457	1408.49008	1836.40696	804.553263
Branching Index	16.1722277	9.77136243	18.1004762	5.27130706
Convex Hull 2D Area	2772.28816	2236.30525	4466.97345	1224.58589
Convex Hull 2D Perimeter	193.448397	187.069167	411.240055	140.202684
Convex Hull 3D Volue	53823.421	36779.5461	66221.4561	13708.5986
Convex Hull 3D Perimeter	7917.55034	8310.318	8781.92446	3556.40305

Table 3.1 | 3D Reconstruction Average Values: Average Values for morphological features for both treatment groups. Grey matter includes a total of 143 cells from A46 and A25. White matter includes a total of 31 cells from cingulum bundle and corpus collosum.

Reconstruction ANOVA Table and Pairwise Comparison Results

Grey Matter (A46/A25)

Morphological Feature	df	F	p value	Mean Difference (Control vs Treated)	p value
Soma Surface Area*	1	5.987	0.016	0.405	0.016
Total Length	1	2.806	0.096		
Total Intersections	1	3.188	0.076		
Average Diameter	1	1.111	0.294		
Quantity*	1	5.705	0.018	0.396	0.018
Nodes	1	1.265	0.263		
Ends	1	1.517	0.22		
Area	1	1.103	0.295		
Branching Index	1	1.003	0.318		
Convex Hull 2D Area	1	1.108	0.294		
Convex Hull 2D Perimeter	1	1.135	0.289		
Convex Hull 3D Volume	1	1.194	0.276		
Convex Hull 3D Perimeter	1	0.562	0.455		

White Matter (Cingulum Bundle/Corpus Callosum)

Morphological Feature	df	F	p value	Mean Difference (Control vs Treated)	p value
Soma Surface Area	1	0.4	0.532		
Total Length *	1	17.637	<0.001	-1.242	0
Total Intersections *	1	7.853	0.009	-0.932	0.009
Average Diameter	1	0.99	0.328		
Quantity	1	0.496	0.487		
Nodes *	1	8.783	0.006	-0.974	0.006
Ends *	1	6.854	0.014	-0.883	0.014
Area *	1	8.397	0.007	-0.957	0.007
Branching Index *	1	9.13	0.005	-0.988	0.005
Convex Hull 2D Area *	1	15.648	<0.001	-1.196	0
Convex Hull 2D Perimeter *	1	14.745	<0.001	-1.173	0.001
Convex Hull 3D Volume *	1	20.038	<0.001	-1.291	0
Convex Hull 3D Perimeter *	1	16.522	<0.001	-1.217	0

Table 3.2 | 3D Reconstruction Statistics Summary: Results of ANOVA assessing differences in morphological features for grey matter ROIs (top) and white matter (bottom). For the grey matter: control reconstructed microglia have larger somas as well increased

quantities of branches compared to treated cells. Within the white matter significant differences were found based on many morphological features, combined these changes suggest that the microglia of CUR treated monkeys are increased in their ramification compared to those from VEH monkeys.

CHAPTER FOUR:

CURCUMIN SUPPLEMENTATION AFFECTS INFLAMMATION WITHIN THE CNS AND PERIPHERY

Introduction

Inflammaging refers to an age-related rise in chronic low levels of inflammation (Franceschi et al., 2000). While the causes of age-related inflammation and oxidative stress are not fully understood, several studies report aging results in a priming toward proinflammatory response coupled to declines in intrinsic anti-inflammatory and anti-oxidative capability of cells (Perry & Holmes 2014, Safaiyan et al., 2016, Sheffield et al., 1998). The protracted and exaggerated inflammatory response observed in aging is associated tissue can exacerbate tissue damage (Franceschi et al., 2000). For this reason, therapeutics which dampen inflammation may present opportunities to mitigate or even reverse the negative consequences associated with inflammaging.

Interest in the use of naturally occurring polyphenols for the treatment of inflammation and disease has grown dramatically in recent years despite having its roots in traditional medicine (Gupta et al., 2012). Curcumin (CUR) the primary active ingredient in the Curcuma Longa or turmeric root is one such polyphenol that has been shown to have potent antioxidative and anti-inflammatory effects. A primary method of action of CUR includes an intrinsic antioxidative function, due to is polyphenol structure CUR is able to donate H+ which can stabilize ROS. Additionally, CUR has been demonstrated to be a potent regulator of nuclear factor kappa-light-chain enhance of activated b cells (NF-kb) and signal transducer and activators of Transcription 1 (STAT1) (Shishodia et al., 2007).

These transcription factors regulate many inflammatory processes that can lead to inflammatory cytokine production and ROS generation. Additionally, CUR has also been observed to increase intrinsic antioxidative response, by stimulating the activity and translation of glutathione synthase (GSH) (Awasthi et al., 2000).

Rodent studies of CUR, have demonstrated improvements in cognition linked to a reduction in oxidative stress within the brain (Kim et al., 2008, Nam et al 2014, Yu et al., 2012). Human studies have been less conclusive in determining positive effects of CUR supplementation on cognition, and effects within the brain are poorly understood (Rainey-Smith et al., 2016, Cox et al., 2015, Cox et al., 2020, Baum et al., 2008, Hishikawa et al., 2012, Ringman et al., 2012). Non-human primates are excellent models to parallel human age related cognitive decline, that importantly allow for collection of brain tissue that at time points that is not readily available in human clinical trials. In addition to NHPs, in vitro models of microglial response have been instrumental in assessing the potential mechanisms by which neuroinflammatory process are dampened by diets rich in antioxidants. (Rutledge et al., 2019).

In the present study, we tested the efficacy of CUR supplementation to decrease makers of inflammation within the brain and serum by reducing NF-kb expression and boosting GSH expression. Additionally, we tested whether CUR supplementation would alter Myelin Basic Protein (MBP) levels within the CSF, to measure if CUR had an effect on mitigating age-related myelin damage or enhancing myelin clearance from the brain. Further, qRT-PCR was used to measure gene expression from fresh frontal lobe samples of middle-aged monkeys treated with either CUR or VEH over a three-year period. While

multiplex ELISA was used to analyze serum cytokine levels and myelin basic proten levels within the CSF. An *in vitro* assay has shown decreased pro-inflammatory responses in LPS stimulated rat HAP microglial cells treated with serum of humans with dietary supplementation of berry fruits with well-known anti-oxidant properties (Rutledge et al., 2019). Here we tested if serum from CUR treated monkeys would exhibit similar protective effects to Rutledge et al., 2019. Nitrite production by HAPI microglia was measured to assess any dampening effects serum pretreatment may have.

Methods

Subjects

For PCR and ELISA experiments tissue (brain, serum/CSF, respectively) from male and female rhesus macaques aged 13.7-21 (equivalent to 41-64.5 in human years; Tigges et al., 1998) were used. For nitrite experiments additional serum samples from untreated young and old control monkeys were included (6.7-24.4 years old, [20.1-73.2 human equivalency). Treated monkeys received daily CUR supplementation (500mg/day) over a 3-year period. All serum and CSF experiments compared bassline samples (collected prior to beginning of treatment) and samples following 2 years of supplementation. The CUR given was a lipidated formulation to increase bioavailability, provided by Verdure Sciences (Noblesville, IN). CUR or VEH treatments were mixed with either 150ml of yogurt or Prima-Burger TM (BioServ, Flemington, NJ). Monkeys were observed to consume the treatments on 98% of days.

Tissue Collection

Briefly, following an induction of ketamine (10mg/kg IM), blood was collected from either left/right femoral vein, placed on ice, aliquoted and stored at -80°C. CSF was also collected following a sedation with of ketamine (10mg/kg IM). Sedated monkeys were placed in a stereotactic head holder in a prone position and cerebral spinal fluid (CSF) was collected from the cisterna magna. The monkey's head was tilted ventrally to allow access to the atlanto-occipital junction and a 23-gauge needle was used to access the cisterna magna to withdraw 2-3cc of CSF. Blood and CSF were collected at regular intervals over the 3-year period of supplementation. For brain tissue collection following and sedation with ketamine (10 mg/kg) and blood collection and CSF collection which monkeys were deeply anesthetized with sodium pentobarbital (25 mg/kg IV to effect). The monkey was placed in a supine position, the chest cavity and the pericardium were opened to expose the heart. Blood was collected from the left ventricle and then monkey was euthanized by exsanguination and perfused trans-cardial fixation, first with Krebs-Heinsleit buffer (4°C, pH7.4, 3-6L), followed by 4% paraformaldehyde (37°C, pH7.4, 8L). During perfusion with Krebs-Heinsleit buffer, following clearance of the vasculature fresh tissue samples were collected prior to the fixation with paraformaldehyde. This fresh tissue was flash frozen on dry ice and stored at -80°C.

ELISA for Cytokines and MBP

With the exception of MBP all other cytokines and transcription factors were detected in the serum using the LEGENDplex multi analyte flow assay kit (740389, Biolegend, San Diego, CA, USA). MBP was detected in the CSF using ELISA (Ansh Labs, Houston, Tx, USA). All kits were used according to the manufacturer's instructions.

LEGENDplex: All samples (baseline, 2 years post treatment) were thawed and diluted 4-fold in Assay buffer. The standard was reconstituted and 6 standards were generated from this by serial 1:4 dilutions. Analyte beads were sonicated for 1 minute. Standards and samples were loaded onto the plate in triplicate. 25ul of analyte beads were added to each standard and sample well. The plate was sealed and covered with aluminum foil, and set on an orbital plate shaker set at 500rpm for 2 hours at room temperature. Plates were washed once with the provided wash buffer and 25ul of detection antibodies were added to each well. A second incubation was performed at 500rpm for 1 hour. Following incubation, detection antibodies (25ul) were added to the plate and allowed to incubate at 500 rpm for 30 min. The plate was then washed 1 with 150ul of wash buffer, the samples were read using a BD LSRII flow cytometer.

MBP ELISA: Many of the steps were similar to the LEGENDplex assay. Key differences include: 1:2 sample dilution. Standards and samples were run in duplicate, incubations were done at 800rpm on an orbital plate shaker at durations specified by the manufacturer. Importantly detection for the MBP ELISA was done using a chromagen detection method. As such following addition of the detection solution and a 20 min incubation, the plate was read on a plate reader (Promega Glowmax Multi Detection Plate Reader, Madison, WI, USA)

qRT-PCR

Fresh frozen tissue from the frontal lobe was used in the study. Tissue biopsies were collected from the brain during perfusion with Krebs's buffer and were kept frozen on dry ice until needed. 100mg of fresh tissue was collected from each animal to extract RNA.

Samples were then individually homogenized using an RNAse free scalpel. Samples were placed into individualized tubes and 1ml of TRIzol (ThermoFisher, Waltham, MA) was added. Samples were triturated, using increasing smaller needles (18G, 20.5G, 23G) and allowed to incubate for 5 min on ice. 0.2 ml Chloroform was then added to each sample, and allowed to incubate for 2 mins at room temperature. Samples were centrifuged for 20 min at 3200G at 4°C and the aqueous layer was removed and two ethanal extractions were performed using first isopropanol, then 70% ethanol. Following extraction, the samples were centrifuged for 10 min at 3200g 4°C. The pellet was then air dried and resuspended in 40ul of PCR grade water. Purity was checked using a NanoDrop (ThermoFisher,Waltham, MA). Extracted RNA was then converted to cDNA utilizing a High Capacity RNA to cDNA kit (ThermoFischer, Waltham, MA). Samples were then normalized to 2ug and qRT-PCR was run using normalized samples with GAPDH in all plates as a housekeeping gene. kiqStart primers (Sigma Aldrich) were used for all genes tested.

HAPI Microglia Assay

Protective effects of serum application were tested on samples from CUR and VEH as well as samples from young and old controls were tested on cultured HAPI rat microglia cells, prepared according to (Rutledge et al., 2019). Briefly, HAPI rat microglia cells were cultured in Dulbecco's modified Eagles medium (DMEME, Invitrogen, Grand Island, NY) containing 10% fetal Bovine serum (FBS), penicillin and streptomycin in a humidified incubator at 5%CO2, 37 ℃. Microglia were transferred to 6 well plates prior to treatment. Cells were incubated in DMEM absent FBS and were pre-treated with serum (10%

concentration) from study subjects for 8 hours. Following CSF pre-treatment, the media was decanted, the cells washed with DMEM. Washed cells were stimulated with lipopolysaccharide (LPS, Sigma-Aldrich, St Louis, MO) at 100 ng ml^{-1}in DMEM overnight.

Following LPS activation the media were collected and aliquoted for use. Extracellular NO production was measured using Greiss reagent (Invitrogen), per the manufacturer's instructions. The plates absorbances were read at 548nm. A standard curve was derived from including samples of known nitrite concentration. The NO concentration was then calculated using the linear equation derived from the standard curve

Analysis

ELISA and HAPI Microglia Assays: Concentration data from LEGENDplex and MBP ELISA as well HAPI microglia assays were analyzed using 2-way ANOVA. For ELISA experiments concentration was the dependent measurement, and time and treatment being the independent factors. For HAPI microglia assay concentration was the dependent measurement, treatment and age were the independent measurements. To compare relative levels of gene expression fold change was calculated using the ddCT analysis method.

Results

ELISA for Cytokines and MBP

With the exclusion of MBP measured in the CSF, all other markers were measured in the serum (Table 4.1). We found no significant effect of treatment on the absolute concentration of any of the markers measured. However, we did find a significant effect of time on IL-23 concentration (Table 4.2). For samples from both treated and control

monkeys there was a significant increase in IL-23 concentration after 2 years compared to the baseline measurement (Figure 4.1).

qRT-PCR

We measured the fold change of gene expression comparing CUR to VEH monkeys, of several genes associated with pro and anti-inflammatory actions and growth, including: granulin precursor (GRN), interleukin 22 (IL-22), Mamu class II histocompatibility antigen (mamuDRA), nuclear factor kalp light chain enhancer of activated b cells (NfkB), tumor necrosis alpha (TNFa), signal transducer and activator of transcription 1 (STAT1), glutathione peroxidases 1/3 (GPX1, GPX3), glutathione s-transferase Pi 1 (GSTP1), heme oxygenase 2 (HMOX2), nuclear factor erythroid 2 like 2 (NFE2l2), superoxide dismutase 1 (SOD1), brain derived neurotrophic factor (BDNF), neurotrophic receptor tyrosine kinase 2 (NTRK2), mechanistic target of rapamycin (MTOR), MTOR associated protein, Eak-7 (MEAK7) and MROP associated protein, LST8 homolog (MLST8). We found no significant effect of treatment on gene expression.

HAPI Microglial Assay

We measured nitrite production by LPS stimulated rat HAPI microglia that were treated with serum from both CUR and VEH monkeys, as well as young and old controls (Figure 4.3). We found no significant effect of either treatment [$F(1,19) = 1.48$, $p=0.24$)] or age [$F(2,19) = 0.15$, $p=0.86$)] on nitrite production.

Discussion

Summary of Results

In the present study, we tested the effect CUR has on inflammatory cytokine concentration within the serum, MBP concentrations within the CSF and inflammation related gene expression within the brain. Further, we tested whether serum from CUR treated monkeys would dampen LPS stimulation *in vitro* rat HAPI microglia cells. We found no significant effect of either treatment nor time on all markers measured except for IL-23. IL-23 was increased in both groups when comparing baseline serum to serum collected 2 years later. qRT-PCR was used to detect changes in inflammation related gene expression in brain tissue collected from treated and untreated monkeys. We found no significant differences in any of the genes examined. Lastly, the serum of CUR and VEH, collected 2 years after beginning supplementation, were applied to rat HAPI microglia as a pretreatment, followed by an LPS stimulation to promote inflammatory response. We found that serum pretreatment from either group had no effect on nitrite production, suggesting that the CUR pretreatment did not have a dampening effect on microglial activation.

Inflammaging

Inflammaging refers to a rise in the chronic low levels of inflammation in age that occurs in the absence of an underlying infection (Franceschi et al., 2000). While the source of this increase in inflammation is not yet fully understood, circulating levels of proinflammatory cytokines such as NF-kb, IL-6, TNF-α have been demonstrated to be strong predictors of mortality in older adults (Varadah et al. 2013). Further this increase in age related inflammation is thought to contribute to cognitive decline (Franceschi et al., 2000, Eiklenboom et al., 2012, Bettercher et al., 2015). Several studies report the efficacy

of CUR to mitigate inflammatory response, but further they show that CUR also improves cognitive function in aged rodents (Sun et al., 2012, Belviranli et al., 2013, Queen & Tollefsbol 2010; Salminen et al., 2008; Sikora et al., 2010a, Sikora et al., 2010b). In particular these studies a highlight a strong effect of CUR on NF-kb, IL-6, TNF-α, and would suggest that CUR is a potent anti-aging nutraceutical. Our laboratory has demonstrated that CUR improves cognitive function in middle aged rhesus monkeys, (Moore et al. 2017). Here we examined if this improvement in cognitive function may be related to changes in circulating cytokines and inflammatory gene expression within the brain. The results from our experiments show that in the rhesus monkey, daily CUR supplementation over a two-year period did not affect circulating cytokine levels, nor did it have a significant impact on inflammatory gene expression in the brain. This may be potentially due to the poor bioavailability of CUR and the dosage used in the current study (Choudary et al., 2015). In rodents CUR supplementation has been shown to exert effects in a dosage dependent fashion. As this is the first study of CUR in NHPs, our dosage of 500mg was based off ongoing clinical trials of CUR in humans, however some studies report no effect on cognition in human subject even at dosages of 4g of CUR (Baum et al., 2008). Additionally, we do not observe a significant effect of time on the majority of cytokines examined. This suggests that perhaps for both CUR and VEH significant levels of circulating cytokines have not yet developed such that a dampening of CUR supplementation can be detected.

Rutledge et al., 2019 demonstrate dampening effects of serum from individuals with dietary supplementation with berry fruits, on in vitro HAPI microglial inflammatory

response. Importantly they show that both the serum of berry fruit supplemented individuals collected during fasting and postprandial timepoints were effective at attenuating microglial response. Key to this observed affect is that in both fasting and postprandial conditions, metabolites of berry fruits were detected, suggesting a persisting circulating level of metabolites that may confer anti-inflammatory effects (Sandhu et al, 2018). Using the same model system as Rutledge et al., 2019, we see no effects of CUR supplementation on attenuation of microglial response to LPS stimulation. Two possible reasons for these results include: 1) CUR in contrast to berry fruits is ineffective at mitigating microglial response. 2) Unlike berry fruits CUR and metabolites did not persist having been metabolized thoroughly and removed from circulation within the serum. As CUR has been shown to be a potent regulator of microglia in vitro, our observed results are likely due to the 2nd of the two options presented. Future studies in which serum from monkeys taking CUR at varying dosages, and serum collection pre and post prandial would then better answer if CUR supplementation is effective at mitigating inflammation.

Conclusions

Aging is associated with increased chronic low levels of inflammation. Here we tested the effects of CUR on dampening circulating inflammatory cytokines, inflammatory gene expression in rhesus monkeys. Further we tested whether serum pretreatment of CUR treated monkeys could attenuate in vitro microglial response to LPS activation. We found no significant effects of CUR on circulating cytokines, inflammatory gene expression nor on in microglial production of nitrite in vitro. These results suggest that further study of

CUR in rhesus monkeys, could benefit from increased dosage, as CUR supplementation in rodents shows dramatic effects on inflammatory signaling.

Average Concentrations

	CUR		VEH	
Inflammatory Marker	**Baseline (pg/ml)**	**2 Years post (pg/ml)**	**Baseline pg/ml**	**2 Years post (pg/ml)**
MBP	6.396586	8.322955	2.976576	5.674753
IL-6	8.322955	12.495	16.75833	13.3175
IL-1β	42.5455	54.024	71.785	35.61125
IL-17α	28.437	47.318	31.6025	31.595
IFN-β	112.87	137.6638	51.4475	49.315
IL-23	54.62	235.0094	22.055	218.5738
TNFα	17.28286	22.41833	22.055	17.102
INF-γ	30.84167	21.59	32.1725	24.375
BDNF	41.78459	29.14492	66.40643	46.50861

Table 4.1 | Inflammatory Marker Concentrations: Average concentration values for markers of inflammation measured at baseline and 2 years after treatment. With the exception of MBP (from CSF samples), all other markers were measured in the serum. No significant differences were observed between groups. IL-23 concentration was significantly increased, for both groups, from baseline measurement (Table 4.2).

Cytokine Analysis ANOVA Tables and Pairwise Comparison Results

		Treatment		Time	
Inflammatory Marker	**df**	**F**	**p Value**	**F**	**p Value**
MBP	1	0.177	0.683	0.895	0.366
IL-6	1	0.008	0.929	0.201	0.664
IL-1β	1	0.393	0.545	1.497	0.249
IL-17α	1	0.734	0.412	0.604	0.455
IFN-β	1	2.112	0.177	0.3	0.596
IL-23	1	0.733	0.412	5.678	0.038 *
TNFα	1	0.064	0.806	0.492	0.499
INF-γ	1	0.033	0.859	0.176	0.684
BDNF	1	1.698	0.222	0.065	0.804

	Mean Difference (Baseline vs 2YRS)	**p value**
IL-23	-281.513*	0.038

Table 4.2 | Inflammatory Marker Statistics Summary: Results from ANOVA comparing the effects of both treatment and time on inflammatory marker concentrations. No effect of treatment was observed. A significant effect of time was observed for IL-23, where, for both groups, concentration increased 2 years after baseline measurement.

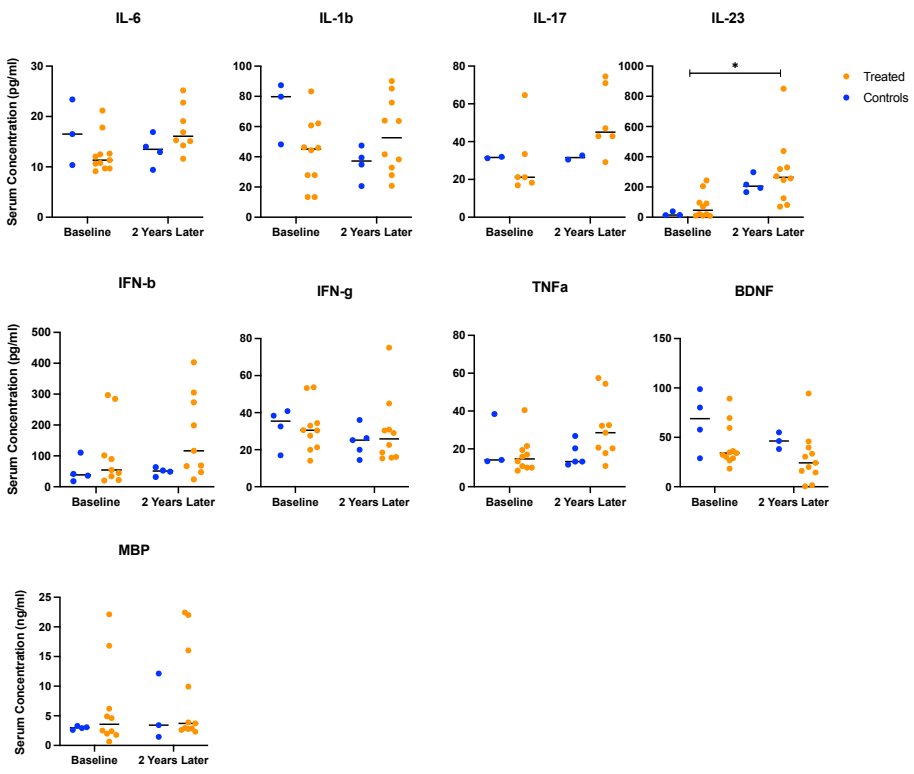

Figure 4.1 | Inflammatory Marker Concentrations: Comparison of changed in inflammatory marker concentration by treatment and time. All markers were measured in the serum, with the exception of MBP which was measured in the CSF. Il-23 demonstrated a significant increase in concentration 2 years after baseline measurement for both groups. No other significant effects were observed.

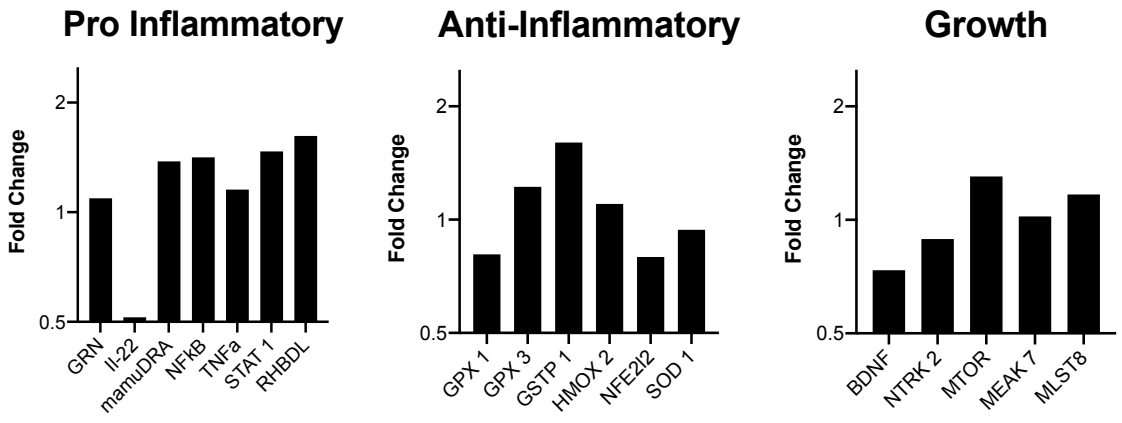

Figure 4.2 | Fold Change of Gene Expression: qRT-PCR and the ddCT method was used to measure fold change of inflammation and growth-related comparing CUR vs VEH. A fold change above 2 or below 0.5 is typically considered significant. No significant group differences were observed in any genes assayed.

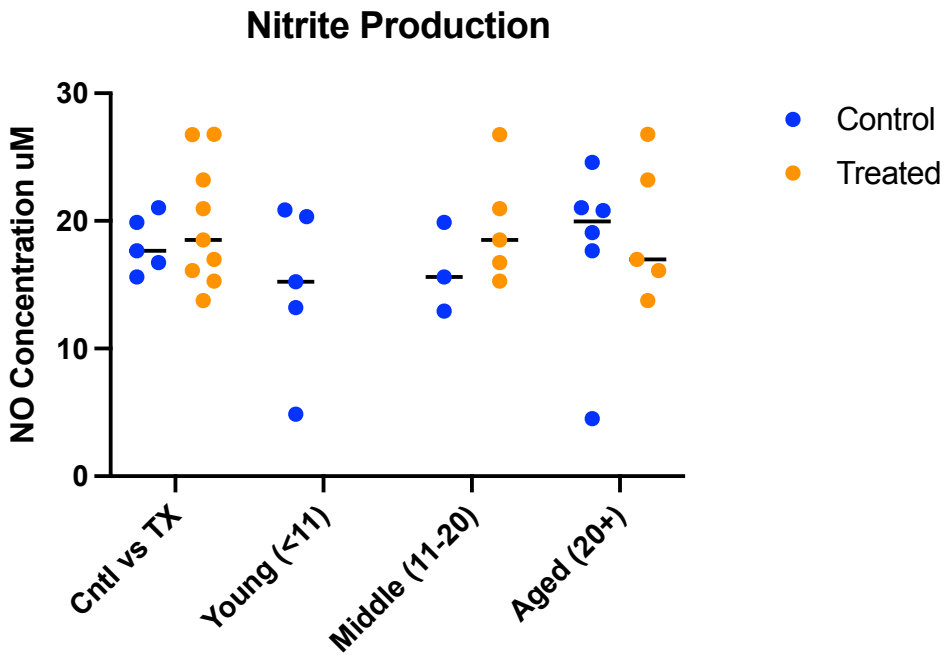

Figure 4.3 | Nitrite Production: Nitrite production by rat HAPI microglial cells treated with serum from CUR and VEH monkeys. No significant effects were observed for either treatment or age group on nitrite production.

CHAPTER FIVE:

CONCLUSIONS AND FUTURE DIRECTIONS

Summary of Findings

Even in the absence a dementia state aging alone can result in cognitive decline (Harada et al., 2013). This cognitive decline may be driven by an accumulation of damage to myelin sheath that is particularly evident in the frontal white matter (Bowley et al., 2010, Peters et al., 2009, Feldman and Peters 1998, Peters and Salthares 2002, Sandell and Peters 2003) A combination of multiple causes likely underlies age related myelin damage however evidence indicates that both inflammation and oxidative stress are key contributors. In the present study we tested the hypothesis that CUR supplementation would improve cognitive function by reducing inflammation. The primary behavioral findings shown here are that CUR supplementation does not improve object discrimination performance, but does enhance reversal learning. Specifically monkeys that received daily oral administration of CUR, made fewer perseverative errors during the 2 round of object reversal testing in comparison VEH control monkeys. To assess changes in inflammation, in the same monkeys, we then examined microglia by quantifying the density, degree of LN3 expression and morphology. We found that CUR supplementation did not alter the density of microglia within the cingulum bundle (CngB), corpus callosum (CC) nor the frontal white matter (FWM). Further CUR treatment did not result in changes in microglial LN3 expression. The microglia of CUR treated monkeys, from both grey and white matter regions, did however exhibit morphological differences, that were more evocative of ramified or surveillance state microglia in comparison to VEH monkeys. To further assess

effects of CUR supplementation on inflammation, we examined the serum for circulating cytokine levels and found no differences as a result of treatment. Additionally, using fresh frozen brain tissue, we measured gene expression of a variety of pro and anti-inflammatory makers, known to modulated by CUR in rodent and culture models, and found no significant changes as a result of treatment. Finally, we examined whether supplementation confers 'protective' changes within the serum following treatment, and if the serum would dampen *in vitro* LPS stimulated microglia nitrite production. We found no significant difference in nitrite production with serum pre-treatment from either CUR or VEH monkeys. While our behavioral findings are consistent with our laboratory's previously published results from this cohort and suggest that CUR supplementation is improving frontal but not temporal lobe function, our initial examination does not reveal a clear and strong dampening of inflammation by CUR.

Curcumin Supplementation in Other Models

CUR is the primary active ingredient in the spice turmeric, and has been widely used in traditional medicines of the east. Since CUR's isolation from turmeric by Vogel and Pelletier in 1815, it has been used in numerous studies with a significant increase in the number of publications relating to its use in the past decade (Gupta et al., 2012). As Sarker and Franks 2018 report, while preclinical studies, primarily in rodents, have demonstrated a clear benefit of CUR supplementation on cognition, the data from human clinical trials, though positive, have been less consistent. This disconnect between rodent and human studies, highlights the difficulty in comparing results across species, but perhaps more importantly raises the issue of why is such a significant difference in efficacy

exists. Though CUR has been studied in a variety of modalities here we will focus on the neuroprotective related effects of CUR observed in rodents, humans and from in-vitro studies.

Curcumin Effects on Rodent Cognition

The effects of CUR on cognition has been studied quite extensively in rodent models of Alzheimer's Disease, accelerated and normal aging as well as induced inflammation. Across these studies there is a great deal of variability in dosage, duration of treatment, delivery and type of CUR administered, however a general consensus emerges that supplementation with CUR can improve cognitive function. In both rat and mouse, amyloid precursor protein (APP) and preselin 1 mutant models of AD, studies demonstrate that, in a dose dependent fashion, CUR improves cognitive function. Specifically, CUR improves working and spatial memory, correlated to a reduction in amyloid-beta (Aβ) aggregation, increased markers of autophagy and decreased circulating inflammatory cytokine levels (Lim et al., 2001, Chen et al 2011). Further, CUR may be effective in treatment of AD pathologies resulting from a combination of effects including the reduction in lipid and protein oxidation, reductions in proinflammatory cytokine production, and metal chelation (Frautschy et al 2001, Lim et al., 2001, Venkatesanad et al 2000, Baum et al 2004, Cole et al 2005). Rodent studies of age-related cognitive decline have also demonstrated promising results from CUR supplementation. Using a neurodegenerative mouse model of cognitive aging and dysfunction, the senescence accelerated prone 8 mouse, Sun et al., 2013 show that CUR supplementation improved Morris Water Maze (MWM) performance, reducing escape latency. Further they

demonstrate that CUR supplementation enhances synaptic plasticity through a restoration of calcium/calmodulin-dependent kinase II and N-methyl D-aspartate, receptors concentrations in the hippocampus in comparison to control mice. Lastly Sun et al., 2013 show that CUR supplementation results in less oxidative stress, as they found treated mice showed reductions in malondialdehyde (MDA), a marker of lipid peroxidation within the hippocampus. Normal aging rodent models show similar improvements in MWM performance due to CUR treatment, correlated to decreases in brain MDA concentrations (Belviranli et al., 2013). While most rodent studies of CUR use the MWM to test cognitive function, reversal learning has also been examined in rat model of autism spectrum disorder, and mouse model of fetal alcohol spectrum disorder (Bhandari and Kuhad 2015, Cantacorps et al., 2020). Both these studies demonstrate an improvement in reversal learning following CUR supplementation correlated to decreases to inflammatory cytokine TNF-a. These in vivo rodent studies confirm a benefit to cognition following CUR supplementation due to reductions in inflammation and oxidative stress.

Curcumin Effects on Human Cognition

Epidemiological studies have revealed a generalized benefit of CUR use, where in cultures who regularly consume turmeric in their diet show lower incidences of Alzheimer's Disease and other dementias (Chandra et al., 2001, Vas et al., 2001, Ng et al., 2006).

There are several ongoing human clinical trials testing the effects of CUR to slow or reverse cognitive decline in both AD and aging models. Baum et al., 2008, Hishikawa et al., 2012, and Ringman et al., 2012 explore the effects of CUR in AD models. All three

of these studies measured cognitive function using the mini-mental state exam which assesses orientation, attention, memory, language and visual spatial skills. All three of these studies report no significant benefit of CUR supplementation. Cox et al., 2015 studied CUR supplementation in healthy individuals aged 60-85 and report significant improvement to sustained attention (digit vigilance task) 1 hour after a single 400mg CUR treatment. Further they show that both acute (single 1 dose, 1-hour post treatment) and chronic (4 weeks daily supplementation) treatment with CUR improved working memory (serial three subtraction). Lastly Cox et al., 2015 report significant improvements in mood following chronic supplementation including decreases in fatigue, and lessened reductions in calmness and contentedness. A partial replication of this study was performed by Cox et al., 2020, in which individuals aged 50-85 were tested following a supplementation over 4 and 12 weeks. In this replication, Cox et al., 2020, report similar findings, with improvements in working memory only after 12 weeks of supplementation following CUR treatment. In contrast to their previous results, while they show improvements in mood, with reductions in fatigue and anger, these effects were only significant at 4 but not 12 weeks of supplementation, this discrepant result is explained by the authors being due to a change in methodology of mood measurement. Curcumin supplementation has also been studied in healthy middle age humans. Santos-Parker et al., 2018 studied the effects of supplementation on motor and cognitive function in healthy middle-aged adults (45-74 years given 2000mg/day). This study used the NIH toolbox to measure cognitive function measuring processing speed, executive function, working memory, episodic memory and language, and found no significant effects of CUR on most domains tested with only slight

improvements to language following 12 weeks of supplementation. In these human clinical studies, in which there is seemingly little to no benefit of CUR. The authors suggest that it likely due to the poor bioavailability of CUR. Importantly these results highlight that few studies report a beneficial effect of CUR on cognitive function in human subjects.

Curcumin Effects on Glia

Rodent *in vivo* and *in vitro* models both demonstrate that CUR can significantly alter the activity of glia. CUR has been demonstrated to modulate microglia gene expression and cytokine production by alteration of mitogen activated regulated kinase pathways and inhibition of NF-kb (Jin et al 2007, Karlstetter et al., 2011, Shi et al., 2015, Yu et al., 2018). These studies demonstrate that CUR is effective at dampening proinflammatory microglial response to multiple stimulating factors including lipopolysaccharide (LPS), Aβ and lipoteichoic acid, resulting in decreased proinflammatory cytokine production (IL)-1β, IL-6, and TNF-α Nitric Oxide synthase. In addition to dampening pro-inflammatory activity, CUR supplementation has been shown to increase anti-inflammatory cytokine production by microglia, increasing heme oxygenase, nrf2 activity (Parada et al., 2015). Additionally, CUR treatment has been demonstrated to alter microglial morphology, reducing soma size, increasing branching nodes reminiscent of ramified microglial morphology, while also reducing density of IBA1+ microglia in the hippocampus of the GFAP-IL-6 mouse (Naeimi et al., 2018, Ullah et al., 2020). Attenuation of proinflammatory cytokines and ROS by CUR has also been demonstrated to impact oligodendrocytes. Curcumin has been shown to reduce lipid peroxidation, increase MBP expression, increase myelin thickness and oligodendrocytic

differentiation (Bernadro et al., 2021, Caillaud et al., 2018, Kaushal et al., 2014, Yu et al 2016).

Curcumin Effects in Rhesus Monkeys

Curcumin has been shown to effectively improve cognition in rodents (Lim et al., 2001, Chen et al 2011, Sun et al., 2013, Bhandari and Kuhad 2015, Cantacorps et al., 2020). *In vitro* and *in vivo* rodent studies further reveal that CUR has potent anti-inflammatory and antioxidative effects, in particular CUR has been shown to mitigate proinflammatory microglial response (Karstetter 2011 Karlstetter et al., 2011, Shi et al., 2015, Yu et al., 2018). Human clinical trials are less conclusive in demonstrating similar cognitive benefits of supplementation (Cox et al., 2015, Cox et al., 2020, Baum et al., 2008, Hishikawa et al., 2012, and Ringman et al., 2012). This may be due to a greater efficacy in metabolism of CUR in humans than rodents but also it has been suggested that rodents are poor models of human inflammatory response (Ireson et al., 2002, Seok et al., 2013). The work done in this thesis and by our laboratory attempts to bridge the gap between the rodent and human literature. This is the first study, to examine CUR supplementation in rhesus monkeys and as such, the data collected here represent novel findings regarding the effects of CUR supplementation in this species.

Behavior

Monkeys and humans both exhibit age related cognitive decline, particularly the domains of executive function and memory (Cahn-Weiner et al., 2000; Fristoe et al., 1997; Lai et al., 1995; Moore et al., 2003, 2006; M. B. Moss et al., 1997; Rabbitt and Lowe 2000; Souchay et al., 2000). In both species, this decline has been observed to occur beginning

near midlife (Albert 1984; Chodosh et al., 2002; Drag and Bieliauskas 2010; Hara et al., 2012; Kwon et al., 2016; León et al., 2016; Light 1991; Simen et al., 2011; Zeamer et al., 2012; Zeamer et al., 2011, Moore et al., 2003, 2006; M. Moss et al., 2007; Rapp and Amaral 1989, 1991; Sridharan et al., 2012; Voytko 1999). The cognitive decline of both species has been typically observed in working spatial memory, while object memory remains largely unaffected (Arnsten and Goldman-Rakic 1990; Bachevalier et al., 1991; Killiany et al., 2000; León et al., 2016; Rapp and Amaral 1989, 1991; Zeamer et al., 2012). Aging does not seem to impact object discrimination ability, when discriminations can be achieved using a single characteristic. However, several studies demonstrate across species (rodent, human and monkeys), deficits in object discrimination tasks, when the objects share features, following damage to the perirhinal cortex or age (Bartko et al., 2007, Bussey et al., 2002, Barense et al., 2005; Barense, Burke et al., 2010, Burke et al., 2011). In particular these studies point to the role and necessity of a functional perirhinal cortex in perception and formation of object memory when objects share overlapping features. The results found during this thesis, show no differences in discrimination performance nor recall following discrimination between CUR and VEH monkeys. As discrimination performance was unaffected by supplementation, it is plausible that CUR is not significantly affecting the functioning of the perirhinal cortex in our model. Further as recall of the discrimination is also not affected by supplementation, it is also likely that the hippocampus is not significantly affected. These data are consistent with previous published results from our laboratory which demonstrated no effect of CUR

supplementation on DNMS performance. Combined these data would suggest that the temporal lobe function is not significantly affected by supplementation in our model.

Killiany et al., 2000 have shown deficits in DNMS are not typically seen in early senescent monkeys (25 years+), and Burke et al 2011 found decreased object discrimination performance in monkeys with an average age of 24.8 years. Here we tested a middle-aged cohort of monkeys, as such, any positive effect of supplementation may be masked by a lack of decline in the abilities tested in either group. Rainey-Smith et al 2016 demonstrate that in human subjects CUR supplementation did not improve the treated groups performance on the Montreal Cognitive Assessment but rather the control subjects performed worse. A future study in which monkeys begin supplementation in middle age and continue supplementation as they reach advanced aged could provide more in depth information of whether CUR supplementation delays or mitigates age related declines in recognition memory and object discrimination testing. A second study in which supplementation begins in advanced aged would help elucidate if CUR supplementation can reverse age related cognitive decline. Another remaining question, is if CUR supplementation would yield the most beneficial effects in subjects that are mildly impaired or subjects with severe cognitive decline, and a study with advanced aged monkeys in which there is presumably a wider spectrum of cognitive decline, would address this question. As noted earlier, CUR treatment in rodent studies of cognition often report a dosage dependent effect. In this current study, we used a single dose across subjects, and a future experiment with different dosages would reveal if dose dependent effects are also true for non-human primates.

Given the age of the monkeys tested in the current study, they are likely to be in the earliest stages of cognitive decline and this may have impacted our ability to detect significant effects of supplementation on the temporal lobe dependent tasks used. Tasks of executive function, particularly those that activate the prefrontal cortex, have been shown to more sensitive at detecting age related deficits as early as middle age (Moore et al., 2003, 2006, Bartus et al., 1979). Several studies have demonstrated that reversal learning, is impaired in aged monkeys relative to young monkeys, with aged monkeys making more perseverative errors (Gray et al., 2017; Munger et al., 2017; Lai et al., 1995; Rapp 1990; Bartus et al., 1979). While the modality and testing parameters of reversal tasks heavily influence the brain regions and structures involved in reversal learning, Jones and Mishkin 1972 show that lesions to the OFC resulted in greater perseverative or stage I errors during object reversals. Here we demonstrate that CUR supplementation reduces perseverative responding during object reversal learning. These results and those published by our laboratory showing improved DRSTsp performance, suggest that CUR is affecting frontal cortical function. In both these sets of data we show that the treatment group experiences improved performance, while the performance of the control monkeys does not change. From this we can conclude that CUR supplementation is enhancing performance on tasks that are reliant on the prefrontal cortex. Future studies of the effects of CUR supplementation in middle age may find more impact of supplementation when utilizing tasks of working memory or executive function, and tasks that heavily engage frontal cortical structures, such as the conceptual set shifting task used by Moore et al., 2005. Lastly in human studies of CUR supplementation, the most consistent positive effect has

been on mood and attention (Cox et al. 2015, Cox et al. 2020). Future studies in NHP should also consider measuring other metrics of cognition including such as fatigue, contentment, and attention.

Inflammation

Rodent and in vitro studies have demonstrated that CUR is a potent regulator microglia and can dampen proinflammatory response. Here we assessed what effects CUR has mitigating inflammation within the brain as well as within the periphery. We show that CUR treatment had no effects on microglial density in the CngB, CC or FWM. Further we show that CUR did not alter inflammatory gene expression within the brain. We also measured MBP within the CSF, which has been shown to be elevated in age but also used as an indicator of myelin damage, and found no effects of supplementation. To assess peripheral inflammation, we measured circulating cytokine levels, and tested if the serum of CUR treated monkeys could dampen LPS stimulation of microglia in vitro. We found no significant effects of CUR (using serum collected after 2 years of treatment) on circulating cytokine levels nor on in vitro stimulation following serum pretreatment. Possible explanations for our results include: 1) the dosage of CUR used in this study was insufficient to cause significant reductions in the metrics we used to measure inflammation. 2) A significant and pronounced shift in inflammation has not yet occurred in our treatment groups which obfuscates any effect of CUR treatment. 3) Our metrics of measuring inflammation are not broad enough to capture effects of CUR treatment.

To address these issues future studies of CUR in rhesus monkeys should consider the following: 1) The use of multiple dosages to determine optimal dosing. In the present

study, we used only a 500mg dose, and future studies should consider the use of a dosage based on individualized animal weights to better control the dosages that animals receive. 2) Testing the effects of CUR in an older cohort of monkeys, that are more likely to exhibit greater degrees of prolonged immune activation. 3) Studying the effects of CUR treatment in induced inflammation monkey model, such as Ji et al 2004 have used, may better illustrate the benefits of curcumin in a pronounced inflammatory state. 4) In contrast to quantifying microglia by morphology or conducting PCR to measure gene expression changes, a more detailed assay using single cell RNA sequencing would yield greater insight into the effects of CUR on inflammation in a cell specific manner. This would also allow for differentiation of CUR effects based on cell type and allow for comparison of CUR on microglia, astrocytes and oligodendrocytes.

Conclusions

This work in this thesis in part, encompasses the first study of CUR supplementation in the rhesus monkey. Age related cognitive decline is linked to an accumulation of white matter damage that may be driven by an increase in inflammation and microglial response (Bowley et al., 2010, Shobin et al. 2017). Here we tested if CUR supplementation would improve cognitive function and attenuate age-related increases in inflammation. We found that in middle age rhesus monkeys CUR treatment improved performance on frontal but not temporal lobe dependent behaviors. While we found that CUR did not affect density of microglia within the frontal white matter regions examined, we did find that CUR altered microglial morphology. This changes in morphology observed would suggest that the CUR treated microglia were more ramified or likely to be

in a surveilling state. We showed that CUR supplementation did not affect circulating cytokine levels in the serum nor did it affect inflammatory gene expression within the brain. While these data show promising effects of CUR on cognition further study is required to better assess its effects on inflammation in monkey models.

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