The role of Creatine Supplementation and mTORC1 Signaling in Ameliorating Cognitive Deficiency in a Neuroinflammatory Rat Model

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by

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The role of Creatine Supplementation and mTORC1 Signaling in Ameliorating Cognitive Deficiency in a Neuroinflammatory Rat Model

Presented by Xuansong Mao

A candidate for the degree of Doctor of Philosophy

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DEDICATION

This dissertation is dedicated to

my remarkable family members,

my precious friends,

and those who encouraged and supported me.

Every challenging work needs self-efforts and guidance from those who were very close to my heart,

Without you, I would not be able to make it.

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List of Abbreviations

AD	Alzheimer's disease
PD	Parkinson's disease
APP	Amyloid precursor protein
PSEN1	Presenilin
PESEN2	Presenilin
Aβ peptide	Amyloid beta peptide
CNS	Central nervous system
SNpc	Substantial nigra par compacta
NMDA	N-methyl-d-aspartate
MCI	Mild cognitive impairment
LPS	Lipopolysaccharides
PAMP	Pathogen-associated molecular pattern
TLR4	Toll-like receptor 4
Iba1	Ionized calcium binding adaptor molecule
MWM	Morris water maze
i.p.	Intraperitoneal
i.c.v.	Intracerebroventricular
BBB	Blood-brain barrier
PCr	Phosphocreatine

CK	Creatine kinase
CRT	Creatine transporter
ROS	Reactive oxygen species
mTOR	Mammalian target of rapamycin
p70S6K	p70 ribosomal S6 kinase 1
eIF4E	Eukaryotic initiation factor 4E
4EBP1	Eukaryotic initiation factor 4E-binding protein 1
ULK1	Unc-51 like kinase 1
BDNF	Brain-derived neurotrophic factor
BMT	Barnes maze test
NOR	Novel object recognition
LTP	Long-term potentiation

Aims of dissertational research

Overall aim

The overall aim of this dissertational research was to determine whether enhanced mTORC1 signaling underlies the neurocognitive effects of Cr supplementation. The work performed using a neuroinflammatory rat model explored the use of Cr supplementation as a potential therapeutic strategy for mild cognitive impairment.

Overall hypothesis

Enhanced mTORC1 signaling is required for Cr supplementation to fully exert its effects to ameliorate the cognitive deficiency elicited by neuroinflammation.

Aim 1:

Clinical studies have shown that Cr supplementation is able to improve cognitive processing under a variety of different paradigms (e.g., aging, traumatic brain injury, sleep deprivation). However, whether Cr supplementation can also be cognitively beneficial in the context of MCI remains unknown. Furthermore, the potential neuro-molecular mechanism(s) underlying Cr supplementation have not been investigated in the past. Therefore, the current aim was to examine the cognitive effects of Cr supplementation in an animal disease model mimicking MCI, along with its associated changes at the molecular level (Figure 1.1).

Outcome #1: Determine whether 6 weeks of Cr supplementation is sufficient to ameliorate cognitive deficiency elicited by neuroinflammation in hippocampus in MCI rats.

Outcome #2: Determine the molecular expression level of effector proteins of mTORC1 signaling and downstream synaptic proteins within dentate gyrus.

Outcome #3: Determine neuronal changes of mTORC1 signaling activity in consequence of 6 weeks of Cr supplementation

Aim 2:

Accumulated evidence supports that enhanced mTORC1 signaling is cognitively beneficial, which can be activated by Cr supplementation in central nervous system. As an extension of aim 1, aim 2 attempted to examine whether enhanced mTORC1 activity is required for Cr supplementation in ameliorating the cognitive deficiency in a neuroinflammatory rat model (Figure 1.1).

Outcome #1: Assess the neurocognitive effects of Cr supplementation when mTORC1 signaling is blocked through its selective inhibitor rapamycin in MCI rats.

Outcome #2: Examine the molecular changes within dentate gyrus and medial prefrontal cortex, respectively, after the blockage of mTORC1 signaling.

Outcome #3: Test if Cr supplementation is sufficient to upregulate mTORC1 signaling activity within cultured PC12 cell neuronal cells.

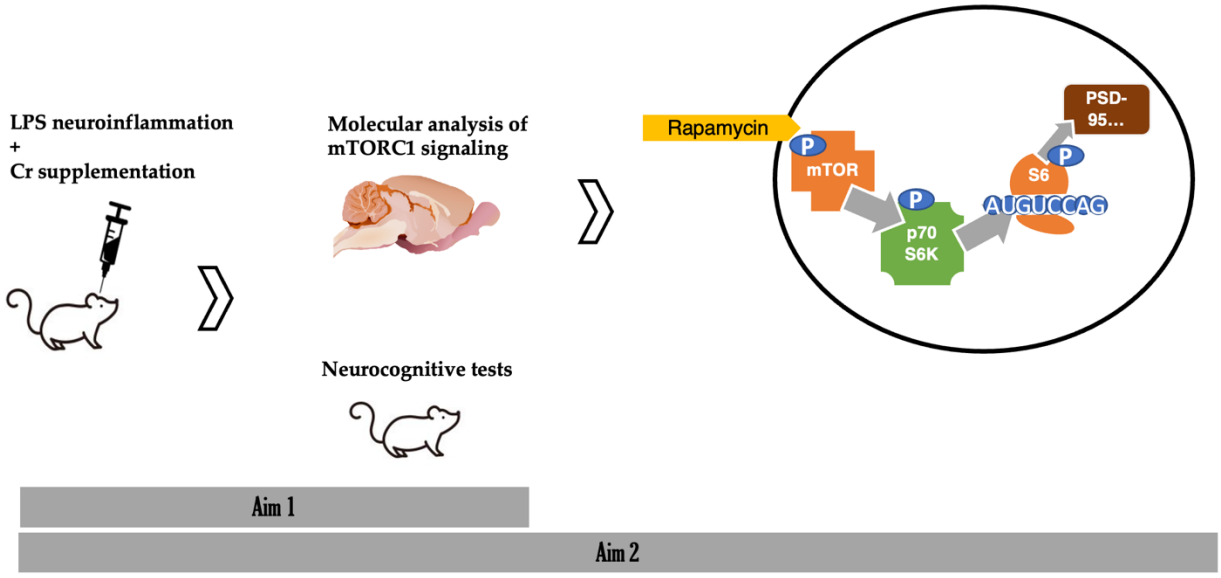

Figure 1.1. Graphical illustration of overall aim for dissertation

Abstract

Mild cognitive impairment (MCI) was defined as a boundary area between cognitive function of natural aging and dementia. Early studies have suggested that MCI could be a therapeutic window for prevention of dementia, given the irreversible nature involved in the pathology of dementia. One promising intervention may appear to be beneficial for MCI is creatine (Cr), which has been proven to be effective for ameliorating cognitive deficiency in general. However, it is unclear whether Cr supplementation could ameliorate cognitive deficiency in MCI. Moreover, the neuro-molecular evidence regarding the positive neurocognitive effects of Cr supplementation is currently lacking, hindering the clinical application. In order to better understand the neurocognitive and neuro-molecular effects underlying the Cr supplementation in the context MCI, I established a neuroinflammatory female Wistar rat model, which resembles the pathology of MCI in humans. Chronic (6-week) supplementation of Cr significantly ameliorated cognitive deficits in rats receiving intracerebroventricular injections of lipopolysaccharides (LPS) that induces neuroinflammation. Molecular analysis revealed that Cr supplementation robustly increased mTORC1 signaling and its downstream synaptic proteins, PSD-95 and synapsin, in dentate gyrus but not in medial prefrontal cortex. Immunohistochemistry analysis revealed upregulation of mTORC1 in $NeuN^+$ neurons. Importantly, selective inhibition of mTORC1 signaling through rapamycin attenuated the protective effects of chronic Cr supplementation in ameliorating cognitive deficits. Lastly, acute Cr treatment (12 and 24-hour) was sufficient to activate mTORC1 signaling within PC12 cells. In conclusion, the present study provides a novel insight to this field and offers a promising therapeutic treatment for cognitive deficiency.

Chapter 1: Introduction

Current consideration of dementia

Defining the dementia

Dementia is a term used to describe a group of symptoms that negatively affect memory and thinking (cognition) ability. Dementia is classified as an irreversible condition in nature once it occurs and the risk of dementia gradually increases during aging [1]. Notably, dementia is not a particular disease, instead, it is any disorder that causes progressive cognitive decline severely enough to disrupt daily life. Among elders, Alzheimer's disease (AD) and Parkinson's disease (PD) are most common subtypes of dementia, while brain tumors or traumatic brain injury are most common triggers for dementia in young adults [2]. Although significant advancements of molecular diagnosis and clinical strategies were seen in past years, currently, there are still more than 55 million people living with dementia worldwide and this number is rapidly growing with nearly 10 million new cases every year announced by World Health Organization (WHO) [3]. In light of pandemic level of dementia, therefore, discoveries of novel biomarkers and clinical therapies become an inevitable practice for improving the overall quality of life of patients and their families. In order to gain a comprehensive understanding of dementia, this section will examine the risk factors behind the dementia, followed by the discussion of major types of dementia (e.g., AD, PD), lastly, current therapeutics for dementia and future directions will be discussed.

Aging, lifestyle, genetic risks associated with dementia

Aging is the greatest risk factor for the incidence of many chronic diseases, including dementia. During the aging process, dysregulated brain energy metabolism occurs whereby it leads to some common features of brain aging (e.g., mitochondrial dysfunction, inflammation, oxidative damage) [4]. The fundamental similarities of those features shared by aging brain and dementia were

observed by clinical investigation [5], where it suggested that dementia was linked to aging at both molecular and cellular levels. Accumulated evidence points out that pathological alterations occurred in the brain causing dementia are largely due to compromised bioenergetics and interrupted neuronal networks during aging process [4]. Studies that utilized postmortem tissue and animal models [6]–[9] have reported the mitochondrial dysfunction, dysregulated intracellular Ca^{2+} homeostasis, elevated oxidative stress level and inflammation as primary triggers that convert “normal aging” into the “dementia” state. Although aging is the primary risk factor for the occurrence of dementia, different types of lifestyles could also be modified factors to influence the risk of dementia.

Lifestyle plays a strong role in shaping the risk of lots of chronic diseases, such as cardiovascular disease, metabolic diseases. It is well known that high fat diet (HFD) could lead to insulin resistance, vascular dysfunction, and inflammation [10], of which might contribute to dementia. Experimental observation in human suggests that HFD sensitizes the immune cells within hippocampus, and subsequently induce elevated proinflammatory cytokines [11], which compromise the synaptic plasticity and neurotransmission leading to cognitive decline. Furthermore, chronic exposure to sedentary lifestyle and lack of social interaction have negative effects on increasing stress and reducing cardiovascular health, which may provide a pathological mechanism for stimulating cognitive impairment and increasing the risk for dementia [12]. Additional factors, such as smoking, insulin resistance, were also extensively studied to accelerate the cognitive impairment and dementia through a variety of mechanisms (e.g., oxidative stress, vascular damage, elevated baseline insulin level, inflammation).

Most patients were clinically diagnosed with the late-onset dementia or sporadic dementia (over 65 years old), which could be determined or influenced by a wide spectrum of risk factors without an evident genetic cause. While a rare form of dementia, namely early-onset dementia, existed

which typically manifests itself younger than 65 years of age. Early-onset dementia is strongly determined by genetic factors, multiple aberrant gene mutations linked to the occurrence of dementia were discovered by genetic studies [13],[14], such as amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2). Those genetic mutations are highly penetrant and people who inherit them are very likely to develop symptoms of dementia at an early life stage. Given the complexity of pathological mechanisms of dementia, and a large population worldwide that were diagnosed with dementia, it is not surprising that tremendous efforts were dedicated to study different subtypes of dementia and their associated pathological mechanisms.

Major subtypes of dementia

Alzheimer's disease

Alzheimer's disease (AD) is the most prevalent cause of dementia, which affects 5.5 million people in the U.S. and approximately 24 million people globally [15]. It is well known that abnormal aggregation of amyloid beta (Aβ) peptide and hyperphosphorylated tau protein in central nervous system (CNS) are two hallmarks of AD [16]. Both Aβ peptide and tau protein can cause neuronal degeneration processes, including neuronal dysfunction, neuronal death and synaptic loss, which lead to cognitive impairment [17]. AD research in the past decades has considered that Aβ peptide is the primary cause of AD and the accumulation of Aβ initiates the deleterious effects, such as hyperphosphorylated tau and other neurodegenerative processes. However, recent evidence from human and animal disease models suggest that AD pathology is not a "linear" model that is predominated by Aβ peptide, rather, the formation of Aβ synergistically interacts with tau protein to activate the pathological cascade to induce symptoms of AD [18],[19]. Furthermore, most aging-related factors, including elevated oxidative stress, cholinergic neuron degeneration, neuroinflammation, calcium imbalance, were also found to be involved in the pathogenesis of AD [20]. Except the central mechanisms that contribute to AD, peripheral

mechanisms could also induce the occurrence of AD. It is now accepted that cardiovascular defects/dysfunction during the midlife could contribute to the late-onset AD. Dementia induced by cardiovascular dysfunction was categorized as vascular dementia, but it has a very close association with AD. Given the complicated neuro-molecular mechanisms underlying the onset of AD and a variety of diverse risk factors associated with AD, therapies aiming to target only a particular mechanism for AD need to be reconsidered, additionally, more comprehensive therapies are required to treat AD.

Vascular dementia

Vascular dementia is the second most frequent subtype of dementia after AD. However, what is worse than AD is that currently there is no licensed treatment for vascular dementia [21]. As mentioned above, although vascular dementia is very similar as AD, cognitive impairment in the context of vascular dementia is more clinically variable than AD. Vascular pathology frequently targets frontostrital neuronal networks, therefore, attention, executive function could be negatively affected during the vascular dementia [22]. In addition, depression and anxiety could be also induced by vascular pathology given that a variety of neural substrates could be affected due to vascular dysfunction [23]. It is well known that aging has a dramatic effect on cardiovascular health, with vascular function gradually declining during aging process [24]. Human clinical studies report that maximal oxygen consumption (VO_{2max}) declines 10% per decade during aging in both men and women regardless of activity level [25]. As adequate blood and oxygen supply to the brain is essential for maintaining the brain health and cognitive capability [26], a comprised cardiovascular system during aging process with additional pathological changes could cause detrimental effects on cognitive functioning. Although epidemiological studies have point out the cardiovascular risks associated with AD, a clear distinction regarding pathological mechanisms and treatment strategies between those two types of dementia are required further investigations.

However, in the absence of effective treatment for those two types of dementia, a preventative therapeutic strategy may need to be considered [27].

Parkinson's disease

Parkinson's disease (PD) is ranked as the second most common neurodegenerative disorder worldwide, and epidemiological studies revealed that the incidental rate is between 100 to 300 per 100,000 individuals [28]. The pronounced hallmark of PD is mainly expressed as the progressive loss of dopaminergic neurons (DNs) in substantial nigra par compacta (SNpc) [29], a brain region located in the basal ganglia circuit, which regulated the voluntary movement in vertebrates. Therefore, DNs degeneration in SNpc results in motor disorders, involving bradykinesia, resting tremor, rigidity and postural instability, observed in PD patients. Unfortunately, however, the same brain lesions caused by DNs degeneration also cause memory decline/impairment that severely compromises functions to maintaining a "normal" lifestyle. Similar as other subtypes of dementia (e.g., AD), PD has distinct pathogenic mechanisms. The familial form of PD was strongly influenced by mutations associated with 3 genes, which are PARK1, PARK2 and PARK5 [30]. Genetic mutation occurred to PARK1 causes misfolded and accumulated α-synuclein proteins, a presynaptic protein with unclear function, also called lewy body [31] , while PARK2 and PARK5 mutations lead to compromised ubiquitin-protease pathway that is incapable of degrading misfolded or damaged proteins [32], facilitating the aggregation of misfolded α-synuclein proteins. The neuro-toxic effects of α-synuclein eventually cause neurodegeneration in SNpc to induce PD. In addition, recent evidence also found that age-dependent increase of oxidative stress and mitochondrial dysfunction promote the neuronal death in SNpc, as dopaminergic neurons are particularly vulnerable to free radical damages [33]. Notably, oxidative stress also interacts with α-synuclein to further exacerbate the progression of PD [34] , making PD not only a genetic-influenced disease, but also an aging-dependent disorder

that impairs both motor and cognitive functions. Another most common subtype of dementia is Lewy body dementia (LBD), which closely resembles PD, as LBD is also associated with abnormal deposits of α-synuclein or Lewy bodies. However, LBD lesions primarily occur in hippocampus, cerebral cortex and brain stem, making its movement disorder synchronized with cognitive impairment [35]. In contrast, cognitive symptoms generally begin to appear 1 year after the appearance of motor disorder in PD due to its pathology primary attacks limbic structures of the brain [36]. Although ongoing investigations are extensively studying different forms of α-synuclein or Lewy-body related forms of dementia, there is currently no effective cure for this type of dementia.

Current therapies and future directions for dementia

It is generally accepted that dementia rate increases as the population aging. Current therapies designed for dementia are primarily focusing on pharmacological approaches and only prescribed for alleviating partial symptoms [37]. Current therapies for AD, (e.g., N-methyl-d-aspartate (NMDA) receptor antagonists, acetylcholinesterase inhibitor, and serotonergic agonist), do not cure AD and prevent the progression of AD [38],[39]. Meanwhile, more advanced therapy targeting Aβ peptide undergoing clinical experiments are still under clinical investigations. Those therapies, although dedicated to overcoming some clinical symptoms to improve the quality of life of patients, ignored the fact that underlying pathogenesis of dementia is multifactorial, which will likely require earlier intervention and multifaceted therapies. For this reason, therapeutics targeting an earlier stage of the disease may provide larger benefits, which may also prevent the progression of diseases. As stated by DeKosky et al. “In the case of AD, a delay in onset by 5 years could translate into a 50% decrease in disease prevalence and, a delay of 10 years would result in virtual disappearance of the disease” [40]. Therefore, the prodromal stage of dementia may deserve more in-depth investigations, and future therapeutics designed for an earlier window of disease might provide more significant effects.

Early "therapeutic window" for treating dementia

Defining mild cognitive impairment

In the last decade, efforts have been made by many clinicians, researchers within "aging and dementia" field to identify a reliable state for controlling disease development. Such identification would allow new insight and therapeutic interventions to be implemented to prevent the progression of dementia. Based on clinical observations that were utilized to characterize cognitive function in normal aging and dementia [41], researchers identified a sizeable population that apparently exhibit a certain extent cognitive impairment between cognitive changes seen in normal aging and dementia state. Now, it becomes clear that this "grey zone" has its ability to later transition into dementia and this transitional phase was named as mild cognitive impairment (MCI). Essentially, MCI is a syndrome characterized by only memory loss beyond the normal aging and educational level [42]. However, other cognitive domains (e.g., executive functioning, language, visual skills) are preserved to be intact [43]. Population-based epidemiological studies estimated that prevalence of MCI ranges from 10% - 20% among elderly individuals older than 65 years of age [44]–[47], and incidental rate of MCI showed significantly high rate, as compared to other neurodegenerative diseases, with an annual rate ranging from 5%-10% in the U.S. [48]. However, it is worth to know that one of core criteria for diagnosing MCI relies on subjective report of cognitive changes. As a result, the overall prevalence and incidental rate of MCI might be significantly underestimated. However, due to the recent advancement in both research and clinical settings for studying MCI, its underlying pattern of disease progression, neuropathological mechanisms, risk factors associated with MCI are began to be revealed.

Pattern of disease progression

After years of research, MCI is now defined as a prodromal stage of dementia, which represents an intermediate state between cognitive changes in the context of normal aging and dementia

(Figure 1.2). It is generally accepted that a significant portion of MCI patients later would progress to dementia, in particularly AD. Population-based studies found that more than 90% of MCI patients who progress to dementia involves clinical signs of AD [49], indicating that there are pathological similarities between MCI and AD. Furthermore, epidemiological studies based on human participants recruited from “Rush Memory and Aging Project”, a project started in 1997 that was designed to identify factors associated with cognitive health [50], examined incidental rate of AD among subjects with MCI. Following only 2.5 years of follow-up, 25.8% of MCI patients developed AD, and this number was predicted to be approximately 80% after 6 years [51]. Despite that partial MCI patients do progress to AD, Jessen et al. [52] found that late-MCI patients have a significantly increased rate of developing AD than early-MCI patients, suggesting that the severity of cognitive impairment of MCI is positively correlated with the risk of AD. Consequently, MCI was suggested to be an ideal early therapeutic window for the prevention of AD by many studies [53],[54] and thus, gaining a deeper understanding of pathological mechanisms underlying MCI turns out to be clinically significant.

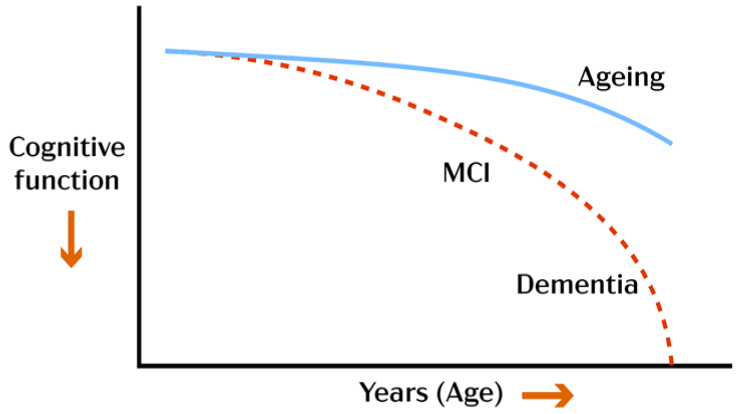

Figure 1.2. Graphic illustration of cognitive function decline as a function of years of age. Blue line indicates the cognitive function decline during the normal aging process, red dash line represents cognitive function decline in the context of mild cognitive impairment (MCI) or dementia, as compared to normal aging.

Inflammation as a central mechanism in mild cognitive impairment

The presence of constant immune response in the central nervous system has been considered as a prominent feature in AD, and neuroinflammation was demonstrated to exacerbate the aggregation of both Aβ plaques and neurofibrillary tangles (tau proteins), two core features of AD brain [55]. Considering the high risk of transitioning from MCI to AD that was mentioned previously, it is not surprising that neuroinflammation could proceed the MCI to initiate a cascade of cellular events to cause cognitive impairment and then, subsequently, dementing the MCI brain. During the aging process, the immune system functions' efficacy declines progressively resulting in immunosenescence that elevates the cellular production of proinflammatory factors (proinflammatory cytokines), such as factor-α (TNF-α), interleulin 1-β (IL1-β) and IL-6 [56],[57]. This elevated neuroinflammation then could lead to mitochondrial dysfunction and excessive production of reactive oxygen species (ROS) through imbalanced mitochondrial respiratory chain [58], which further stimulates and reinforces the release of proinflammatory factors through innate

immune system [59]. This process results in a vicious cycle which gradually remodels the immune function favoring a proinflammatory cellular environment, and causes tissue and cellular damages [60]. In diagnosed MCI patients, proinflammatory cytokines (e.g., TNF-α) were found to be significantly higher than in healthy age-matched controls, through cytokine array [61]. Meta-analysis covering 28 studies summarized that neuroinflammation level in MCI patients, although was lower and less disperse compared to AD, was significantly higher relative to controls [62], implying that neuroinflammation is involved in the pathogenesis of MCI, which highlights their inevitable role of participating in the disease development and progression [63]. In fact, transition from immunosenescence to pathological neuroinflammation is a complicated process which will require complex cellular and molecular cascades involving immunological cells, such as microglia, to initiate the neuroinflammation [64]. Current evidence pointed out that chronic neuroinflammation is through an enhancement in microglial activation and elevated production of proinflammatory cytokines released by hyperactive microglia. Now, it is generally agreed that hyperactive microglia would result in neuroinflammation which increases the vulnerability of CNS to neurodegenerative diseases, such as MCI.

Neuroinflammation model for studying mild cognitive impairment

As now it is a common sense that neuroinflammation induced cellular and molecular changes cause cognitive impairment, in particularly memory decline, animal disease models were generated for mimicking neuroinflammation to allow investigators to study disease pathological mechanisms and therapeutic treatments [65]. Lipopolysaccharides (LPS) are large molecules and major component of the outer membrane of Gram-negative bacteria, where LPS consist of a lipid and a polysaccharide composed of O-antigen to play an essential role in modulating interactions between bacteria and host through mediating responses of the host immune system [66]. Due to the conserved presence of LPS at the surface of many bacterial pathogens, molecular signaling mechanisms have been evolved by host immune system in order to recognize the presence of

LPS, as a pathogen-associated molecular pattern (PAMP), for detecting bacterial pathogens. Within the host immune cells, toll-like receptor 4 (TLR4) is the primary responsive receptor for recognizing the LPS in order to initiate signaling cascades to activate innate immune responses. Within the CNS, TLR4 are highly expressed by microglia [67], an innate immune cell, to first respond to the conserved feature of LPS for a defense purpose. However, chronic activation of microglia caused by persistent activation of TLR4 leads to dynamic transformation of phenotypes of microglia that are closely associated with the initiation of neuroinflammation, which will finally lead to neuronal dysfunction and loss. For this prominent feature of LPS, LPS have been thus utilized widely by researchers to develop neuroinflammation animal model for studying diseases and treatments. In mice, systemic LPS administration significantly impairs spatial memory during the Morris water maze (MWM) behavior test and increases protein expression of ionized calcium binding adaptor molecule (Iba1), a marker for activated microglia, in mice hippocampus [68]. In the same study, the comparison made between intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) injections of LPS suggests that both i.p. and i.c.v. injections of LPS result in great memory loss (cognitive impairment). However, cognitive impairment might be even more severe after i.c.v. injections than i.p. injections, which could be due to the relative resistance of blood-brain barrier (BBB) to i.p. injections of a low dose of LPS compared to the same dose of i.c.v. injections [69]. Furthermore, i.c.v. injections of a single dose of LPS in rats have been also shown to be effective in inducing neuroinflammation in both hippocampus and prefrontal cortex brain regions, concurrent with deficits observed during the spatial memory tests [70]. Furthermore, LPS animal model has several advantages over other models. First of all, LPS elicits high levels of proinflammatory cytokines production shortly after its administration, and this rapid action leads to dose-dependent effects for neuroinflammation [71]. In addition, a large body of studies utilizing rodents found high reproducibility of LPS's ability to elicit neuroinflammation and subsequent cognitive impairment [65],[72], suggesting the reliability and stability of this particular "neurotoxin". Lastly, the technique ease of using LPS (e.g., solvent, temperature) favors the preparation of LPS

under the lab environment. In summary, modern research relies on animal disease models to study disease mechanisms and therapeutic approaches, LPS were utilized on rodents to mimic neuroinflammation for inducing cognitive impairment. Therefore, studies for which investigators want to pursue neuroinflammation-induced neurodegenerative diseases may utilize LPS because of its prominent features and suitability for pre-clinical assessment of cognitive impairment.

Creatine: a metabolic agent that improves cognitive function

The story of creatine

Since the discovery of phosphocreatine (PCr) and creatine kinase (CK) reaction in 1927 and 1934, respectively [73], many efforts thus have been given to creatine (Cr) for its biochemical, physiological and pathological characteristics. Cr is a naturally occurring organic compound that can be synthesized primarily in liver kidneys and pancreas, from amino acids glycine and arginine. After production of Cr, it is transported via blood and circulates throughout the body to arrive tissues, such as skeletal muscle and brain, dependent on a specific transportation system. The specific transporter (CRT) for cellular uptake of Cr is encoded by SLC6A8 gene within target tissues [74], mutations occurred to SLC6A8 gene results in a disease called creatine transporter defect, which is inherited in a X-linked manner primarily happened in brain [75],[76]. When Cr arrives the cell, a phosphate bond from ATP produced by mitochondria is transferred to Cr to from the PCr through a coupled reaction (CK reaction) mediated by CK for storage as a high energy reservoir, while when energy demand is increasing, the phosphate bond from PCr is rapidly donated to ADP to regenerate ATP. This CK reaction is critical for skeletal muscle to efficiently utilize energy to initiate muscle contraction and enhance contractility, unlike energy produced by mitochondria and glycolysis, ATP resource produced by CK reaction provides high rates of energy transfer under the anaerobic condition for immediate energy support [77].

Beyond the well-described role of Cr in providing energy source for skeletal muscle, it also plays a critical role in brain functions. The elementary enzymes for endogenous Cr synthesis were found

to exist in the CNS and creatine transporters were also found at the BBB, neurons and some glial cells [78], implying that Cr is essential for regulating some significant cellular and molecular events. In fact, research outcomes from studies of animal models have shown that Cr is especially important for maintaining normal brain function, development, and neuroprotection. For example, genetic deletion of brain type creatine kinase in mice compromises normal brain development, with abnormal mossy fiber field observed, which was shown to associate with slower acquisition of spatial task [79]. Furthermore, brain Cr content was found to be reduced following the mild traumatic brain injury (mTBI) [80], and this reduced Cr content in the brain may even deteriorate the energy shortage after nerve damage, mitochondrial dysfunction and oxidative stress caused by mTBI. Consequently, in animal experiments mimicking mTBI, researchers found that Cr supplementation not only ameliorated the cognitive impairment induced by mTBI, but also rescued cortical damage following mTBI ranging from 36% to 50% in rodents [81],[82]. This neuroprotective effect of Cr was then hypothesized to mediate the mitochondrial membrane potential and thus, to upregulate the mitochondrial bioenergetics. Furthermore, they also observed decreased ROS within mitochondria and mitochondrial transition permeability. Collectively, Cr and Cr supplementation seemingly has a wide spectrum of effects on maintaining and improving brain functions, despite the limited amount of research and data.

Creatine and cognitive function

Within recent years, interest in the cognitive potential of Cr starts to expand the field and, currently, there are growing body of studies beginning to assess the cognitive effects of Cr in the context of different states. McMorris et al [83] firstly examined the Cr supplementation among elderly population. In this human trial, Cr supplementation (20 g) was given daily to participants for 7 days and they found that Cr supplementation significantly improved spatial recall, long-term memory and some other cognitive domains. In contrast to elderly population, young participants who consumed Cr (0.03 g/kg/day) for 6 weeks showed no cognitive improvement compared to

their age-matched controls [84]. However, this obvious discrepancy across two age groups could be due to different dosage protocols and duration used by different studies. Interestingly, Benton et al. [85] demonstrated that Cr supplementation (20 g/day) for 5 days lead to improved memory in vegetarians, while this improved effect of Cr was not observed in omnivores by using the same administration protocol, suggesting that Cr may have a stronger effect among those who are naturally more susceptible to experiencing the energetic shortage. In support of this, Cr supplementation has been found to be effective of ameliorating cognitive performance in individuals who underwent 24 h and 36 h sleep deprivation [86],[87], which could cause reduced blood flow and hypoxia. In addition, 7 days of Cr supplementation prior to the acute oxygen deprivation restored hypoxia-induced cognitive impairment, and this restoration has been found to associated with increased corticomotor excitability [88], again confirming that Cr has its ability to rescue oxygen utilization during demanding tasks. Collectively, although studies that examined Cr supplementation have generated some controversies regarding Cr's efficacy of improving cognitive processing in human trials, mainly between experimental subjects' ages, it seems that Cr is more effective of eliciting cognitive benefits when cognitive challenges are demanding or under certain disease conditions.

Some animal studies have also been done to examine the cognitive effects of Cr and its potential underlying neuro-molecular mechanisms. Pioneering work done by Snow et al. [89] indicated that 8 weeks of chronic dietary Cr supplementation significantly enhanced spatial memory in wild-type (WT) mice, which was concurrent with elevated levels of proteins that were implicated in cognition (e.g., Egr2, CaMKII), additionally, mitochondrial electron transport chain complex I proteins, fission protein Drp1 along with mitochondrial respiration were also found to be increased accompanying Cr supplementation. Although causalities were not to be investigated in this study, a wide range of neuro-molecular effects of Cr were implicated. In their follow-up study where transgenic AD mice were used [90], Cr supplementation was proved to be effective of ameliorating

cognitive impairment in female AD mice associated with increased CREB phosphorylation, and CaMKII protein expressions, again, implicating the capabilities of Cr in mediating neurocognition possibly in a multifaceted fashion. More specifically, in a severe traumatic brain injury (TBI) rat model induced by fluid percussion, 3 consecutive days of Cr supplementation were observed to greatly decrease the thiobarbituric acid reactive species (TBARS) and protein carbonyl content [91], two assays performed to evaluate the cellular oxidative stress level. In oxidatively injured cultured cells, Cr significantly attenuated oxidative stress level within 4 different cell lines. Furthermore, mutations of mitochondrial DNA induced by ultraviolet (UV) radiation exposure were normalized by Cr supplementation paralleled by restored level of oxygen consumption, mitochondrial membrane potential, and ATP production [92]. Collectively, all those data and experiments suggest a possibility of future practical use of Cr for diseases where their pathologies involving oxidative stress and mitochondrial dysfunction as causal factors.

Creatine, synaptic plasticity, and cognition

Synaptic plasticity refers to the activity-dependent modifications to the strength of synaptic transmission at existing synapses, which is by far considered as a basis for learning and memory [93]. There are different forms of synaptic plasticity that either enhance or depress the synaptic strength [94]. Among synaptic plasticity, long-term potentiation (LTP) is widely recognized as one of the cellular mechanisms to mediate learning and memory function, and it defines a persistent strengthening of synaptic transmission between contacting neurons [94]. Therefore, an increased LTP may imply an improvement in learning and memory. Due to the great potential of Cr in enhancing cognitive function revealed by human and animal studies, it is possible that one of neuro-molecular mechanisms underlying Cr's neurocognitive effects is through modulating synaptic plasticity of existing neurons. In fact, in-vivo studies [98],[89],[90] revealed that chronic Cr supplementation significantly upregulated postsynaptic density protein 95 (PSD-95) protein expression level, a well-defined scaffolding protein that is required for forming LTP, in mice

hippocampus [95],[96]. Furthermore, behavioral tests performed by those studies suggest that there is a correlation between Cr elicited increase of PSD-95 protein in hippocampus and enhanced memory function. Additionally, neuronal cell culture experiment [97] also suggests that 12-hr of Cr treatment robustly increased PSD-95 protein expression per dendrite within neurons compared to controls, again confirming the potential capability of Cr of enhancing LTP in vivo. Interestingly, maternal supplementation of Cr through drinking water (1%) in rats was found to significantly enhance the dendritic tree development and LTP, observed from CA1 pyramidal neurons of newborn pups at the weaning age (21 days from postnatal day 0) [98]. For further investigating the long-lasting modifications that maternal Cr supplementation may possess, CA1 neurons of adult rats (8-10 weeks of age) born from dams with Cr supplementation were again studied [99]. In this study, CA1 neurons of offspring from Cr supplemented adult rats exhibited an enhanced neuron excitability along with an increased LTP, suggesting that the synaptic effect of maternal Cr supplementation can persist into the adulthood. Although studies confirmed that Cr has a direct effect on enhancing synaptic strength, which in turn could contribute to improved learning and memory, there are still some knowledge gaps remain to be filled to fully understand the cellular actions of Cr.

mTOR signaling pathway

Biology of mTOR signaling pathway

The mammalian target of rapamycin (mTOR) signaling plays a critical role of regulating cell metabolism, growth, proliferation and survival [100]. Within the cell, mTOR signaling senses growth factors, energy status, oxygen levels, and amino acids to determine the subtle changes occurred to cellular environment and make adaptive regulations to downstream effectors. Additionally, mTOR signaling also functions to regulate protein synthesis-dependent plastic changes within neuronal cells to mediate learning and memory function [101]. Now, mTOR is well-recognized to have two multiprotein complexes, namely mTORC1 and mTORC2, which have

different downstream substrate specificities and thus, different cellular functions. mTOR1 signaling is comprised of five protein subunits: mTOR, which is the catalytic subunit; regulatory-associated protein of mTOR (Raptor), which is unique to mTORC1 complex; proline-rich AKT substrate 40kDa (PRAS40); DEP-domain-containing mTOR-interacting protein (Deptor); and mammalian lethal with Sec13 protein 8 (mLST8) [100]. Within the mTORC1 protein complex, Raptor interacts with mTOR protein to regulate mTORC1 protein complex activity through modulating recruitment of substrates [102]. Solid evidence [103],[104] showed that cellular knockdown of Raptor blocked phosphorylation of downstream effectors of mTORC1 upon nutrient stimulation, additionally, mutation of Raptor at its phosphorylation site Ser^{863} reduced overall mTORC1 activity both in vivo and in vitro. While under the nutrient deprived condition, the total amount of Raptor bound to mTOR protein increased remarkably that inversely correlated with a lower kinase activity of mTOR and vice versa [104], implying that the interaction between Raptor and mTOR gates mTORC1 signaling activity. In contrast, mTORC2 has several same protein subunits as mTORC1, but it has a subunit unique to mTORC2 called rapamycin-insensitive companion of mTOR (Rictor). The current knowledge regarding mTORC1 is quite more thorough in comparison to mTORC2. Although evidence existed to reveal that mTORC2 is highly involved in the control and maintenance of the actin cytoskeleton [105], its upstream regulatory mechanisms and cellular functions still remain to be investigated. As a result, the current topic will specifically focus on mTORC1 as its relative importance relates to learning and memory function.

mTORC1 signaling pathway: a master regulator of cell growth and metabolism

It is now clear that mTORC1 regulates both cellular anabolic process, such as synthesis of proteins, and catabolic processes, such as cellular autophagy. However, much of the knowledge gained from research are a result of the use of rapamycin, a selective inhibitor for mTORC1 complex. After entering the cell, rapamycin binds to FK506-binding protein of 12 kDa (FKBP12),

which then bind to FKBP12-rapamycin binding domain of mTOR protein to prevent the recruitment of downstream substrates [106]. As FKBP12-rapamycin binding complex cannot physically interact with mTORC2, it is suggested that rapamycin is highly selective for inhibiting mTORC1. Therefore, on the basis of this pharmacological tool, lots of discoveries regarding mTORC1 were found in the past decades.

There are two main substrates for controlling protein biosynthesis downstream to the mTORC1 signaling, the p70 ribosomal S6 kinase 1 (p70S6K) and the eukaryotic initiation factor 4E(eIF4E)-binding protein 1 (4EBP1). Upon phosphorylation of threonine 389, p70S6K protein is activated to further lead to activation of S6 ribosomal protein, which increases protein synthesis at the ribosome [107]. Additionally, activation of p70S6K also promotes mRNA biogenesis and translation of ribosomal proteins [108]. For years, the phosphorylation status of threonine 389 on p70S6K has been used as a biomarker for assessing the activation status of mTORC1 signaling. While the phosphorylation of 4EBP1 protein releases its binding with eIF4E, allowing eIF4E to initiate cap-dependent protein translation [109]. Except those two substrates for regulating protein synthesis, mTORC1 signaling also regulates cellular autophagy through modulating activity of unc-51 like kinase 1 (ULK1), an autophagy initiating molecule. It was reported by several groups that inhibition of mTORC1 leads to increased ULK1 activity [110]–[112]. Reversely, activated mTORC1 phosphorylates ULK1 to prevent its binding with other substrates and inhibit autophagy [113]. In mammalian cells, mTORC1 protein complex constitutively integrates the cellular nutrients and energy level to make cellular decisions. When energy or nutrients level is high, mTORC1 signaling is activated and promotes cell proliferation, growth, and protein biosynthesis, while inhibiting cellular autophagy, favoring anabolic process. However, under the energy or nutrient deprivation condition, such as fasting, mTORC1 is inhibited and promotes catabolic process to maintain cellular homeostasis [114].

mTORC1 signaling in learning and memory

Due to the multifunctional roles of mTORC1 signaling in mammalian cells, mTOR has been stated to involve in regulation of many physiological processes including neurocognition. When researchers begin to explore the cellular mechanisms that regulate the memory formation, the role of translational regulatory mechanisms necessary for the formation of memory was also realized [115], therefore, a cellular signaling pathway that regulates translational activity would be important for forming new memory. In a study designed for investigating molecular regulator of hippocampus-dependent long-term memory formation [116], researchers initially found concurrent increases of phosphorylation of mTOR and p70S6K proteins with a memory training regime. Later in their next experiment, the memory formation was hindered by pharmacological inhibition of mTORC1 through applying rapamycin in hippocampus prior to memory training. Consequently, the authors declared that mTORC1 is a major and necessary mechanism for memory consolidation. Additionally, rapamycin administration in mice was reported to cause deficiency in memory retention after learning the maze task, which extends the previous finding and supports that mTORC1 signaling play a critical role for both memory formation and retention [117]. The acquisition of maze task was not impaired by inhibiting mTORC1 signaling reported by this study, however, it is possible that this study underestimates the effect of mTORC1 signaling during the acquisition phase because mice, as compared to rat, has a much slower rate to acquire spatial memory tasks [118]. While it seems that the neurocognitive effects of mTORC1 signaling expand cross a wide range of different brain regions and cognitive domains, research done to investigate the molecular mechanisms underlying formation and retention of fear memory found a critical role of mTORC1 signaling also in amygdala [119]. In this study, injection of rapamycin into rats' amygdala prevented the formation of fear memory after fear conditioning and in addition, blockage of mTORC1 signaling in amygdala also attenuated the fear memory recall in previously trained rats who have already stored fear memory. While even though it is clear that mTORC1 signaling is critical and, in some circumstances, necessary for new memory formation, a more

specific explanation for how does mTORC1 signaling contribute to memory formation is required. Previously, immunostaining studies indicated that the distribution of effector proteins for mTORC1 frequently overlap with the postsynaptic protein, PSD-95, and the presynaptic protein, synapsin [120], implying that the neurocognitive mediator's role of mTORC1 is possibly through modulating translational activities of functional synaptic proteins and, thus synaptic plasticity. Experimental disruption of mTORC1 in hippocampal slices suggests that LTP expression induced by high-frequency stimulation was significantly reduced by applying rapamycin. Also, synaptic potentiation induced by brain-derived neurotrophic factor (BDNF) was blocked by rapamycin treatment [120]. Collectively, those experimental observations demonstrate an essential role of mTORC1 signaling in linking new protein synthesis dependent synaptic events to functional outcomes. Although downregulation of mTORC1 impairs learning and memory function proved by many experiments, studies also showed that hyperactivation of mTORC1 signaling lead to cognitive deficiency, normally observed in the aging brain and some neurodegenerative diseases, such as AD. Currently, hyperactivation of mTORC1 signaling pathway is considered as a major pathological mechanism of eliciting AD. For example, enhanced activation of mTORC1 signaling during aging could contribute to overwhelmed production of Aβ protein, which impairs synaptic plasticity and memory [121],[122]. Additionally, hyperactivated mTORC1 signaling also suppresses physiological level of autophagy, causing failure of degrading protein aggregates, to form a vicious cycle to exacerbate the pathological progression of AD. Interestingly, however, one group reported that downregulated mTORC1 signaling was found to associate with impaired synaptic plasticity, in hippocampal slices isolated from AD transgenic mouse and from WT slices directly exposed to Aβ treatment [123]. This finding conflicts with others' findings where hyperactivation of mTORC1 signaling was found during the pathology of AD [124]. Furthermore, the previous group also reported that both pharmacological and genetic upregulation of mTORC1 signaling rescued the impaired synaptic plasticity, or LTP, in AD mouse [124], further contending

their suggestion that mTORC1 is dysregulated to cause impaired synaptic plasticity in the context of AD.

In summary, mTORC1 signaling pathway plays an important role in regulating many aspects of neuronal activities and balancing cellular environment. Given the dual role of mTORC1 signaling in mediating learning and memory, it seems that mTORC1 signaling could be a therapeutic target for ameliorating learning and memory related dysfunction. However, precise regulation of mTORC1 signaling and extra cautiousness would be also required for maintaining an optimal cognitive capacity.

Chapter 2: Creatine supplementation upregulates mTORC1 signaling and markers of synaptic plasticity in the dentate gyrus while ameliorating LPS-induced cognitive impairment in female rats

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Abstract:

Mild cognitive impairment (MCI) designates the boundary area between cognitive function in natural aging and dementia, and this is viewed as a therapeutic window to prevent the occurrence of dementia. The current study investigated the neurocognitive effects of oral creatine (Cr) supplementation in young female Wistar rats that received intracerebroventricular injections of lipopolysaccharides (LPS) to mimic MCI. Neuromolecular changes within the dentate gyrus were analyzed following behavioral testing. We also investigated both neurocognitive and neuromolecular changes following Cr supplementation in the absence of LPS in young female Wistar rats to further investigate mechanisms. Interestingly, based on trial 2 of Barnes maze test, Cr supplementation ameliorated spatial learning and memory deficit induced by LPS, shown by decreased latency time and errors to reach the escape box ($p < 0.0001$, n=12) during the Barnes maze test. Cr supplementation also attenuated recognition memory deficit induced by LPS, shown by increased amount of time taken to explore the new object ($p = 0.002$, n=12) during novelty object recognition testing. Within the dentate gyrus, Cr supplementation in LPS injected rats upregulated mTORC1 signaling ($p = 0.026$ for p-mTOR phosphorylation, $p = 0.002$ for p-p70S6K phosphorylation, n=8) as well as the synapsin ($p = 0.008$) and PSD-95 synaptic proteins ($p = 0.015$, n=8), in comparisons to LPS injected rats. However, Cr supplementation failed to further enhance spatial memory and recognition memory in the absence of LPS. In conclusion, Cr ameliorates LPS-induced cognitive impairment in a rodent MCI model. Mechanistically, these phenotypic effects may, in part, be mitigated via an upregulation of mTORC1 signaling, and an enhancement in synaptogenesis in the dentate gyrus. While preliminary, these findings may inform future research investigating neurocognitive effects of Cr for MCI patients.

Introduction

Dementia is a comprehensive descriptor for various combinations of symptoms, which include deficits in memory, problem solving, thinking skills, or language [125]. The most prevalent dementia type is Alzheimer's Disease (AD). AD has been categorized into three developmental phases that are preclinical, mild cognitive impairment (MCI) AD, and dementia AD [126]. Hence, MCI transcends between the preclinical and dementia AD stages. Although results from the Rush Memory and Aging Projects showed 42% of the MCI patients developed dementia after a median of ~3 yrs with 38% of patients reverting back to normal at the same time [127], studies have shown that the AD pathology is irreversible in nature once it occurs [128],[129]. This implies the clinical significance of using MCI as an early therapeutic window for populations who are vulnerable to AD. However, therapeutic interventions for mitigating the progression of MCI are currently lacking.

Creatine (Cr) is a naturally occurring compound that is synthesized from the amino acids glycine and arginine, primarily in liver and kidneys. When Cr is stored, it is converted to high energy form of phosphocreatine (PCr), which provides immediate energy supply by donating its phosphate to regenerate ATP through the creatine kinase (CK) reaction, as energy demand increases [130]. Although Cr supplementation has been widely used as an ergogenic aid for professional and recreational athletes for decades [130]; there has been recent interest in examining its efficacy in enhancing cognition [131],[132]. In a double-blinded designed human study, Rae et al. [133] found that six-weeks of Cr supplementation significantly improved working memory and intelligence score. McMorris and colleagues [83] further revealed a significant effect of Cr on enhancing cognition in elderly participants after only one week of Cr supplementation. Other studies have assessed the cognitive-enhancing properties of Cr under stressed conditions. For example, in 24 h and 36 h sleep-deprived individuals, Cr supplementation significantly augmented cognitive performance compared with placebo-control [86],[87]. In addition, 7 days of Cr supplementation

has been shown to restore the acute hypoxia-induced decrements in cognitive performance in healthy young adults [88].

While promising, the neuro-molecular mechanisms associated with the cognitive benefits underlying Cr supplementation are not well established. Mammalian target of rapamycin complex 1 (mTORC1) is a protein complex that is composed of mTOR, Raptor, mLST8, Deptor and PRAS40, and mTORC1 functions to sense and integrate nutritional and environmental cues, such as amino acids, growth factors, stress level, and energy status, to regulate many essential anabolic processes. Upon activation, mTORC1 complex promotes protein synthesis mainly through phosphorylating downstream 70-kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP) [100]. Studies have implied that mTORC1 signaling may be a mechanism involved in mediating the cognitive enhancement of Cr supplementation. For example, it has been shown that the inhibition of mTORC1 signaling via rapamycin in hippocampal preparations reduced long-term potentiation (LTP) induced by high-frequency stimulation and BDNF [120]. It has also been shown that learning tasks induce a rapid increase in the phosphorylation status of mTOR (Ser 2448) and its specific substrate p70S6K in the hippocampus, while bilateral infusion of rapamycin into CA1 region of the hippocampus diminishes the mTORC1 pathway and memory formation [116], suggesting a key role of mTORC1 signaling in mediating memory formation and learning. Others have reported Cr supplementation in mice increases hippocampal mTORC1 signaling [134],[135]. Collectively, these findings suggest mTORC1 signaling could be a candidate pathway that underlies the cognitive benefits of Cr.

Herein, we utilized an LPS-induced rodent MCI model, which exhibits impaired spatial and recognition memory, published previously by our laboratory [136], to examine potential cognitive effects of Cr supplementation. Furthermore, we examined neuro-molecular mechanisms in the dentate gyrus resulted from and associated with Cr supplementation, this being a sub-region of

hippocampus known to play a fundamental role in hippocampus-dependent learning and memory [137],[138]. In the current study, we hypothesized that 6 weeks of oral Cr supplementation (at a dosage of 1.542 g/kg/day for the first week and 0.385 g/kg/day for following 5 weeks) to female rats would (a) ameliorate the LPS-induced cognitive deficits and (b) increase mTORC1 signaling and markers of synaptic plasticity (pre-synaptic synapsin and post-synaptic PSD-95 proteins) in the dentate gyrus. We also tested the effects of Cr supplementation without LPS to further explore the cognitive effects and mechanisms associated with Cr supplementation.

Materials and Methods

2.1. Animals and Experimental Design

Experimental protocols described herein were approved by the University of Missouri Animal Care and Use Committee (protocol code: 10111, date of approval: 11th October 2019). Female Wistar rats (150–200 g, 49 days of age) were bred at the University of Missouri, and individually housed under controlled conditions (12 h: 12 h light/dark cycle, 24 °C). During the entire study, rats were provided food and water (Formulab Diet 5008; Purina, St. Louis, MO, USA) ad libitum. Animals were randomly assigned into specified experimental groups, with each experiment using a separate sample of animals. In experiment 1 (LPS experiment in Figure 2.1), 7-week-old female rats were randomly divided into three groups to determine whether and how Cr affected cognitive deficits induced by LPS. These groups included: (a) vehicle injected (Veh), (b) LPS injected (LPS), and (c) LPS injected with 6 weeks of oral Cr supplementation (LPS + Cr) (n = 12 rats/group). In experiment 2 (non-LPS experiment in Figure 2.1), 7-week-old female rats were randomly divided into two groups to examine the cognitive and neuro-molecular effects of Cr without LPS. These groups included (a) placebo and (b) oral Cr supplementation (Cr) (n = 12 rats/group).

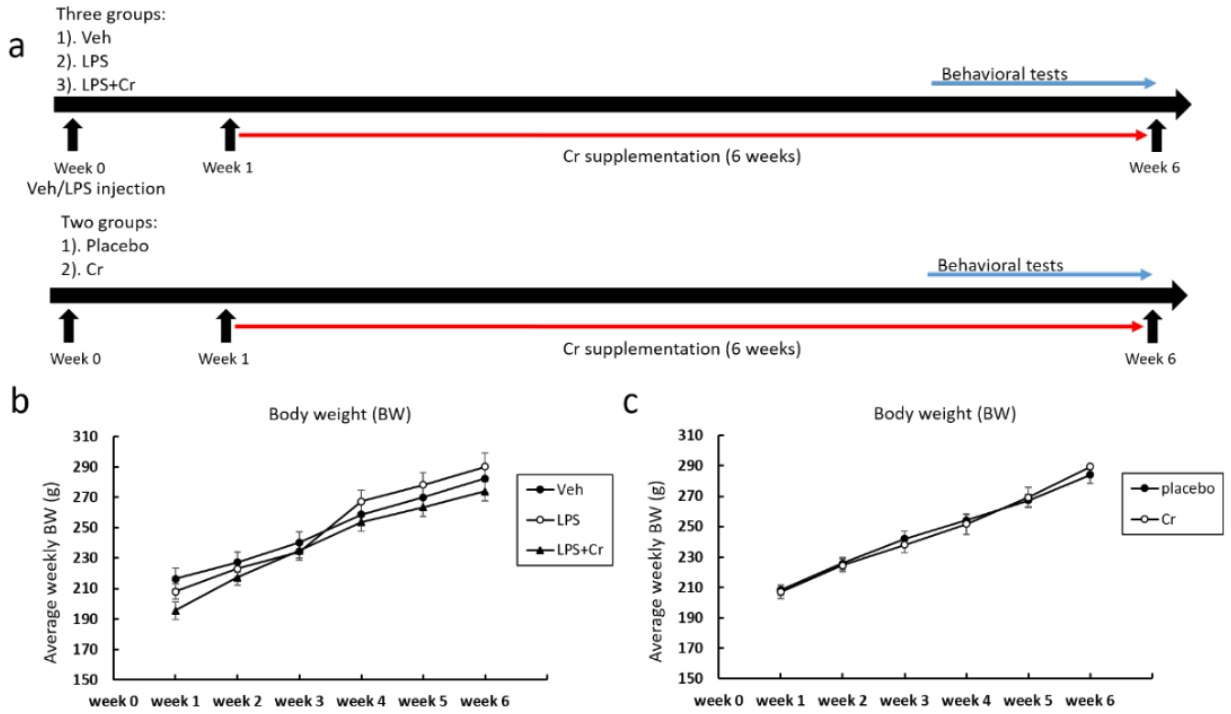


Figure 2.1. Experimental timeline, changes of bodyweight across the experiment. (a) Experimental timeline for the study, where the LPS experiment (experiment 1) has three groups: Veh, LPS and LPS + Cr. All groups underwent either Veh or LPS injection through stereotactic surgery. Creatine (Cr) supplementation or placebo started at week 1 lasting for 6 weeks, and behavioral tests occurred at the last five days of the experiment; Non-LPS (experiment 2) experimental rats were divided into two groups: placebo and Cr group. All rats started Cr or placebo beginning week 1 lasting for 6 weeks, and behavioral tests occurred at the last five days of the experiment. (b,c) Average weekly BW for each week of the study in both LPS (b) and non-LPS (c) experiment. Results are presented as mean ± SEM (n = 12/group).

2.2. Creatine Supplementation

Creatine (Dymatize, Kings Mountain, NC, USA) was administered to animals daily through drinking water, with standard water utilized as placebo. Notably, fresh bottles were prepared daily. To make the study more similar to human supplementation studies, rats were given a “loading” amount of Cr for the first week at a dosage of 1.542 g/kg per day and, for the following weeks, a maintenance dosage of 0.385 g/kg per day. These doses were based on the normalization of body surface area (BSA) between the rat and human species [139] and were equivalent to an 80 kg human consuming 20 g/day Cr for the first week and 5 g/day Cr for following weeks. Preliminary data were used to determine average daily water consumption for calculating daily Cr administration, and water consumption and body weight were monitored and adjusted throughout the study to confirm proper dosage.

2.3. Surgery and Induction of MCI

The MCI model is discussed in greater detail elsewhere [136],[140] and involves intracerebroventricular (i.c.v.) injections of LPS (Sigma, St. Louis, MO, USA). Briefly, rats were anesthetized with 2% isoflurane and positioned into a stereotaxic frame (David Kopf Instruments, Tunjunga, CA, USA). LPS (4.54 μg/μL) or vehicle (sterile saline/10% artificial cerebrospinal fluid) was loaded into a 25 μL Hamilton syringe (Hamilton Co., Renom, NV, USA). To perform the injections, syringes were mounted to an infusion pump (Harvard Apparatus, Holliston, MA, USA), and injectors were positioned into lateral ventricles using the coordinates (in mm relative to Bregma): anteroposterior 0.8, mediolateral ±1.5, and dorsoventral −3.8. Injections performed bilaterally were controlled at a rate of 1 μL/min for a total of 5 min (45.4 μg LPS total per animal) and, following injections, injectors remained in place for another 5 min to ensure that LPS/vehicle was properly diffused. After the successful completion of injections, incisions were closed with tissue adhesive (Vetbond, 3M, Maolewood, MN, USA) and rats were allowed to recover on a

32 °C heating pad until ambulatory. Rats were then returned to their individual home cages for continued monitoring.

2.4. Behavioral Tests

2.4.1. Barnes Maze Test

In order to measure the spatial memory and learning ability of rats, Barnes maze testing was performed. The apparatus for Barnes maze was comprised of a rotating circular gray platform (122 cm in diameter) with 20 holes (each being 10 cm in diameter) evenly distributed. A dark escape box (30 cm in length × 15 cm wide × 13 cm height) was placed beneath one of the 20 holes. The design of the Barnes maze apparatus was based on rodents' aversion to an open field and to allow rodents to learn and memorize the location of the escape box [141]. In the testing room, temperature, sound, and light were controlled throughout the whole experiment. Testing was performed during the rats' light cycle for five consecutive days with two trials per day, which is a standard setup of Barnes maze test protocol for rats [141]. Before the first trial on the first day of testing, each rat was gently placed into the escape box for 2 min to associate the escape box as a safe environment. When each trial of Barnes maze began, rats were first placed in the center of the platform covered by a start box for 30 s and then allowed to freely explore the platform for 5 min. Each trial ended when the rat entered the escape box on its own or the 5 min trial ended. If the rat did not find or enter the escape box at the end of the 5 min trial, it was gently guided into the escape box. Once the rat entered escape box, it was allowed to stay inside for another 30 s in order to reinforce the association between escape box and safe environment. After the completion of each trial, rats were returned back to their home cages. The platform was designed in a fashion to eliminate odor cues between rats, and 70% ethanol was also used to deodorize the platform between rats. During the testing phase, the time cost to locate and step into the escape box (latency) and total amount of errors made (nose pokes into holes without the escape

box underneath) were recorded. AnyMaze tracking software (Stoelting, Wood Dale, IL, USA) was utilized to record Barnes Maze testing data.

2.4.2. Novel Object Recognition Test

To evaluate the recognition memory of rats, the novel object recognition test was performed. The novel object recognition test was based on the innate preference of rodents to explore a new object rather than a familiar one, thus allowing for the testing of memory after initial exposure to an object for familiarization [142]. Testing was comprised by three consequent phases: habituation, familiarization, and testing. During the habituation phase, rats were allowed to freely explore the testing field (60 cm in length × 60 cm width × 46 cm height) for 5 min without any object presented. Familiarization occurred 24 h after habituation where rats were given a maximum 10 min to explore two identical objects, which were set 20 cm away from each other and 5-cm away from one wall. Then, testing took place one hour later at which rats were returned back to the testing field with one of familiar objects replaced by a novel object. Again, a maximum 10 min was given during the testing phase. The experiment was stopped when rats had explored objects for a total of 20 s or when the 10 min time period was over. AnyMaze tracking software was used to record the time spent on exploring either object. Temperature, sound, and light were controlled throughout the whole experiment, and upon the completion of each testing, 70% ethanol was used to eliminate the odor.

2.5. Euthanasia and Tissue Harvesting

Experimental rats were euthanized on next following day of the last day of the behavioral test via carbon dioxide asphyxiation, followed by quick removal of their brains. Dentate gyrus punches that were 2 mm thick and 3 mm in diameter were taken from coronal brain slices by using a brain matrix (Braintree, Braintree, MA, USA). Isolated dentate gyrus punches were then frozen with liquid nitrogen and stored at −80 °C until processing.

2.6. RNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction (RT-PCR)

RNA isolation, cDNA synthesis, and RT-PCR were performed as described before by our laboratory [26]. Briefly, dentate gyrus punches were placed in TRIzol (Invitrogen, Carlsbad, CA, USA) with RNase-free stainless beads and homogenized for 1 min at 25 Hz for three times via the Tissuelyer (Qiagen, Germantown, MD, USA). The TRIzol protocol was then carried out according to manufacturer's instructions to obtain an RNA pellet. RNA pellets were dissolved in RNase-free water for quantification by using the Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA). Prior to cDNA synthesis, RNA was treated with DNase I (Thermo Scientific, Waltham, MA, USA) followed by DNase I inactivation with EDTA for 10 min at 65 °C. Thereafter, DNA-free RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). For RT-PCR, 15 µg of cDNA from each sample was assayed in duplicate by using gene-specific primers (Table 2.1) and SYBR green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). mRNA expression values were presented as 2ΔCT whereby ΔCT = 18S CT-gene of interest CT

Table 2.1. Primers used for RT-PCR.

Gene	Forward (5′→3′)	Reverse (5′→3′)	Accession No.
18S	GCTCGCTCCTCTCCTACTTG	CGATAAATGCACGCGTTCCCC	NR_046237.2
TNF-α	AACACACGAGACGCTGAAGT	TCCAGTGAGTTCCGAAAGCC	NM_012675.3
IL-1β	TGACTTCACCATGGAACCCG	GACCTGACTTGGCAGAGGAC	NM_031512.2

2.7. Immunoblotting

Immunoblotting was performed as previously described [143] to examine select cell signaling and synaptic protein markers in the dentate gyrus after the experimental interventions described above. In short, dentate gyrus tissue was homogenized in Radioimmunoprecipitation (RIPA) buffer [50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 1 × protease + phosphatase inhibitor cocktail] using a Tissuelyser (Qiagen) for 1 min at 25 Hz for three times. The homogenate was then centrifuged at 12,000× g for 10 min, and the supernatant was extracted. Protein concentrations were then determined through the bicinchoninic acid (BCA) assay (Piece Biotechnology, Rockford, IL, USA). Then, 27 µg of protein from each sample was loaded onto 4–15% Criterion TGX gels (Bio-Rad, Hercules, CA, USA), and electrophoresis was ran at 200 V for 1 h. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) and incubated with Ponceau S (Sigma, St Louis, MO, USA) to verify the equal loading among all lanes. After that, 5% nonfat milk in Tris-buffered saline + 0.1%, Tween20 (TBS-T) was used as a blocking agent. Primary antibodies (rabbit polyclonal) for glial fibrillary acidic protein (GFAP) (dilution 1:2000; catalog #12389, Cell Signaling, Danvers, MA, USA), phosphorylated 70kDa ribosomal protein S6 kinase (p-p70S6K) (dilution 1:1000, catalog #9205, Cell Signaling, Danvers, MA, USA), 70kDa ribosomal protein S6 kinase (p70S6K) (dilution 1:1000, catalog #9202, Cell Signaling, Danvers, MA, USA)), phosphorylated mammalian target of rapamycin (p-mTOR) (dilution 1:1000, catalog #2971, Cell Signaling, Danvers, MA, USA), mammalian target of rapamycin (mTOR) (dilution 1:1000, catalog #2972, Cell Signaling, Danvers, MA, USA), postsynaptic density protein-95 (PSD-95) (dilution 1:1000, catalog #3409, Cell Signaling, Danvers, MA, USA), Synapsin (dilution 1:1000, catalog #2312, Cell Signaling, Danvers, MA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (dilution 1:20,000, catalog #5174, Cell Signaling, Danvers, MA, USA) were diluted in TBS-T with 5% BSA and applied to membranes overnight at 4 °C. The following day, horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution 1:1000; Cell Signaling) diluted in TBS-T with 5% non-fat milk were

applied to membranes for one hour at room temperature. Next, enhanced chemiluminescence (ECL) substrate solution (Pierce Biotechnology, Biotechnology, Rockford, IL, USA) was applied to membranes for two minutes. A gel documentation system (Kodak 4000R imager and Molecular Imagery Software; Kodak Molecular Imaging Systems, New Haven, CT, USA) was then used to capture digital images of each membrane. Associated software was used to determine band densities, and these values were normalized to the values of GAPDH to obtain final expression values.

2.8. Statistical Analysis

All analytical procedures were performed using SigmaPlot, version 14.0 (Systat Software, Inc., Chicago, IL, USA). Two-way ANOVAs [Trials (Trial1-10) × Treatment (Veh vs. LPS vs. LPS + Cr)] were performed on dependent variables obtained from the LPS experiment. Likewise, two-way ANOVAs [Trials (Trial1-10) × Treatment (Placebo vs. Cr)] were performed on dependent variables from the non-LPS experiment. Mauchly's tests of sphericity were performed on latency time and errors, and both tests yield p value < 0.05. Thus, Greenhouse–Geisser corrections were applied when reporting the trial and interaction p values. Holm–Šídák post hoc analyses were applied for multiple comparisons. Statistical analyses on mRNA expression, band densities, and novel object recognition test were conducted using one-way ANOVAs followed by Holm–Šídák post hoc analyses for the LPS experiment. Student's t tests were used to assess group differences for the non-LPS experiment. All values are expressed as the mean ± SEM, and statistical significance was established as $p \leq 0.05$ for all analyses.

Results

3.1. Experiment 1

In order to verify LPS-induced inflammation in the dentate gyrus, we performed RT-PCR to examine pro-inflammatory genes and western blot for GFAP protein expression (Figure 2.2). For TNF-α mRNA (Figure 2.2a), Veh vs. LPS was 1.00 ± 0.08 vs. 4.85 ± 0.83-fold, respectively, and

Veh vs. LPS + Cr was 1.00 ± 0.08 vs. 5.75 ± 0.86-fold, respectively ($p < 0.0001$ for both comparisons). For IL-1β mRNA (Figure 2.2a), Veh vs. LPS was 1.00 ± 0.09 vs. 32.81 ± 6.01-fold, respectively, and Veh vs. LPS + Cr was 1.00 ± 0.09 vs. 40.33 ± 5.75-fold, respectively ($p < 0.0001$ for both comparisons). For GFAP protein (Figure 2.2b), Veh vs. LPS was 1.00 ± 0.11 vs. 1.59 ± 0.13-fold, respectively ($p = 0.004$), and Veh vs. LPS + Cr was 1.00 ± 0.11 vs. 1.76 ± 0.25-fold, respectively ($p = 0.006$). There was no significant difference observed between LPS and LPS + Cr group for the assayed pro-inflammatory mRNA markers ($p = 0.244$ for TNF-α mRNA, $p = 0.408$ for IL-1β mRNA) or GFAP protein levels ($p = 0.743$). Overall, these data suggest that LPS injection was sufficient to induce neuro-inflammation in the dentate gyrus.

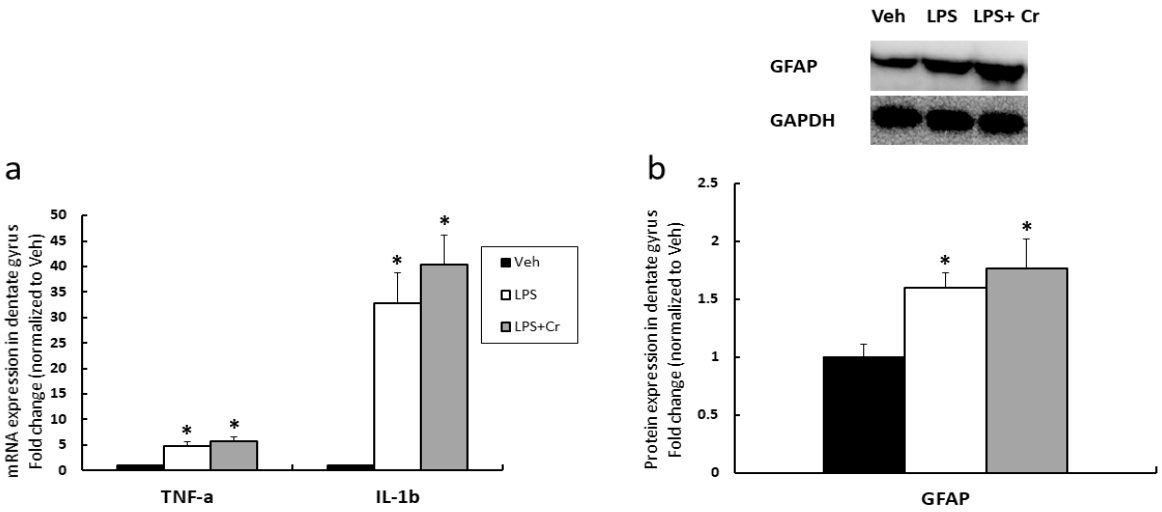
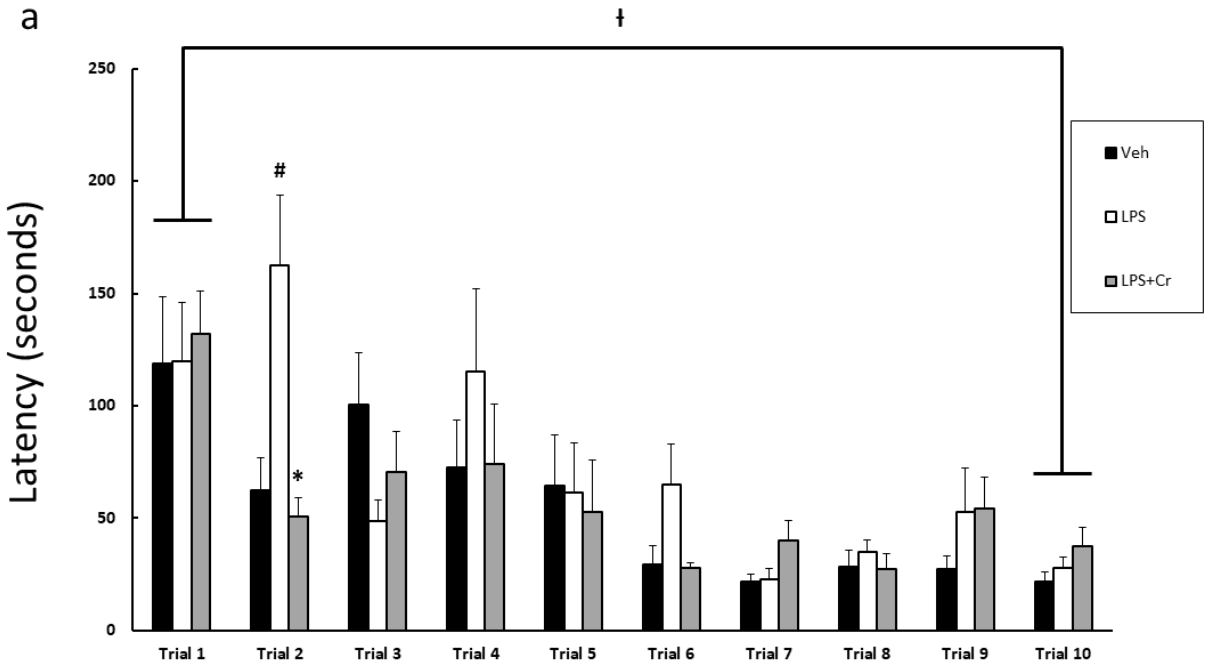


Figure 2.2. Proinflammatory markers and, marker for the activation of astrocytes in the dentate gyrus of Veh, LPS, and LPS + Cr groups. (a) Proinflammatory TNF-α, IL-1β mRNA expression level in the dentate gyrus. (b) GFAP protein expression in the dentate gyrus. Results are presented as mean ± SEM (n = 8). * $p < 0.05$ significantly different from Veh group.

The effects of Cr supplementation on spatial memory deficit induced by LPS is presented in Figure 2.3a,b. Significant interactions existed between group and trials for both latency ($F(9,152) = 2.105$, $p = 0.030$) and errors ($F(10,170) = 1.861$, $p = 0.049$) during the Barnes Maze testing. During trial 2, which followed the initial trial (trial 1), the LPS group revealed a higher average latency time (in seconds) of locating the escape box Figure 2.3a, compared with both Veh and LPS + Cr group; Veh vs. LPS was 62.5 ± 14.3 vs. 162.4 ± 31.2, respectively ($p = 0.0001$); LPS vs. LPS + Cr was 162.4 ± 31.2 vs. 50.7 ± 8.5, respectively ($p < 0.0001$). No significant difference between Veh and LPS + Cr ($p = 0.644$) suggests that LPS induced a spatial memory deficit shown by a slower rate of learning Barnes maze test in contrast to the Veh, and this deficit was attenuated with Cr supplementation. The LPS group also exhibited a higher number of errors made before locating the escape box at the trial 2, compared with both Veh and LPS + Cr group (Figure 2.3b), Veh vs. LPS was 4.3 ± 0.9 vs. 10.8 ± 2.3, ($p = 0.0003$), and LPS vs. LPS + Cr was 10.8 ± 2.3 vs. 2.9 ± 0.6, ($p < 0.0001$). Similar to latency, there was not a significant effect between Veh and LPS + Cr group ($p = 0.412$). Interestingly, all groups significantly decreased their latency and number of errors between trial 1 to trial 10 ($p < 0.0001$, Figure 2.3a,b), indicating intact spatial memory learning in all groups. Taken together, these data suggest that Cr was able to ameliorate the spatial memory deficit induced by LPS by undergoing the spatial learning at a faster rate. Data in Figure 3c. show results from the novel object recognition test. In contrast to the Veh and LPS + Cr group, the LPS group showed a significantly decreased preference (in percent) to the novel object (Figure 3c), Veh vs. LPS was 0.65 ± 0.06 vs. 0.44 ± 0.05 ($p = 0.015$), and LPS vs. LPS + Cr was 0.44 ± 0.05 vs. 0.73 ± 0.04, respectively ($p = 0.002$). There was no significant effect between Veh and LPS + Cr detected ($p = 0.260$). Again, these data suggest that Cr supplementation was able to ameliorate the recognition deficit induced by LPS.


a
Latency (seconds)
250
200
150
100
50
0
#
*
†
Veh
LPS
LPS+Cr
Trial 1
Trial 2
Trial 3
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Trial 5
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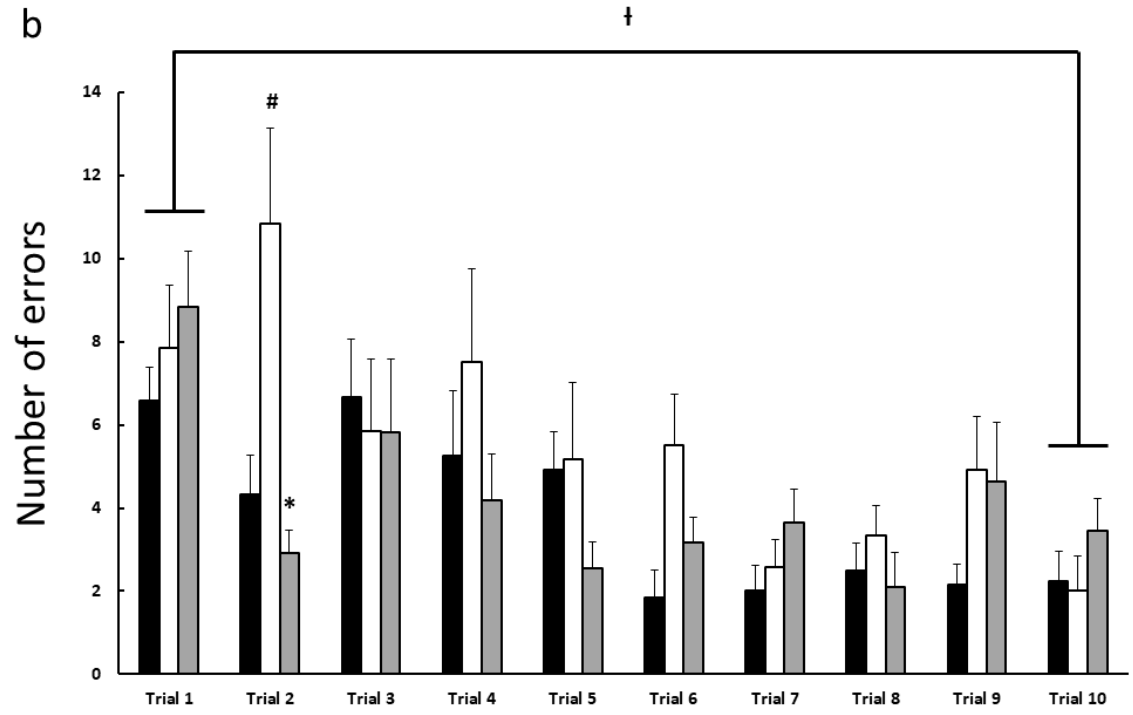
Trial 9
Trial 10


b
Number of errors
14
12
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8
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Trial 1
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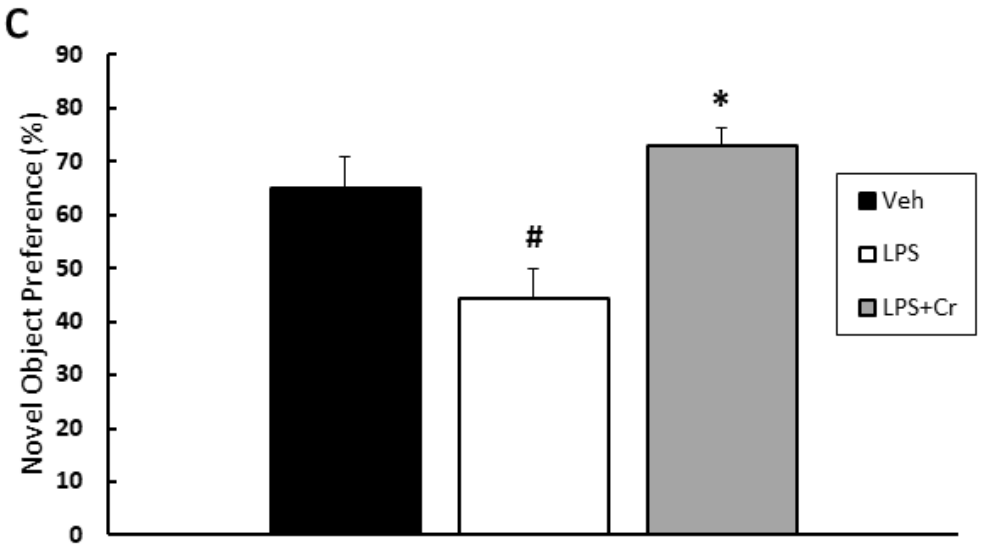


Figure 2.3. Effects of 6 wks of creatine (Cr) on spatial memory and object recognition memory deficits induced by LPS. **a** Average latency time (seconds) to locate the escape box during five days of Barnes maze testing (2 trials/day, 10 trials in total). **b** Number of errors made before locating the escape box during five days of Barnes maze testing (2 trials/day, 10 trials in total). **c** Preference of novel object (%) during the Novelty object recognition test. Results are presented as mean ± SEM (n=12). ł $p < 0.05$ significantly different from trial 1 for both Veh, LPS and LPS+Cr groups. # $p<0.05$ significantly different from Veh group. * $p < 0.05$ significantly different from LPS group.

To detect the molecular signaling changes in the dentate gyrus induced by 6 weeks of Cr supplementation, we next examined the dentate gyrus for mTORC1 signaling markers as well as select synaptic proteins (Figure 2.4). Both the Veh and LPS groups presented the similar expression patterns of all assayed mTORC1 signaling proteins. However, when adding Cr to LPS, LPS + Cr group had significantly higher expression patterns versus the Veh and LPS groups. Pathway increases were (a) 1.93 ± 0.33-fold to Veh for mTOR protein phosphorylation (p = 0.025 to Veh, p = 0.026 to LPS); (b) 1.73 ± 0.16-fold to Veh for p70S6K protein phosphorylation (p = 0.003 to Veh, p = 0.002 to LPS); (c). 1.22 ± 0.07-fold to Veh for PSD-95 protein (p = 0.033 to Veh, p = 0.015 to LPS); and (d) 1.22 ± 0.05-fold to Veh for synapsin protein (p = 0.026 to Veh, p = 0.008 to LPS) (Figure 2.4). Of note, there was no significant difference between groups for total proteins (Figure 2.4a). Taken together, these data suggest that 6 weeks of Cr supplementation increased mTORC1 signaling and the expression of select synaptic proteins in the dentate gyrus.

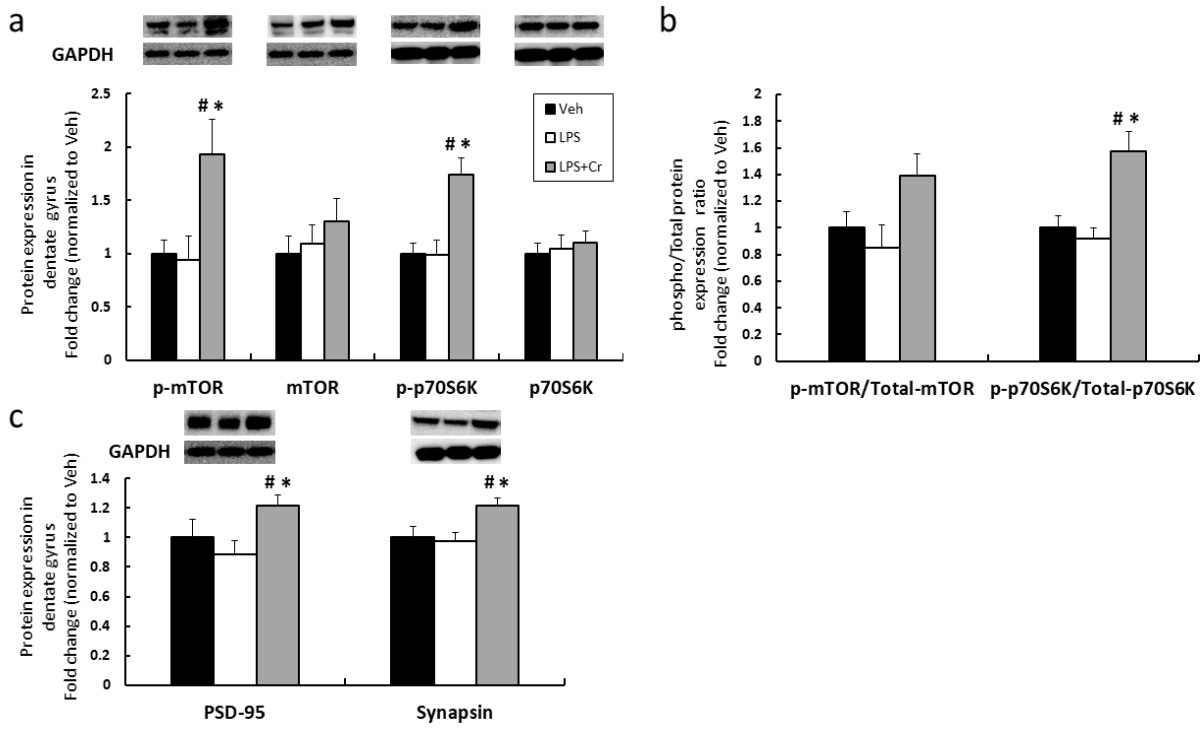
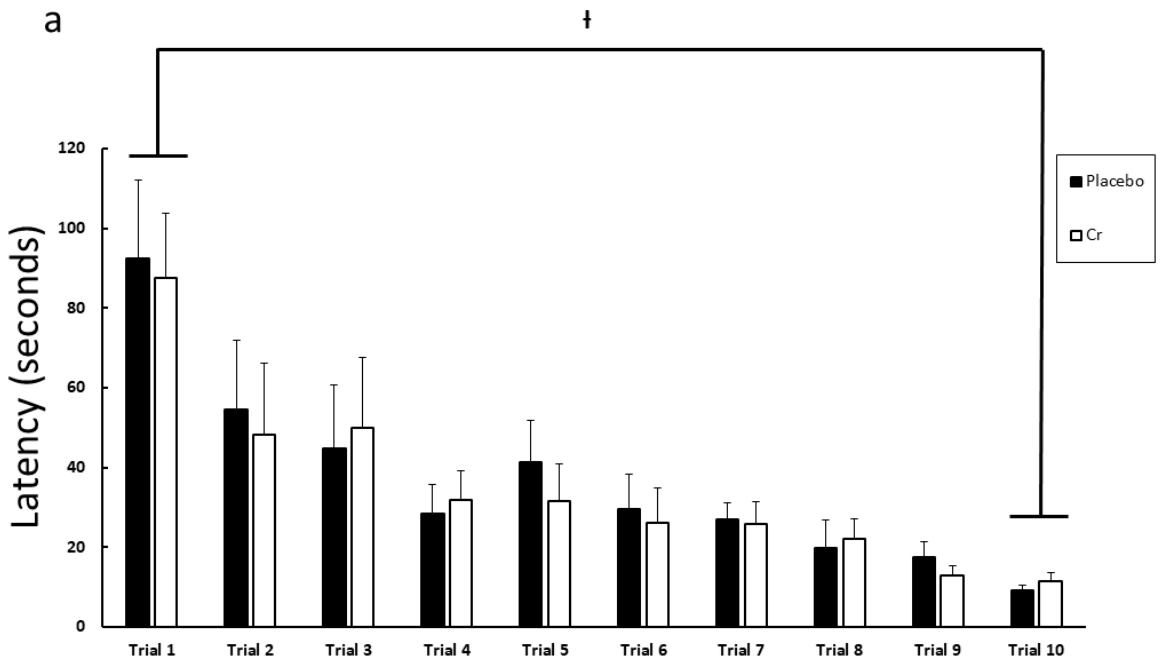


Figure 2.4. Effects of 6 wks of Cr supplementation on molecular signaling in dentate gyrus following LPS. **a** Proteins expression level of mTORC1 signaling pathway. **b** Ratio of phosphorylation /total mTORC1 signaling proteins. **c** Synaptic proteins downstream to the mTORC1 signaling. Results are presented as mean ± SEM (n=8). # $p < 0.05$ significantly different from Veh group. * $p < 0.05$ significantly different from LPS group.

3.2. Experiment 2

Experiment 2 was performed without the surgical injection of LPS in order to determine if Cr supplementation alone affected some of the aforementioned performance outcomes and molecular variables. The Barnes maze and novel object recognition testing were performed for both placebo (control) and Cr supplementation (Cr) groups (Figure 2.5). For the Barnes maze testing, two-way ANOVA revealed that there was not a statistically significant interaction between groups and trials for both latency and errors (Figure 2.5a,b). However, a statistically significant difference existed between trial 1 and trial 10 within the placebo and Cr groups, with $p < 0.0001$ for both latency and errors (Figure 2a,b). For the novel object recognition test, there was no significant group difference ($p = 0.740$) observed (Figure 5c), suggesting that Cr supplementation did not affect test outcomes. Taken altogether, these data suggest that 6 weeks of Cr supplementation was not able to further enhance cognition under the condition without LPS.


a
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Latency (seconds)
120
100
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Placebo
Cr
Trial 1
Trial 2
Trial 3
Trial 4
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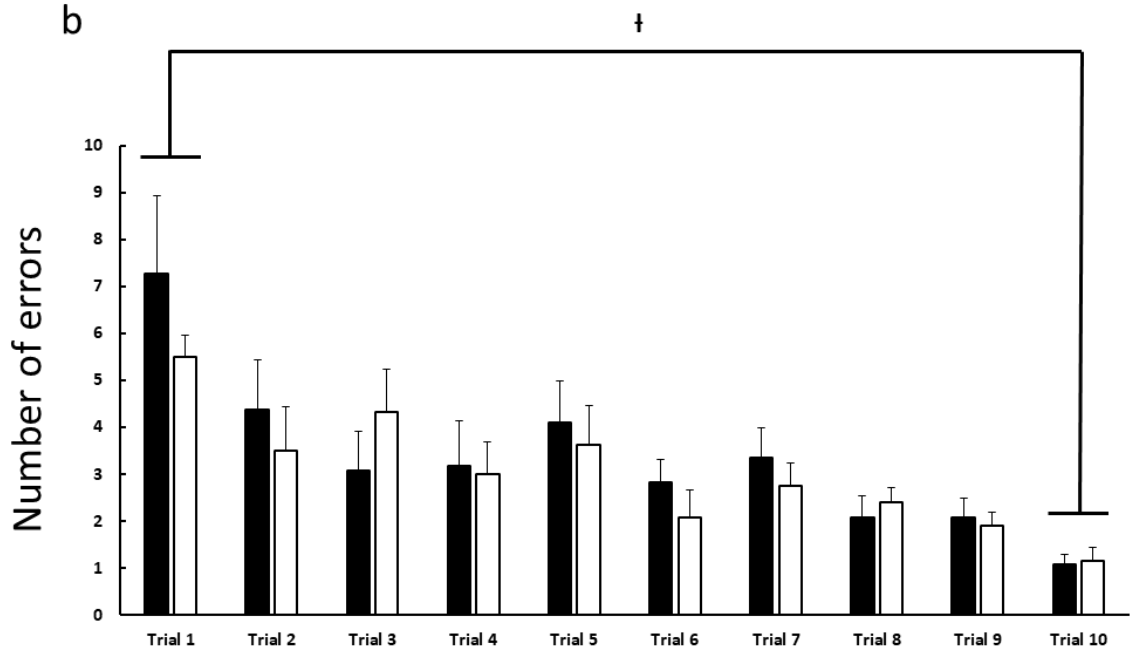
Trial 6
Trial 7
Trial 8
Trial 9
Trial 10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Number of errors
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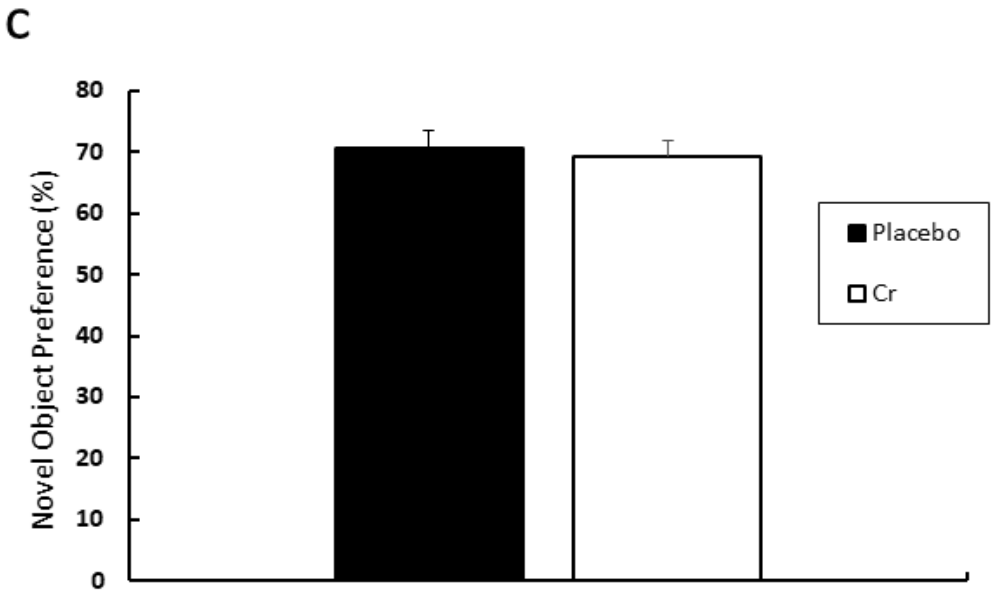

Figure 2.5. Effects of 6 wks of Cr supplementation on spatial memory and recognition memory without LPS. **a** Average latency time (seconds) to locate the escape box during five days of Barnes maze testing (2 trials/day, 10 trials in total). **b** Number of errors made before locating the escape box during five days of Barnes maze testing (2 trials/day, 10 trials in total). **c** Preference of novel object (%) during the Novel object recognition test. Results are presented as mean ± SEM (n=12). ł $p < 0.05$ significantly different from trial 1 for both placebo and Cr groups.

Western blotting was performed to examine the protein expression mTORC1 signaling markers and downstream synaptic proteins in the dentate gyrus between the placebo and Cr groups (Figure 2.6). In contrast to the placebo group, Cr group revealed significantly increased mTOR phosphorylation (placebo vs. Cr was 1.00 ± 0.15 vs. 1.87 ± 0.15 fold, respectively, p = 0.003), p70S6K phosphorylation (placebo vs. Cr was 1.00 ± 0.18 vs. 1.76 ± 0.14 fold, respectively, p = 0.006) (Figure 2.6a), PSD-95 (placebo vs. Cr was 1.00 ± 0.13 vs. 1.45 ± 0.18 fold, respectively, p = 0.039), synapsin (placebo vs. Cr was 1.00 ± 0.23 vs. 1.49 ± 0.10 fold, respectively, p = 0.030) (Figure 2.6c). There were no significant changes observed for total proteins (p = 0.692 for mTOR, p = 0.553 for p70S6K) (Figure 2.6c). Additionally, the ratio between p-mTOR/mTOR was significantly higher in Cr group in contrast to placebo group (placebo vs. Cr was 1.00 ± 0.26 vs. 1.92 ± 0.21 fold, respectively, p = 0.008) (Figure 2.6b). Again, these data suggest that Cr was able to elevate the mTORC1 signaling and the expression of synaptic proteins.

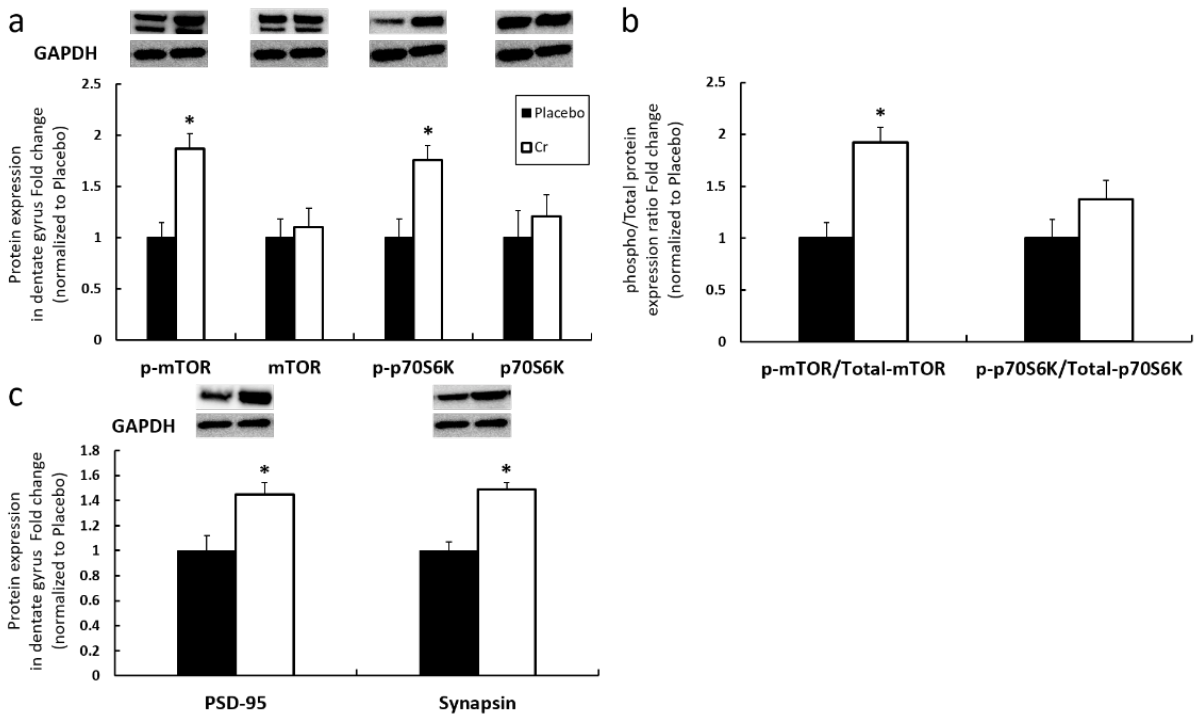

Figure 2.6. Effects of 6 wks of Cr supplementation on molecular signaling in dentate gyrus without LPS. **a** Proteins expression level of mTORC1 signaling pathway targets. **b** Ratio of phosphorylation /total mTORC1 signaling proteins. **c** synaptic proteins downstream of mTORC1 signaling. Results are presented as mean ± SEM (n=8). * $p < 0.05$ significantly different from placebo group

Discussion

Several studies have suggested that creatine (Cr) is a candidate for enhancing cognition [131],[132]. Herein, we tested the hypothesis that oral administration of Cr would ameliorate cognitive deficits induced by LPS in female rats. Our data suggests that 6 weeks of Cr supplementation ameliorated spatial and recognition memory deficits in the presence of LPS. Additionally, we found that ameliorated cognition was concurrent with an increase in mTORC1 signaling within the dentate gyrus. We have also identified that spatial and recognition memory were not further enhanced by Cr supplementation in the absence of LPS, even though mTORC1 signaling was still elevated within the dentate gyrus.

In experiment 1, we utilized LPS to mimic mild cognitive impairment (MCI), given that this model is known to cause neuro-inflammation and cognitive dysfunction [68],[144],[145]. To verify the presence of neuro-inflammation within the dentate gyrus of LPS injected rats, proinflammatory transcripts were quantified through RT-PCR. Similar to other findings [68], our results confirmed that TNF-α and IL-1β mRNAs in dentate gyrus were significantly increased by i.c.v. LPS injections relative to the Veh group. However, Cr supplementation failed to decrease the increased expression of inflammatory transcripts induced by LPS. Notably, this finding does not agree with prior evidence suggesting Cr supplementation has anti-inflammatory effects [82]. The mechanism by which LPS induces pro-inflammatory cytokine production in the brain is through NF-κB signaling activation, caused by toll-like-receptor 4 (TLR-4) activation in microglia [68]. However, we did not perform experiments to examine this signaling in microglia. Thus, this needs to be further investigated. We also assayed glial fibrillary acidic protein (GFAP) levels as a marker for reactive astrocytes [146]. Consistent to our proinflammatory mRNA data, GFAP protein was elevated in all LPS injected groups, regardless of the Cr supplementation. Proinflammatory cytokine (e.g. TNF-α) secretion following LPS are known to trigger reactive astrocytes [147]. Based on our data, it is possible to speculate that activated astrocytes lead to amplified neuro-

inflammation and cause functional changes within the neuro-environment [148]. However, again, Cr did not seem to mitigate this effect. Overall, our data suggest that LPS was sufficient to induce the neuro-inflammation for studying cognitive impairment in the current study; however, Cr seemingly ameliorates cognitive impairment through non-inflammatory pathways.

The Barnes maze and novelty object recognition tests were performed to evaluate the cognitive effects of Cr in the presence of LPS, as they strongly correlate with hippocampus-dependent memory [141],[142]. According to our behavioral data, Cr supplementation ameliorated the impaired acquisition of spatial learning task compared to the LPS only group, shown by its shortened latency and less errors made to reach the escape box at trial 2 of the training regime. Additionally, all groups of rats in the Barnes maze test, including the LPS group, improved their spatial memory from the first trial of their training, implying that the enhanced spatial memory caused by Cr could have resulted from an improved learning ability in contrast to the LPS group. In the object recognition test, Cr supplementation ameliorated recognition memory compared to the LPS only group shown by the increased amount of time taken to explore the new object, which provides a reliable index of recognition memory [142]. This finding agrees with both animal and human studies regarding the ability of Cr supplementation to affect cognition [83],[149],[150]. However, evidence regarding whether Cr is sufficient to ameliorate cognitive impairment in disease models are relatively lacking. Therefore, our study provides preliminary data in this area, and warrants future research.

Our finding of upregulated mTORC1 signaling concurrent with ameliorated cognition caused by Cr supplementation is in line with the notion that mTORC1 signaling plays an essential role in regulating learning and memory [101]. Numerous behavioral studies have reported that the selective inhibition of mTORC1 by rapamycin impairs learning and memory formation [117],[119],[151]. Mechanistically, studies indicate the involvement of mTORC1 signaling in regulating long-term potentiation in the hippocampus [120],[123], an event that links modifications

in synaptic strength to long-lasting behavioral changes. Thus, the regulation of synaptic plasticity via mTORC1 signaling may underlie hippocampus-dependent learning and memory formation. The synapse-associated proteins, particularly the pre-synaptic synapsin and post-synaptic PSD-95, promote synaptic plasticity and neuronal excitability [95],[152]. In line with our finding of increased mTORC1 signaling, we also found upregulation of synapsin and PSD-95 proteins expression with Cr supplementation. Overall, our above findings suggest that mTORC1 signaling may be critical for Cr to exert its effects to overcome the LPS-induced cognitive impairment by promoting synaptic function and plasticity. The upstream mechanism that facilitates activation of mTORC1 via Cr supplementation was not investigated in this study. However, it is possible that Cr upregulates mTORC1 signaling by stimulating insulin-like growth factor (IGF-1) secretion, which binds to the IGF-1 receptor and triggers downstream phosphatidylinositol-3-kinase (PI3K)-AKT signaling activation leading to the phosphorylation of mTOR [153],[154]. Noticeably, there is a conflicting role regarding the mTORC1 signaling in mediating cognition. In the aging brain, pathological hyperactivation of mTORC1 leads to the accumulation of β-amyloid peptide (Aβ) and dysregulated autophagy, fostering the cognitive impairment that is associated with neurodegenerative diseases [155]. Therefore, precise regulation of mTORC1 signaling may be required for different stages of life or diseases. Taken together, ameliorated cognitive impairment via Cr supplementation with a concomitant upregulation of mTORC1 signaling within the dentate gyrus suggests a potential link. Further research will be required to investigate whether mTORC1 signaling is required for Cr to fully exert its cognitive effects.

Experiment 2 examined whether Cr enhances spatial memory and recognition memory in the absence of LPS. Our data suggests that Cr supplementation for 6 wks failed to enhance both spatial and recognition memory compared to placebo group. This result conflicts with other findings in which 6 wks of Cr supplementation was reported to enhance spatial learning and memory in 7-months-old wild-type (WT) mice as assessed with the Morris water maze (MWM)

test [89]. Additionally, our findings do not agree with data suggesting Cr supplementation enhances recognition memory in healthy aged mice [150]. However, tests performed by those studies were carried out at an older stage of life compared to the current study. It is possible that those conflicting outcomes are a result of different age of animals being utilized. In our study, animals supplemented with Cr without LPS injections may have a “ceiling effect” for cognition due to their young age that prevent them from being further affected with Cr supplementation. As supported by other studies, Cr may only improve cognition when cognitive function is impaired [84]. Interestingly, in the current study, Cr supplementation still augments mTORC1 signaling within the dentate gyrus, even though the cognitive function was not altered.

A limitation of the present study is that the estrus cycle was not controlled throughout the experiment, and this has been shown to influence cognition [156]. Future studies are also needed to investigate whether mTORC1 signaling is required for Cr to fully exert its effects in the presence of LPS. Additionally, an optimal Cr dosing protocol for improving cognition needs to be studied given that heterogenous dosages exist in the literature. A key limitation to our cognitive tests is that, although Barnes maze and novel object recognition tests were performed to evaluate spatial and recognition memory, there are still other areas of cognitive functioning remained to be tested in order to gain a more comprehensive understanding of the cognitive effects of Cr supplementation.

In summary, we show that 6 wks of Cr supplementation ameliorates cognitive deficits induced by LPS in female rats, potentially through the activation of mTORC1 signaling and the upregulation of synaptic proteins within the dentate gyrus. While 6 wks of Cr supplementation does not further enhance cognitive function in the absence of LPS, there is a concurrent upregulation of mTORC1 signaling and synaptic proteins within the dentate gyrus.

Chapter 3: mTORC1 signaling is required for Cr supplementation to fully ameliorate the cognitive deficits in a neuroinflammatory rat model

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*Corresponding Author

Abstract:

Creatine (Cr) is an organic compound that favors to supply energy for sudden energy demand. In recent years, Cr has emerged as a potential therapeutic strategy to mitigate cognitive impairment, however, its underlying mechanisms remain relatively lacking. In our previous study, Cr supplementation ameliorated cognitive deficiency in a neuroinflammatory rat model, which was associated with an upregulated mTORC1 signaling activity within dentate gyrus. Consequently, the present study attempted to examine the involvement of mTORC1 signaling behind the Cr supplementation in a model of neuroinflammation elicited by lipopolysaccharides (lps). In order to induce neuroinflammation and cognitive impairment, lps (45.4 ug/animal) was injected into lateral ventricles. Cr supplementation and rapamycin (a selective inhibitor for mTORC1 signaling), were administered for 6 weeks. Our data showed that Cr supplementation significantly ameliorated neuroinflammation induced cognitive deficiency, an effect that was partially abolished by rapamycin. Further molecular analyses revealed that mTORC1 signaling activity and its downstream synaptic proteins (e.g., PSD95, synapsin) were enhanced following Cr supplementation in dentate gyrus, but not in medial prefrontal cortex. While rapamycin significantly downregulated mTORC1 signaling activity, that was enhanced by Cr supplementation in dentate gyrus. Additionally, we also showed that Cr treatment for 12- and 24-hours were sufficient to directly upregulate mTORC1 signaling activity within PC12 cells. Altogether, our results indicate that a full presence of mTORC1 signaling is required for Cr supplementation to fully ameliorate the cognitive deficiency in a rat model of neuroinflammation. Although preliminary, the current study provides potential basis of neurocognitive and neuro-molecular evidence regarding the use of Cr as a therapeutic strategy for future clinical trials.

1. Introduction

Dementia is by far one of the most devastating syndromes that include a wide spectrum of clinical manifestations, such as loss of memory, impairments in learning and thinking [157]. However, dementia caused by neurodegenerative diseases, for example, Alzheimer's disease and vascular dementia, are irreversible in nature [158]–[160]. Given the overall population worldwide with dementia is predicted to be approximately 75 million by 2030 [161], it is urgently required to develop early therapeutics to overcome this pandemic rate of disease. A transitional state namely mild cognitive impairment (MCI) representing a stage of cognitive function between normal aging and dementia existed, at which it was supported as a therapeutic window for early treatment [162],[163]. Previous studies [136],[164],[165], including our own, utilized animal models to mimic MCI have discovered promising future interventions for ameliorating cognitive impairment. As such, an in-depth understanding of underlying neuro-molecular mechanisms is required for future clinical trials.

Creatine (Cr), an ergogenic aid for professional athletes and bodybuilders, was proved to be cognitively beneficial under various pathologies and disorders [81]–[83],[86],[89],[90]. In our previous publication [165], 6 weeks of Cr supplementation was found to significantly ameliorate the spatial and recognition memory deficits caused by neuroinflammation in female Wistar rats. Additionally, such cognitive effects of Cr were also found to associate with a concurrent upregulation of mammalian target of rapamycin complex 1 (mTORC1) signaling in hippocampal dentate gyrus.

Within cells, mTORC1 signaling plays a crucial role in integrating nutritional and environmental stimuli to regulate cellular metabolism, growth, proliferation, and survival, primarily through regulating protein homeostasis [104],[105],[113]. Upon activation, mTORC1 phosphorylates downstream substrates, 70-kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP), which are critical cellular effectors for protein

biosynthesis [100]. In past decades, tremendous new discoveries regarding mTORC1 signaling and neurocognition were made by using rapamycin, a potent and selective inhibitor of mTORC1 protein complex [166],[167]. Through utilizing rapamycin, lots of studies demonstrated that an intact mTORC1 signaling activity is necessary for optimal learning and formation of memory in many brain regions that relate to cognitive processing [116],[117],[119],[120],[168]. While this neuro-cognitive control of mTORC1 signaling was suggested to be, at least in part, through the modulation of synaptic plasticity (e.g., long term potentiation) at preexisting synapses [101],[115],[120],[169], where modifications of the strength of synaptic transmission occur. Meanwhile, many forms of synaptic plasticity require de novo protein synthesis that relies on the activity of protein translational machinery regulated by molecular signaling transduction. Given the crucial role of mTORC1 signaling in controlling translational machinery, prevailing evidence pointed out that mTORC1 signaling is an essential molecular transducer that couples protein synthesis to synaptic events during the plastic behavioral changes, such as learning and memory [101]. Notably, such mTORC1-dependent protein translation involves regulation of pre-synaptic synapsin and post-synaptic PSD-95, which are synapse-associated proteins critical for mediating neuronal excitability and synaptic plasticity [95],[152],[170].

Based upon our previous findings [165], the present study aimed to test the role of mTORC1 signaling during the amelioration of cognitive impairment caused by Cr supplementation. For doing this, intracerebroventricular (i.c.v.) injections of lps was performed in female Wistar rats to elicit persistent neuroinflammation to mimic the pathology of MCI, which was reported to cause robust deficiency in learning and memory [67],[68],[70]. Following modeling, rapamycin was continuously infused into brain during the entire period of Cr supplementation. Collectively, based on previous observations, we hypothesized that mTORC1 signaling is required for Cr supplementation to fully exert its neuro-cognitive effects to ameliorate cognitive impairment

induced by neuroinflammation. Additionally, at the end of study, we also tested whether Cr could directly activate mTORC1 signaling within neuronal cells when only neuronal cell type presents.

2. Materials and Methods

2.1. Animals and Experimental Design

Experimental protocols used in the present study were proved by the University of Missouri Animal Care and Use Committee (protocol code: 10111, date of approval: 9th August). Female Wistar rats (~150 g, 42 days of age) used in this study were purchased from Charles River (Charles River Laboratories, Wilmington, MA). All experimental animals were housed individually upon their arrival under controlled conditions (12-h: 12-h light/dark cycle, 24°C) with food (Formulab Diet 5008, Purina, St. Louis, MO) and water provided ad libitum. One week period of acclimation to the lab environment was given to those animals before the start of experiment. At 7 weeks of age (49 days of age), all animals were randomly divided into 4 experimental groups to determine the role of mTORC1 signaling for creatine (Cr) to ameliorate the cognitive impairment in the context of neuroinflammation induced by intracerebroventricular (i.c.v.) injections of lipopolysaccharide (lps). Experimental groups (n=10 animals/group) include a). lps injected (lps+placebo+veh), b). lps injected with 6 weeks of Cr supplementation (lps+Cr+veh), c). lps injected with 6 weeks of Cr supplementation and rapamycin (Rapa) treatment (lps+Cr+Rapa), d). lps injected with 6 weeks of Rapa treatment (lps+placebo+Rapa). Additionally, we have added a non-lps (n=10) injected group to verify the effects and validity of lps-induced neuroinflammation model. The authors complied with the National Institutes of Health's *Guide for the Care and Use of Laboratory animal*s and thus, efforts were made to minimize the suffering and the total number of experimental animals.

2.2. Stereotaxic surgery

2.2.1. Induction of neuroinflammation

The neuroinflammation model induced by i.c.v. injections of lps (Sigma, St. Louis, MO) is described in greater details elsewhere [171]. Briefly, on surgery day, rats at 7 weeks of age (~150 g) were anesthetized with 2% isoflurane through nasal inhalation. After shaving of their heads, animals were positioned into a stereotaxic frame (David Kopf Instruments, Tunjunga, CA) and a small incision (~ 1.5 cm) was made over the midline of the skull to expose bregma. After that, two small holes were drilled through the skull above the lateral ventricles. To perform the injections, a 25-µl Hamilton syringe (Hamilton Co., Renom, NV) containing lps (4.54 µg/ul) dissolved in sterile saline was mounted to an infusion pump (Harvard Apparatus, Holliston, MA, USA). Injectors of syringes then were slowly positioned into lateral ventricles by using the coordinates (relative to Bregama): anteroposterior (AP) +0.8 mm, mediolateral (ML) ±1.5 mm, and dorsoventral (DV) -3.8 mm. Importantly, injections were performed bilaterally for each animal at a controlled rate of 1 ul/min for a total of 5 min, therefore, a total amount of 45.4 ug of lps was delivered into ventricles for each animal. Following injections, injectors were remained within the ventricles for additional 3 min to ensure that lps was thoroughly diffused.

2.2.2. Pharmacological inhibition of mTORC1 signaling and osmotic pump implantation

In order to pharmacologically inhibit the mTORC1 signaling pathway for a prolonged period (6 weeks), osmotic pumps were used in the present study for continuously delivering rapamycin, a selective inhibitor of mTORC1 signaling [172]. Osmotic pumps (Model 2006, Alzet, Cupertino, CA) were preloaded with either rapamycin (Enzo Life Sciences, Farmingdale, NY) or veh. Rapamycin solution was prepared by dissolving rapamycin in a 40% DMSO/ 60% PEG400 solution to make the final concentration at 10mM. Then, osmotic pumps were coupled to pre-cut brain infusion cannula (3.8 mm, P1 Technologies, Roanoke, VA) through polyethylene (PE)- 60 tubing (Braintree, MA). Prefilled osmotic pumps were then primed in sterile 0.9% saline at 37°C for 60 hours prior to the implantation, in accordance with the manufacturer's instructions (Alzet, Cupertino, CA). Immediately following the lps injections, a blunt scissor was used to create a

subcutaneous pocket from the base of the neck to the scapulae area to receive osmotic pumps, and infusion cannula were inserted into one side of lateral ventricles. Dental cement and skull screws were used to secure infusion cannula in place. After that, skin incisions were sealed with tissue adhesive (Vetbond, 3M, Maolewood, MN, USA) and topical Neosporin was applied around the surgical incision. Animals were then allowed to recover on a 32°C heating pad until ambulatory and, finally, returned to their home cages.

2.3 Creatine supplementation

Creatine (Dymatize, Kings Mountain, NC, USA) was prepared and given to animals daily through drinking water, with standard drinking water used as placebo. Notably, to make the experiment more translatable to human conditions and similar to human supplementation studies, doses were converted from human to rat species based on the normalization of body surface area (BSA) through the following formula: [animal dose (mg/kg) = human dose (mg/kg) * (human K_m / animal K_m)], where K_m values [K_m = body weight (kg) / BSA (m^2)] are based on average BSA calculations for a given species [173]. Therefore, a “loading” dosage of Cr (1.542 g/kg/day) for the first week and a maintenance dosage of Cr (0.385 g/kg/day) for following 5 weeks were used as our supplementation protocol in the present study, which were equivalent to an 80 kg human consuming 20 g/day Cr for the first week and 5 g/day for following 5 weeks. In addition, a preliminary test was done to estimate the daily water consumption level for preparing proper amount of Cr supplementation, water consumption level and body weight were monitored weekly and Cr amount was adjusted accordingly throughout the study.

2.4. Behavior tests

2.4.1. Barnes Maze Test

Barnes maze test was performed at the end of the present study to assess the spatial memory, learning and cognitive flexibility. The apparatus built for Barnes maze was comprised of a 360°

rotating circular gray platform (122 cm in diameter) with 20 evenly distributed holes, which were identical to each other in their shape and size. A black escape box (30 cm in length X 15 cm wide X 13 cm height) was placed below the surface of the platform under one of those 20 holes. Additionally, a bright LED light was built above the maze to illuminate the platform to serve as an aversive stimulation. Within the behavioral testing room, light, sound and temperature were controlled throughout the study to keep consistency in between different phases of Barnes maze test. Our protocol of Barnes maze test consisted of 3 phases: acquisition phase, acquisition probe trial phase and reversal learning phase. The acquisition phase consisted of two trials per day for four consecutive days. Only the first trial on day 1 was preceded by a habituation session in which each animal was gently placed in the escape box for 2 min. Subsequently, rats were placed in the middle of the platform covered by a start box, after 30 sec delays, the start box was lifted, and rats were allowed to freely explore the platform for a maximum 5 min. Each trial was ended once the rat localized and entered the escape box. However, if the rat did not enter the escape box up to the 5 min limit, it was gently guided into the escape box. Once the rat entered the escape box, it was allowed to stay inside for 30 to 60 sec before returning to the home cage. Notably, the escape box was held at a fixed position during the acquisition phase and the platform was rotated 90° clockwise between each test, therefore, the hole contained escape box varied between each testing rat. Following the last trial of acquisition phase, the acquisition probe trial was conducted. During the probe trial, the escape box was removed from the platform and the total duration of the test was set up to 90 sec. When probe trial started, rats were allowed to freely explore the platform, and the time spent within the zone that previously contained escape box was measured. The purpose of this trial was designed to assess whether rats trained from acquisition phase still retain the spatial memory to locate the zone that previously had the escape box, when the box was no longer presented. 24 hours after the probe trial, reversal learning phase was started, during which the escape box position was shifted 180° from its previous location in the acquisition phase. The reversal learning phase was performed for three consecutive days with two trials per

day, other procedure parameters (e.g., duration of trials, 30 sec in start box) were as same as in the acquisition phase. 70% ethanol was used to clean and eliminate the odor between testing rats throughout the whole experiment.

2.4.2. Novel Object Recognition test

The novel object recognition (NOR) test was designed to assess the recognition memory of rodents, which was based on the innate preference of rodents to explore a novel object instead of a more familiar one. The NOR test consisted of three sessions: habituation, familiarization, and testing. During the habituation session, rats were firstly placed into the testing field (60-cm in length X 60-cm width X 46-cm height) without any object presented and allowed to freely explore for 5 min in order to acclimate to the testing field. Familiarization session took place 24 hours after the habituation session, where rats were given a maximum 10 min to explore two identical objects (each object was placed 5 cm away from the side wall and 20 cm away from each other). Two types of objects were provided for familiarization in a rotational manner to eliminate bias. Testing session occurred 1 hour after the familiarization session, at which rats were returned to the testing field with one familiarized object replaced by a novel object. During the test, rats were allowed to freely explore for a maximum 10 min, however, the experiment was stopped if rats had explored both objects for a total 20 sec. Again, light, sound and temperature were controlled throughout the whole experiment, and 70% ethanol was utilized to clean and eliminate the odor between testing rats.

2.5. Euthanasia and Tissue Harvesting

Rats were euthanized via carbon dioxide asphyxiation in a euthanasia chamber between 14:00 – 16:00. Rats' brains were quickly extracted through a bone rongeur and rinsed in ice-cold 0.9% saline. Dentate gyrus (DG) and medial prefrontal cortex (mPFC) that were 2 mm thick and 3mm in diameter were extracted from coronal brain slices via a brain matrix (Braintree, MA). Extracted

brain tissues were then frozen with liquid nitrogen immediately and stored at -80°C until processing.

2.6. RNA isolation, cDNA synthesis, and Real-Time Polymerase Chain Reaction (RT-PCR)

In short, isolated brain tissue punches were placed in TRIzol (Invitrogen, Carlsbad, CA) with RNase-free stainless beads and homogenized through a Tissuelyer (Qiagen, Germantown, MD) for 1 min at 25-Hz for three times. The TRIzol protocol was performed in accordance with manufacturer's instructions. Following that, RNA pellets were dissolved in RNase-free water (30 µl) for quantification via the Nanodrop 1000 (Thermo Scientific, Waltham, MA). Then, RNA was treated with DNase I (Thermo Scientific, Waltham, MA) followed by DNase I inactivation with EDTA for 10min at 65°C. DNA-free RNA was then reverse transcribed through using a High-Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). For RT-PCR, cDNA (15 µg) from every sample was assayed in duplicate with gene-specific primers (Table 3.1) and SYBR green supermix (Bio-Rad, Hercules, CA). Expression values of mRNA were presented as 2^ΔCT whereby ΔCT = 18S CT - gene of interest CT.

Table 3.1. Primers used for RT-PCR.

Gene	Forward (5′→3′)	Reverse (5′→3′)	Accession No.
18S	GCTCGCTCCTCTCCTACTTG	CGATAAATGCACGCGTTCCCC	NR_046237.2
TNF-α	AACACACGAGACGCTGAAGT	TCCAGTGAGTTCCGAAAGCC	NM_012675.3
IL-1β	TGACTTCACCATGGAACCCG	GACCTGACTTGGCAGAGGAC	NM_031512.2

2.7. Immunoblotting

Isolated brain tissues were homogenized in Radioimmunoprecipitation (RIPA) buffer [50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 1 × protease + phosphatase inhibitor cocktail] through a Tissuelyer (Qiagen, Germantown, MD) for 1 min at 25 Hz for three times. The homogenized samples were then centrifuged at 12000 X g for 10 min, and the resultant supernatant was extracted for further processing. Protein concentrations were then determined through the bicinchoninic acid (BCA) assay (Piece Biotechnology, Rockford, IL, USA). Prepared protein was loaded onto 4-15% Criterion TGX gels (Bio-Rad, Hercules, CA) running electrophoresis at 200 V for 1 hour. Gels contained proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). After that, 5% nonfat milk diluted in Tris-buffered saline + 0.1% Tween20 (TBS-T) was applied on PVDF membranes for blocking. Primary antibodies (rabbit polyclonal) for phosphorylated 70kDa ribosomal protein S6 kinase (p-p70S6K) Thr389 (dilution 1:1000; catalog #9205, Cell Signaling, Danvers, MA), 70kDa ribosomal protein S6 kinas (p70S6K) (dilution 1:1000; catalog #2708, Cell Signaling, Danvers, MA), postsynaptic density protein-95 (PSD-95) (dilution 1:1000; catalog #3409, Cell Signaling, Danvers, MA), Synapsin (dilution 1:1000; catalog #2312, Cell Signaling, Danvers, MA), ionized calcium-binding adaptor molecule 1 (Iba-1) (dilution 1:1000; catalog #17198 ,Cell Signaling, Danvers, MA), glial fibrillary acidic protein (GFAP) (dilution 1:2000; catalog #12389, Cell Signaling, Danvers, MA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (dilution 1:20000; catalog #5174, Cell Signaling, Danvers, MA) were diluted in 5% BSA TBS-T, and treated to membranes overnight at 4°C. On the next day, horseradish peroxidase (HRP)-conjugated secondary antibodies (dilution 1:1000; Cell signaling) diluted in TBS-T with 5% non-fat milk were applied to membranes for one hour at room temperature. Following that, enhanced chemiluminescence substrate was applied to membranes, and a gel documentation system (Kodak 4000R imager

and molecular imagery software, Kodak molecular imaging systems, New Haven, CT, USA) was then used to take images of each membrane.

2.8. PC12 Cell Culture and Differentiation

Our protocol for establishing PC12 cell was described previously [140]. PC12 cells were purchased from (American Type Culture Collection, Manassas, VA). Briefly, cells were towed and housed in a BL2 room in a humidified incubator, which was maintained at 37°C with (5% CO_2, 95% air). Cells then were seeded from a maintenance plate onto 0.01% collagen-coated 6 well plate supplemented with 2.5 mL growth media (10% horse serum and 5% fetal bovine serum) each well. Growth media was changed to differentiation media (0.5% fetal bovine serum in RPMI 1640 + 50 ng/mL neural growth factor) with 2.5 mL per well on day 2. After that, media was changed every 72-hour, and cells were plated at an appropriate density (~1.25× 106 cells/mL). On day 9, following media change, Cr treatment began.

2.9. Creatine treatment for cell culture

The concentration and duration of Cr treatment were decided based on a pilot study (data not shown). Creatine was dissolved in a differentiation media (without neural growth factor) to make the desired concentration (100 mM). Cr treatment was divided into control condition with only differentiation media, 12-hour time point with Cr solution 12-hour after the control began, 24-hour time point with Cr solution at the same time as control began. Each condition had 6 wells (n=6). At the conclusion of 24 hours, cells were washed with PBS twice and lysed with RIPA buffer made with (protease + phosphatase inhibitor cocktail). Then cells were conducted through the immunoblotting protocol (see in 2.7).

2.10 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (GraphPad, San Diego, CA). In two-variable experiments, two-way ANOVAs was used to evaluate the significant differences

between experimental groups. In one-variable experiments more than 2 groups, one-way ANOVAs were utilized to evaluate the significance of differences among experimental groups. Turkey's *post-hoc* analyses were conducted for multiple comparisons. Student's *t* tests were used to evaluate significance of differences between 2 groups. All values are expressed as the mean ± SEM and, statistical significance was established as $P < 0.05$.

3. Results

3.1. Body weight and food consumption throughout the experiment

Two-way ANOVAs was used to analyze body weight and food consumption throughout the entire experimental period [group (4 groups) × time (week 1 – week 6)]. Body weight analysis revealed a main effect of time ($F(2.267,79.36) = 442.8$, $P < 0.0001$) (Figure 3.1B). *Post hoc* analysis found that the rest of weeks (week 2 – week 6) had significantly increased body weight compared with week 1, within each individual group (Figure 3.1B). There was no group difference between detected ($F(3,35) = 0.4764$, $P = 0.701$). Food consumption analysis revealed no significant difference in comparison to week 1 and, found no group difference ($F(3,35) = 0.4428$, $P = 0.7239$) (Figure 3.1C).

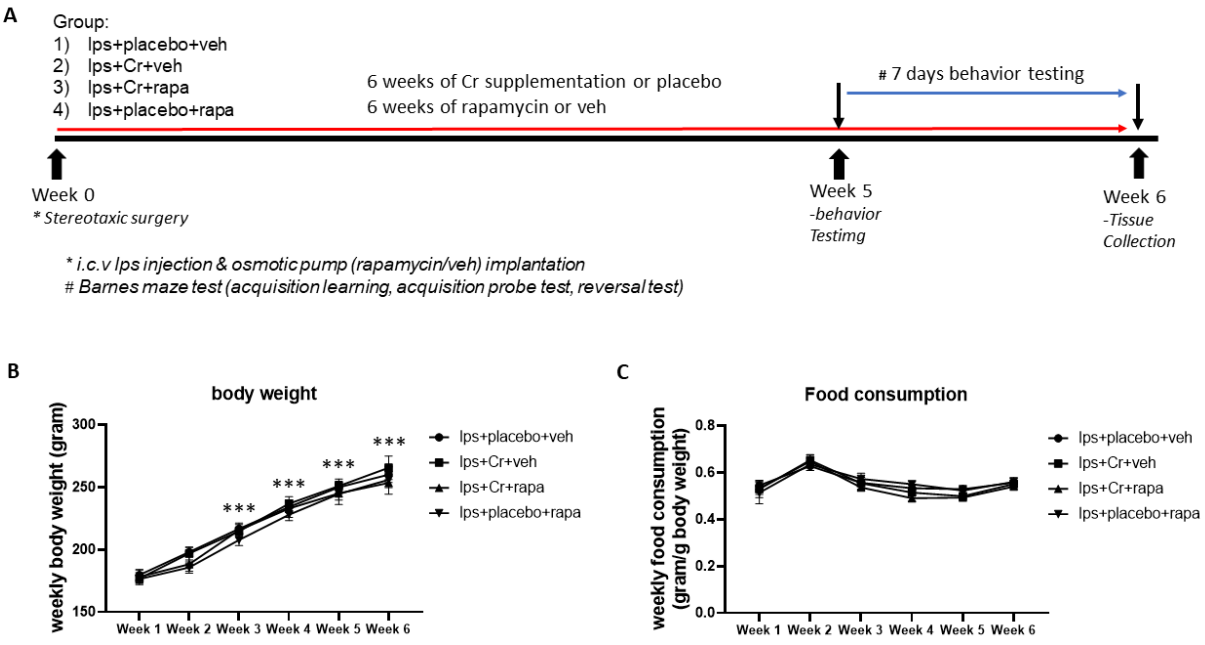

Figure 3.1. Experimental timeline, body weight and food consumption throughout the entire experiment. A: timeline for the present study, where 4 groups were produced at the beginning of experiment. Following that, each individual group received treatment or drug correspondingly for 6 weeks. At the end of 6 weeks period (at week 5), behavioral tests were conducted to assess cognitive function. B: Average weekly body weight for individual group, *** significantly different from week 1 ($P < 0.0001$). C: Average weekly food consumption for individual group. All values are expressed as

3.2. Rapamycin attenuates cognitive amelioration induced by Cr supplementation

To assess the spatial learning and memory, Barnes maze test was conducted. During the first phase (acquisition learning phase), two-way ANOVAs [treatment (Cr supplementation/placebo) × drug (rapamycin/veh) revealed significant interaction between treatment and drug at trial 2 ($F(1,35) = 18.38$, $P = 0.0001$), trial 3 ($F(1,35) = 13.98$), $P = 0.0007$) and trial 4 ($F(1,35) = 7.346$, $P = 0.010$) (Figure 3.2A). At trial 2, groups received Cr supplementation demonstrated a significantly diminished latency to reach the escape box compared to group only received placebo. Meanwhile, both groups who received rapamycin (rapa in Figure 3.2) were impaired, shown by significantly higher latency, with respect to lps+Cr+veh group (Figure 3.2A). At trial 3 and 4, both groups showed diminished latency when compared to lps+placebo+veh group. A two-way ANOVA was used to examine the significance of difference during the acquisition probe test (treatment × drug), which revealed no interaction between treatment and drug ($F(1,35) = 3.041$, $P = 0.090$), also, there was no group difference detected (Figure 3.2B). In reversal learning phase, two-way ANOVAs (treatment × drug) revealed significant interaction between treatment and drug on day 1 ($F(1,35) = 28.20$, $P < 0.0001$) and day 2 ($F(1,35) = 9.0.92$, $P = 0.005$). Both groups were significantly improved, shown by significantly decreased latency, when compared to lps+placebo+veh group (Figure 3.2C). However, groups received rapamycin were significantly impaired with respect to group received Cr supplementation without rapamycin (lps+Cr+veh) (Figure 3.2C). Additionally, in order to also assess the recognition memory, novel object recognition test was conducted to test if there is any group difference. Two-way ANOVA test showed that there was a main effect of treatment ($F(1,35) = 7.268$), $P = 0.011$). More specifically, Cr supplemented group showed significantly increased amount of time taken to explore the novel object in comparison to lps+placebo+veh group, this significant effect disappeared in Cr supplemented group received rapamycin (lps+Cr+rapa) (Figure 3.2D). Overall, learning was

effective for both groups at end of the test for both acquisition and reversal learning phase (Figure 3.2A&C).

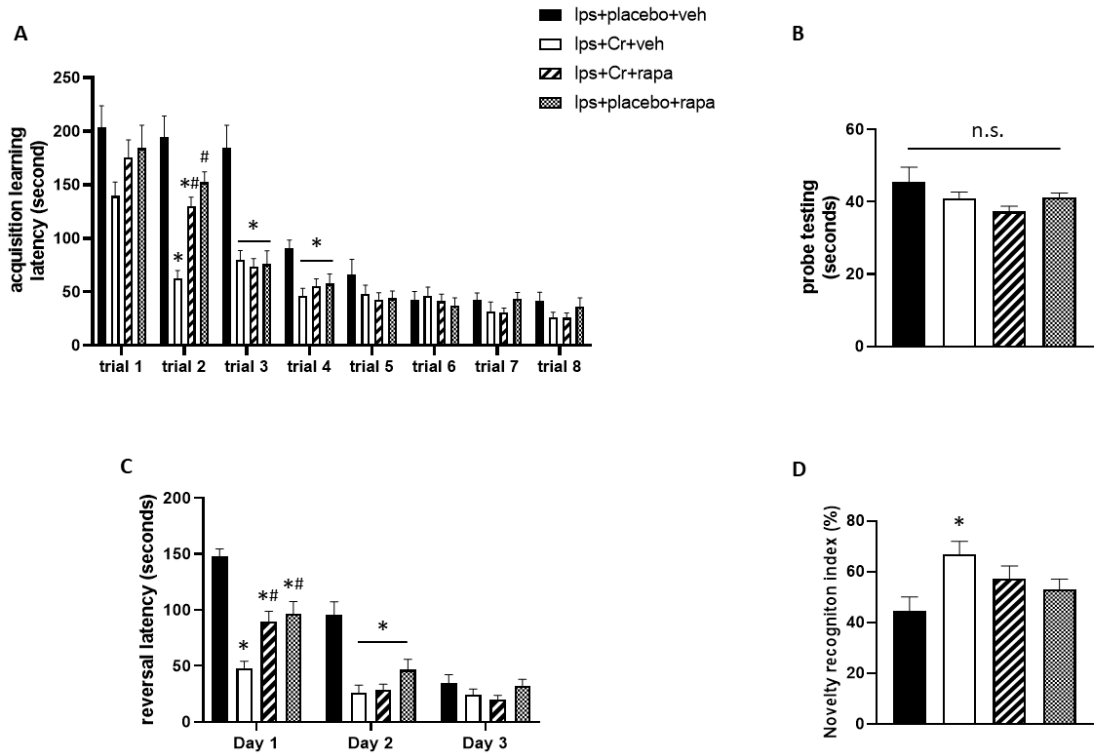

Figure 3.2. Behavioral assessment of cognitive effects of Cr supplementation and rapamycin. A: Latency of reaching escape box during the acquisition learning phase. B: Acquisition probe test for memory retention. C: latency of reaching escape box during the reversal learning phase. C: Percentage of total amount of time spent with novel object. * significantly different from lps+placebo+veh group, $P < 0.05$. # significantly different from lps+Cr+veh group $P < 0.05$, n.s.: not significant. All values are expressed as mean ± SE.

3.3. Rapamycin inhibits the activation of mTORC1 signaling induced by Cr supplementation in dentate gyrus

To evaluate the activity of mTORC1 signaling following Cr supplementation and rapamycin, two-way ANOVAs were used to examine the proteins expression of mTORC1 signaling and its downstream substrates. Within dentate gyrus, analysis of phosphorylation of p70S6K (p-p70) protein revealed a statistical insignificance of interaction ($F(1,20) = 3.520$, $P = 0.0566$), however, *post-hoc* analysis showed that Cr supplementation significantly increased p-p70 protein expression ($P = 0.032$), while this increased p-p70 protein expression was significantly downregulated in the presence of rapamycin ($P = 0.0008$) (Figure 3.3A). Additionally, analysis of total p70S6K (total-p70) revealed no significance of difference between groups (Figure 3.3B). There was a main effect of drug on the ratio of phosphorylation of p70S6K to total p70S6K (p-p70/total-p70) ($F(1,20) = 13.71$, $P = 0.001$). Analysis of downstream synaptic proteins showed that there was a significant interaction of treatment and drug on PSD-95 protein ($F(1,20) = 6.455$, $P = 0.020$), but insignificant interaction on synapsin protein ($F(1,20) = 3.520$, $P = 0.075$) (Figure 3D&E). *Post hoc* analysis revealed that, for both PSD-95 and synapsin proteins, Cr supplementation significantly increased their expression level and rapamycin downregulated Cr supplementation-induced increases of synaptic proteins (Figure 3.3D&E). Collectively, those data confirmed that the activation of mTORC1 signaling within dentate gyrus was inhibited by rapamycin. Wihtin medial prefrontal cortex, analysis detected a main effect of drug on p-p70 protein ($F(1,20) = 37.86$, $P < 0.0001$) (Figure 3.3F) and a main effect of drug on p-p70/total-p70 ($F(1,20) = 25.09$, $P < 0.0001$) (Figure 3.3H). There was no other significance of difference was detected within medial prefrontal cortex.

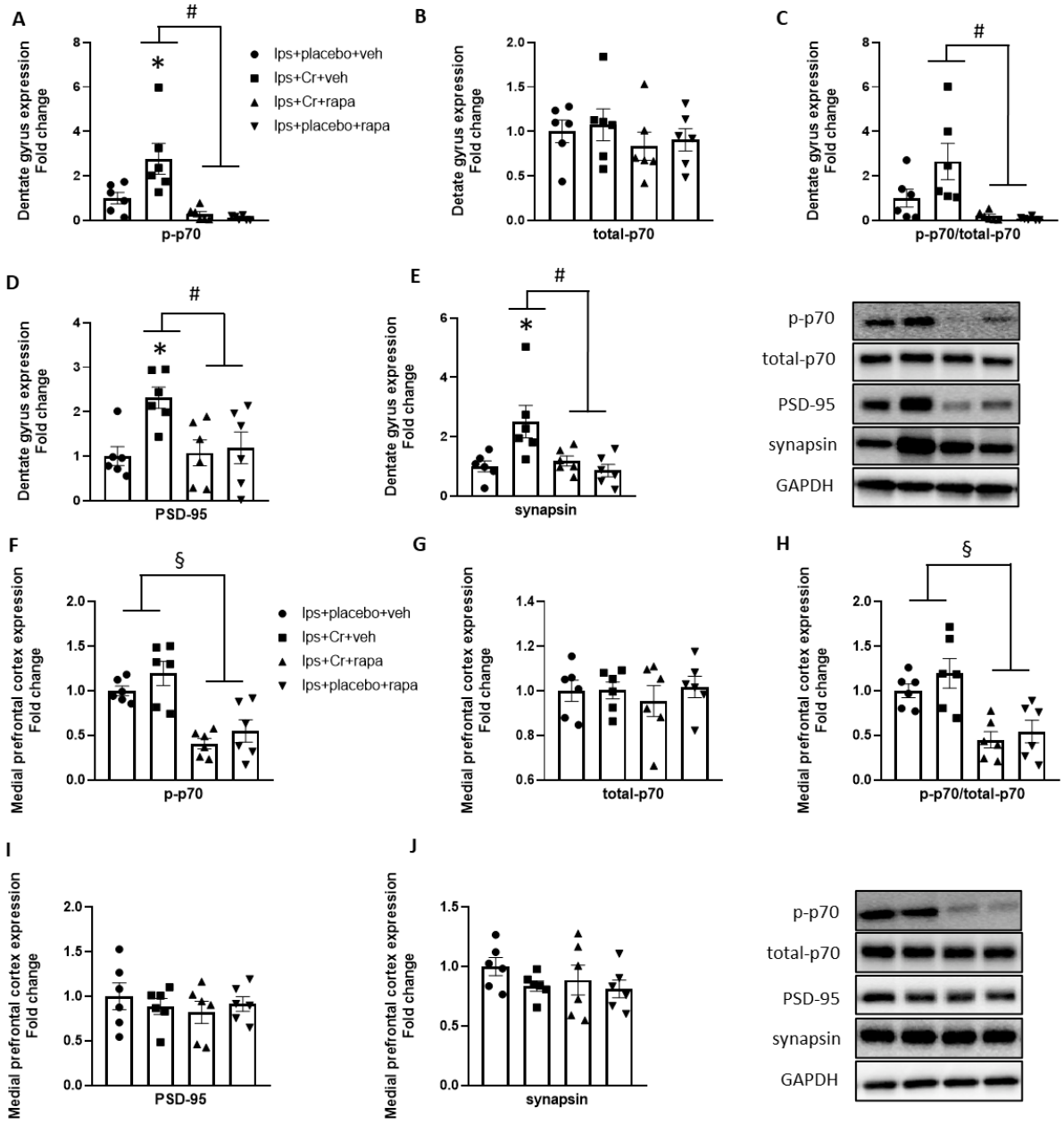

Figure 3.3. Assessment of molecular changes in dentate gyrus and medial prefrontal cortex. A-C: phosphorylation of p-p70S6K protein (p-p70), total p-p70S6K protein (total-p70) and ratio between p-p70 to total-p70 protein within dentate gyrus. D-E: PSD-95 and synapsin proteins expression level within dentate gyrus. F-H: p-p70 protein, total-p70, p-p70/total-p70 protein within medial prefrontal cortex. D-E: PSD-95 and synapsin proteins expression level within medial prefrontal cortex. * significantly different from Ips+placebo+veh, $P < 0.05$. # significantly different from Ips+Cr+veh, $P < 0.05$. § significantly different from groups, $P < 0.05$. All values are expressed as mean ± SE.

3.4 Rapamycin attenuates lps induced neuroinflammation

To evaluate the neuroinflammation, two-way ANOVAs were conducted to examine the ionized calcium binding adaptor molecule1(Iba1) protein expression and pro-inflammatory transcripts level in both dentate gyrus and medial prefrontal cortex, respectively (Figure 3.4). Analysis of Iba1 revealed a main effect of drug in both dentate gyrus ($F(1,20) = 48.27$, $P < 0.0001$) (Figure 3.4A) and medial prefrontal cortex ($F(1,20) = 31.69$, $P < 0.0001$) (Figure 3.4D). *Post-hoc* analysis showed that groups received rapamycin significantly attenuated Iba1 protein expression with respect to groups without rapamycin ($P < 0.0001$ for both comparisons). Correspondingly, further analysis of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) transcripts also revealed a main effect of drug in both dentate gyrus and prefrontal cortex, which were downregulated by rapamycin when compared to groups without rapamycin ($P < 0.0001$ for both comparisons) (Figure B,C,E,F).

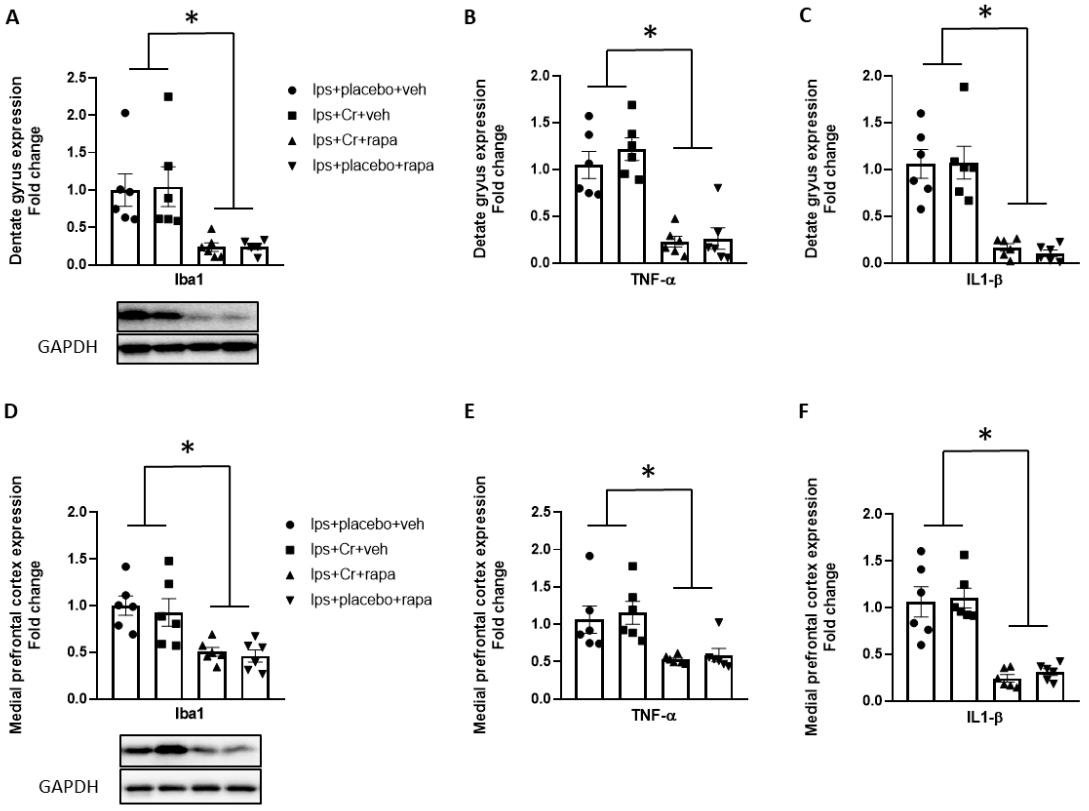

Figure 3.4. Marker for reactive microglia (Iba1) and proinflammatory transcripts within dentate gyrus and medial prefrontal cortex. A-C: Iba1 protein expression, TNF-α and IL-1β transcripts level in dentate gyrus. D-F: Iba1 protein expression, TNF-α and IL-1β transcripts level in medial prefrontal cortex. * significantly different from each other, $P < 0.05$. All values are expressed as mean ± SE.

3.5. Cr supplementation is sufficient to upregulate mTORC1 signaling activity in PC12 cells

To test if Cr supplementation can directly activate mTORC1 signaling within neuronal cell type, Cr treatment was applied to PC12 cells for 12- and 24-hours, respectively. Protein phosphorylation of p70S6K was used as a readout for mTORC1 signaling. Analysis through one-way ANOVA found that both 12- and 24-hours treatment of Cr significantly increased p-p70 protein expression level with respect to control condition (12-hours vs. control, $P = 0.004$; 24-hours vs. control vs. control, $P < 0.0001$) (Figure 3.5A). Furthermore, ratio between p-p70/total-p70 was significantly elevated following 24 hours Cr treatment ($P = 0.002$) (Figure 3.5C). There was no group difference detected between 12- and 24-hours Cr treatment regarding protein expression of p-p70 ($P = 0.140$) (Figure 3.5A) and ratio between p-p70/total-p70 ($P = 0.147$) (Figure 3.5C). Additionally, there was no significance of difference between groups for total-p70 protein (Figure 3.5B).

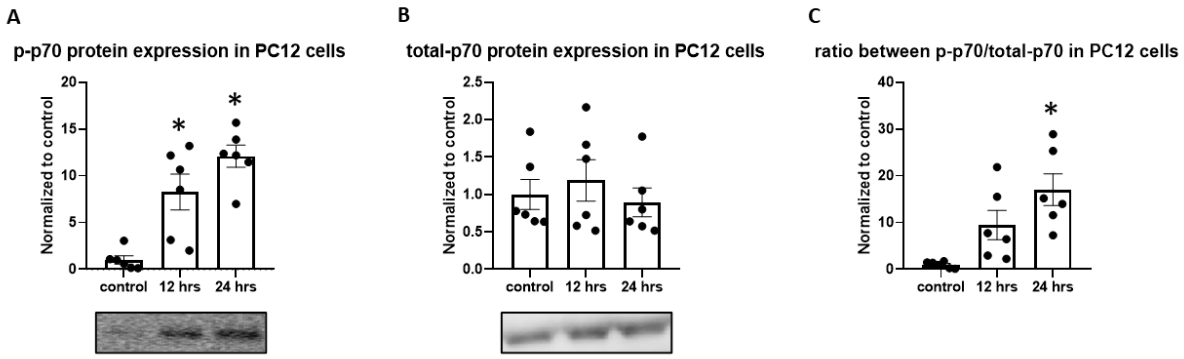

Figure 3.5. Activity of mTORC1 signaling in PC12 cells following 12- and 24-hours Cr treatment. A: protein phosphorylation of p70S6K (p-p70). B: Total p70S6K protein expression (total-p70). C: ratio between p-p70/total-total-p70. * significantly different from control, $P < 0.05$. All values are expressed as mean ± SE.

4 Discussion

Although much effort has been spent to investigate the neurobehavioral outcomes of creatine (Cr) under a variety of paradigms [131],[132],[174], exact signaling and molecular mechanisms underlying neurocognitive effects of Cr are relatively lacking. Through the inhibitory effect of rapamycin against mTORC1 signaling during the entire period of Cr supplementation (6 weeks), the present study suggests that an intact activity of mTORC1 signaling is required for Cr to exert its full effects to ameliorate the deficiency in learning and memory elicited by neuroinflammation. More specifically, our observation also suggests that the full presence of mTORC1 signaling activity in dentate gyrus (DG), but not medial prefrontal cortex (mPFC), is possibly required for Cr to exert its cognitive benefits. Meanwhile, cell culture experiment illustrated that Cr is sufficient to upregulate the mTORC1 signaling activity in neuronal cells when only neuronal cell type presents. While preliminary, the present study examined the potential linkage between Cr supplementation and mTORC1 signaling under the neurocognitive paradigm, which will pave the way for future clinical studies to validate the use of Cr as a treatment for cognitive deficiency in the context of MCI.

In recent years, neurobehavioral tests became a necessary part of neuroscience field, allowing researchers to couple the molecular hypothesis to behavioral observations. Barnes maze task is a valuable tool for assessing spatial learning, memory retention and cognitive flexibility through different testing phases [141]. In the present study, Cr supplementation significantly improved the acquisition learning and cognitive flexibility, shown by a faster rate of targeting the escape box (2^{nd} trial of acquisition and reversal learning phase). While the pharmacological inhibition of mTORC1 signaling through rapamycin attenuated Cr supplementation's effects on acquisition learning and cognitive flexibility. Interestingly, although neurocognitive effects of Cr were compromised by rapamycin, group (lps+Cr+rapamycin) received both Cr supplementation and rapamycin still demonstrated an improved learning and cognitive flexibility, when compared to

group (lps+placebo+veh) only received placebo and veh. This result suggests that Cr supplementation could partially rely on pathways other than mTORC1 to ameliorate the cognitive impairment. Meanwhile, rapamycin was reported to downregulate inflammation through suppressing NF-κB signaling, which plays a pivotal role in mediating lps induced neuroinflammation in microglial cells [175]–[178]. Therefore, a genetic knockdown of mTORC1 in a specific cell type (e.g., neuron) might help to avoid rapamycin induced anti-inflammatory effects, which would allow a more objective observation toward the cognitive effects of Cr in the absence of mTORC1 signaling. Additionally, despite the initial learning difference, there was no difference between tested groups after 2nd day of both acquisition and reversal learning phase, indicating that both groups learned tasks.

It is noteworthy that the memory retention was not affected by either Cr supplementation or rapamycin in the present study, possibly due to that all tested grouped acquired the task prior to the acquisition probe trial. This outcome is consistent with others' findings [179], in which experimental groups who learned to localize the escape box at the end of the acquisition phase were also able to locate the hole which previously contained the escape box, during the following probe trial. However, the probe trial was conducted shortly (4~6 hours) after the last trial of acquisition phase in the current study, which may largely rely on the working memory [180]. Several groups contended that acquisition probe trial conducted 24 hours after the last acquisition trial may ensure that spatial memory (short-term memory) is tested [181]–[183].

Relevant studies have reported that Cr supplementation is sufficient to upregulate synaptic proteins (e.g., PSD-95) and long-term potentiation, a form of synaptic plasticity, in brain regions that relate to cognitive processing [89],[90],[97]–[99]. However, neuro-molecular mechanisms by which Cr supplementation exert its synaptic effects remain unclear. The reversal of Cr supplementation induced increase in presynaptic synapsin and postsynaptic PSD-95 proteins by rapamycin observed in dentate gyrus, suggests that mTORC1 signaling in dentate gyrus is a

molecular transducer coupling Cr supplementation to synaptic events, and possibly required by Cr to elicit neurocognitive effects. Thus far, our result is consistent with previously existing evidence that Cr possibly modulated mTORC1 signaling and downstream synaptic proteins to mediate brain health [134],[135]. In contrast, mTORC1 signaling was not altered by Cr supplementation and synaptic protein changes were not observed in medial prefrontal cortex, suggesting that mTORC1 signaling in medial prefrontal cortex is possibly not required for Cr supplementation to ameliorate cognitive impairment induced by neuroinflammation. However, some hypothesis should be made to try to explain this result: (i) mTORC1 signaling and mTORC1-dependent synaptic changes were reported to respond rapidly within prefrontal cortex [184],[185], therefore, a 6 weeks of Cr supplementation protocol used in the present study may restrain the observation from those changes that have happened earlier; (ii) tissues with higher baseline level of Cr concentration will have a lower accumulation of Cr after its supplementation [186]. Although currently Cr concentration in medial prefrontal cortex relative to other brain regions is unclear, a high resting metabolic rate within medial prefrontal cortex compared to other brain regions possibly implies a high baseline level of Cr storage for high energy demand [187]; (iii) our results do not rule out the possibility that Cr supplementation may have other effects in medial prefrontal cortex. Recent evidence indicate that Cr supplementation also attenuates the oxidative stress, and improves mitochondrial respiratory function [89],[91], which could play critical roles in mediating neurocognition.

The connection between Cr supplementation and neuro-behavioral outcomes in this study was examined through using selective mTORC1 inhibitor rapamycin. In fact, rapamycin was previously reported to play an anti-inflammatory role [175],[176]. During the process of neuroinflammation elicited by lps, microglia are activated to secrete proinflammatory cytokines (e.g., TNF- α, IL-1 β), which would lead to neuronal damage, synaptic loss and thus, cognitive impairment after the prolonged activation of microglia [67]–[69]. In the present study, rapamycin administration

significantly downregulated activated microglia and downstream inflammatory cytokines expression, which might help to explain the improved learning and memory within rapamycin treated groups. However, given the high dose of lps (45.4 ug/rat) utilized in this study, it is uncertain as to what extent that the neuroinflammation was downregulated by rapamycin.

Although currently it is clear that Cr supplementation can upregulate mTORC1 signaling and synaptic proteins in the brain, whether Cr can directly affect mTORC1 signaling within neuronal cells remains unknown. In our study, Cr robustly increased the phosphorylation of p70S6K (Thr389), a readout for mTORC1 signaling, in PC12 cells, indicating that Cr is sufficient to directly activate mTORC1 signaling within neurons. While preliminary, those data may help inform future study to investigate how upstream mechanisms lead to the activation mTORC1 signaling following Cr supplementation.

In summary, the present study revealed a potential neuro-molecular mechanism underlying the Cr supplementation to improve cognitive function. Here, we demonstrated that mTORC1 signaling is required for Cr supplementation to fully ameliorate the cognitive deficiency in a model of neuroinflammation, an effect that may be mediated within dentate gyrus. Lastly, we also illustrated that Cr treatment is sufficiently to upregulate mTORC1 signaling activity within neuronal cell type. Collectively, these data suggest that Cr supplementation might be a promising intervention to treat cognitive impairment.

Chapter 4: Conclusions & Future Directions

Overview of dissertation

The key goal of this dissertation was to investigate the role of mTORC1 signaling behind the creatine (Cr) supplementation in improving brain health, with a particular focus on neurocognitive effects. For doing this, a neuroinflammatory rat model was generated first by injecting lipopolysaccharides (LPS) into lateral ventricles to elicit chronic neuroinflammation for mimicking mild cognitive impairment (MCI). In chapter 2, Cr supplementation was prepared through diluting in drinking water and provided to rats in a daily basis for 6 weeks. Following that, neurocognitive tests were performed to evaluate the learning and memory ability, and a series of molecular assays were performed to assess the potential molecular changes linked to changed cognitive processing. In chapter 3, the procedure of conducting LPS and Cr supplementation were kept same as in chapter 2, however, a selective inhibitor of mTORC1 signaling (rapamycin) was infused into brain during the entire 6 weeks period of Cr supplementation to testing the underlying molecular mechanism. By performing a series of experiments, the present dissertation provides the scientific evidence to fill a knowledge gap of this field and, will pave the way for future clinical trials to use Cr as a therapeutic treatment for cognitive impairment.

Results gained from chapter 2 verified that 6 weeks of Cr supplementation significantly ameliorated the neuroinflammation (LPS-elicited neuroinflammation) induced cognitive impairment, via performing Barnes maze test (BMT) and Novel object recognition (NOR) test. This improved cognitive effects was found to associated with a concurrent upregulation of mTORC1 signaling in the dentate gyrus of experimental subjects. Additionally, chapter 2 also examined the neurocognitive and neuro-molecular effects of Cr supplementation in the absence of neuroinflammation (without LPS). Results from this experiment demonstrated that, although 6 weeks of Cr supplementation failed to further enhance neurocognition, mTORC1 signaling within dentate gyrus was still upregulated by Cr supplementation.

Given above outcomes of which mTORC1 signaling was upregulated by Cr supplementation within dentate gyrus, following immunohistochemistry (IHC) assays were performed to examine whether increased mTORC1 signaling is expressed in neuronal cell type. IHC analyses (data shown Figure 4.2 & 4.3) revealed that, Cr supplementation significantly increased p70S6K protein phosphorylation (p-p70), a readout for mTORC1 signaling, within neuronal cells in dentate gyrus area.

Based on previous findings, chapter 3 examined the role of mTORC1 signaling behind the Cr supplementation in ameliorating cognitive impairment elicited by neuroinflammation. My experimental results demonstrated that an intact activity of mTORC1 is required for Cr supplementation to fully exert its effects to ameliorate the cognitive impairment caused by neuroinflammation. Moreover, the molecular analysis performed within dentate gyrus and medial prefrontal cortex suggested that mTORC1 signaling within dentate gyrus might be responsible for the positive effects of Cr supplementation on neurocognition, whereas mTORC1 signaling within medial prefrontal cortex seemingly is not required for Cr supplementation to ameliorate cognitive impairment. However, my experimental results do not exclude the possibility that mTORC1 signaling within other brain regions related to cognitive processing is also required for Cr supplementation to ameliorate neuroinflammation-induced cognitive impairment. A graphic overview of my dissertational findings is presented in Figure 4.1.

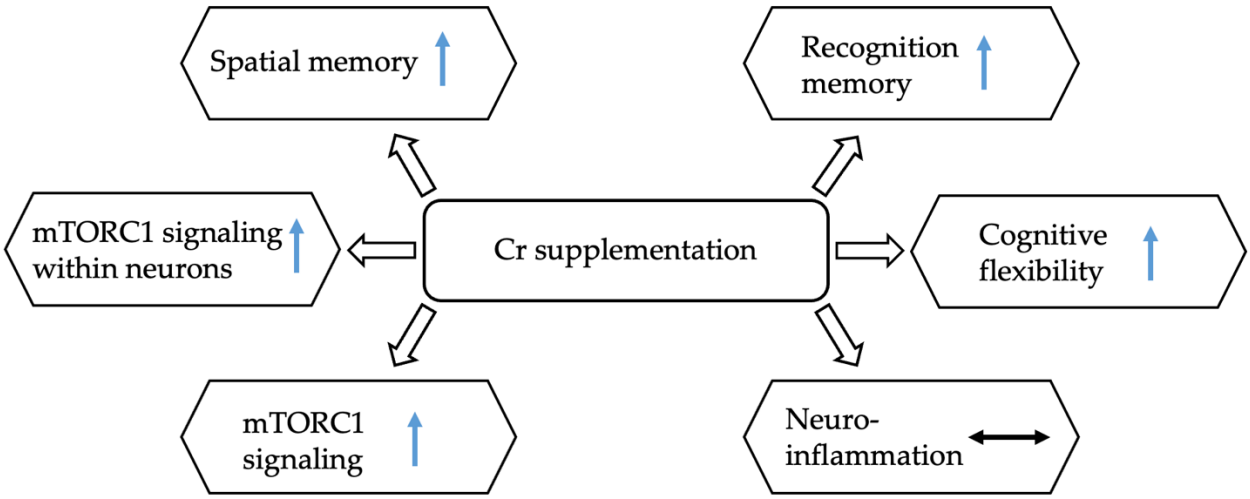

Figure 4.1. Graphic demonstration of dissertational findings. Blue arrow indicates increase, black left-right arrow indicates no change.

The present dissertational work gained insights of Cr supplementation as a potential therapeutic treatment for mild cognitive impairment and, revealed potential neuro-molecular mechanisms underlying neurocognitive or neurobehavior effects of Cr supplementation. Collectively, the current dissertational outcomes, combined with previous clinical studies, suggest that Cr supplementation and mTORC1 signaling are promising future areas of research for advancing therapeutics aiming to treat mild cognitive impairment or improve cognition.

Cr supplementation attenuates cognitive deficiency in a neuroinflammatory rat model

Numerous human clinical studies reported that Cr supplementation is able to enhance cognitive processing under a variety of different paradigms [81]–[83],[85],[86],[88], including sleep deprivation, oxygen deprivation, aging, etc… However, whether Cr supplementation is also effective in ameliorating or restoring cognitive deficiency in the context of mild cognitive impairment or dementia remains to be tested [131],[132]. In chapter 2 and 3, intracerebroventricular (i.c.v.) injections of LPS to elicit chronic neuroinflammation was utilized to

mimic the pathology of mild cognitive impairment (MCI) [188],[189]. Our results showed that LPS-induced neuroinflammation significantly impaired acquisition learning, spatial memory, and recognition memory, which are important cognitive domains. Notably, chapter 2 and 3 demonstrated that, after 6 weeks of Cr supplementation via drinking water administration, acquisition learning and spatial memory deficit caused by neuroinflammation was significantly attenuated. Additionally, recognition memory deficit caused by neuroinflammation was also ameliorated by Cr supplementation, thus supporting the overall hypothesis with respect to Cr's neuro-cognitive effects. It is worth to note that results gained from neuro-cognitive tests in chapter 1 were attempting to fill the research gap in this field where the relationship between Cr supplementation and MCI is still elusive. Additionally, experimental results were based on a neuroinflammatory female rat model under a highly controlled laboratory condition, which might be highly different than actual human clinical studies where dietary style and exercise level could be contributing factors to influence the effects of Cr supplementation [85],[87]. However, to the best of my knowledge, this is the first study designed to examine the influence of Cr supplementation on cognitive perspective of MCI, which would fuel more in-depth investigations in the future.

In chapter 3, Cr supplementation not only attenuated neuroinflammation induced spatial acquisition learning and memory deficits, but also exhibited an ameliorating effect toward the cognitive flexibility shown by the reversal learning phase of Barnes maze test. This is interesting because spatial learning and memory are highly correlated with hippocampus-dependent functions [141], in contrast, cognitive flexibility involves an even broader category of neural substrates and networks, requiring several aspects of executive functions (e.g., working memory) [190]. As a result, some neuro-behavioral outcomes of chapter 3 implied that different brain regions might be involved in ameliorated cognitive deficiency as a consequence of Cr supplementation.

In addition to study the neurocognitive effects of Cr supplementation in the presence of inflammation, chapter 1 also examined whether Cr supplementation could further enhance cognitive function without neuroinflammation. Neuro-behavioral results revealed that 6 weeks of Cr supplementation was not capable of further increasing learning and memory in comparison to the control group of young adult rats. This result conflicts with some others' reports in which chronic supplementation of Cr has been shown to increase cognitive performance in mice [89],[150]. However, there are some potential reasons to explain this obvious discrepancy: (i). aged animals were used by those studies, from which those animals may already exhibit cognitive impairment due to natural aging process; (ii). Dietary supplementation of Cr was conducted by those experiments, though it is currently unknown as to whether dietary administration of Cr is more efficient than ingestion of Cr through drinking water; (iii). Different dosages or dosing protocol of Cr supplementation were used between studies. While in support of my findings, human clinical studies [84],[191] demonstrated that Cr supplementation does not improve cognitive function in young adults, although Cr supplementation has obvious cognitive benefits in elderly population. This suggests that Cr supplementation may have a more profound cognitive effect under the cognitive demanding condition.

Despite research efforts, including the present dissertation, have been devoted to study the cognitive effects of Cr supplementation, experiments designed to assess the cognitive benefits of Cr supplementation can be divided into two time-dependent categories: (i). chronic administration (6 weeks+) and, (ii). Semi acute (one week) administration (between 5 – 7 days). Yet, no research has examined the acute effects of Cr supplementation on cognitive functions over the ensuing hours following initial administration. Furthermore, most studies were designed to investigate the therapeutic effects of Cr supplementation under the cognitive deficiency, less effort has been spent to investigate whether Cr supplementation could be preventative to the cognitive deficiency. As such, future research is needed to address those questions for a more comprehensive

understanding concerning Cr supplementation. Moreover, heterogenous dosages of Cr supplementation existed in literatures where most of those dosing protocols were largely based on studies that reported positive effects of Cr supplementation on skeletal muscle. Therefore, optimal dosing protocols for Cr supplementation in neurocognitive field needs to be developed.

mTORC1 signaling partially mediates the neurocognitive effects of Cr supplementation

Cr has long been believed to be an energy buffer for providing instant energy demand in skeletal muscle, especially during the high-intensity exercise [192]. In recent decades, Cr and its potential neurocognitive effects have attracted much attention, from which this topic then has gained fruitful results from human clinical experiments. However, due to the limitation of human studies that only allow investigators to measure neurobehavioral changes associated with Cr supplementation, molecular signaling transduction mechanisms underlying Cr supplementation are relatively lacking. Thus, results gained from this dissertational work attempt to fill the knowledge gap in relation to this field.

Based on accumulated evidence in the past, the upregulation of mTORC1 signaling pathway was found to directly increase two essential synaptic proteins, presynaptic protein synapsin and postsynaptic protein PSD-95, which are crucial neural basis for establishing long-lasting synaptic changes (e.g., long term potentiation, LTP) [193],[194]. The upregulation of mTORC1 signaling was also verified to enhance LTP particularly in hippocampus and, thus it constitutes an essential cellular signaling transduction mechanism for forming learning and new memory [195]. Due to this reason, in chapter 2, the finding of increased mTORC1 signaling and synaptic protein expression in hippocampal dentate gyrus was further hypothesized to mediate the cognitive benefits of Cr supplementation. Subsequently, through utilizing the rapamycin to block mTORC1 signaling during the entire period of Cr supplementation, chapter 3 revealed an inevitable role of mTORC1 in constituting the full neurocognitive effects of Cr supplementation. This result is consistent to others' reports where the inhibition of mTORC1 signaling was found to reduce the beneficial

effects of Cr supplementation [134],[196]. Although those reports were based on totally different scenarios, when put together, those studies imply that Cr supplementation may possess a wide range of effects including brain heath, and mTORC1 signaling might be the common signaling transducer mediating those beneficial effects behind Cr supplementation. In comparison, the mTORC1 signaling in the medial prefrontal cortex was not altered by Cr supplementation. In fact, medial prefrontal cortex, a subregion of prefrontal cortex, is involved in numerous cognitive functions, including working memory, spatial and long-term memory, attention, and executive function [197]. It was hypothetical that, except dentate gyrus, medial frontal cortex also participates in ameliorated neurocognition following Cr supplementation. Apart from the negative results regarding mTORC1 signaling found within medial prefrontal cortex in chapter 3, it does not rule out the possibility that Cr's cognitive effects are through alternative pathways in medial prefrontal cortex. Based on preliminary data, future studies could perform additional medial prefrontal cortex-dependent cognitive tasks to narrow down the effects of Cr supplementation in this cognitively important brain region.

The use of rapamycin could be a potential limitation in chapter 3, although lots of findings from mTORC1 signaling were mostly based on the utilization of rapamycin. First of all, rapamycin does not completely inhibit the activity of mTORC1 signaling [198]. In chapter 3, rapamycin reduced phosphorylation of p70S6K (used as a readout for mTORC1 signaling) to ~ 80%, implying that Cr supplementation could still be capable of activating mTORC1 signaling, to a certain degree, to elicit behavioral consequences. Therefore, it might be arbitrary to conclude that mTORC1 signaling is only partially required by Cr supplementation to gain neurocognitive effects. Secondly, rapamycin was initially considered as a selective inhibitor for mTORC1 signaling, prolonged exposure to rapamycin was found to block the assembly of mTORC2 protein complex in certain metabolic tissues [199]. Paradoxically, mTORC2 signaling are quite sensitive to rapamycin in some cell types, while remain resistant in certain cell types [200]. Future studies need to address

this issue in order to gain a better scientific view regarding the role of mTORC1 signaling in different cell types. Lastly, rapamycin itself has some unwanted side effects, which include immunosuppression, glucose intolerance, and disruption of lipid homeostasis [201]. Those side effects may adversely affect the neurocognitive functions and neuro-molecular activities in the brain. For example, glucose overload was found to lead to impaired memory, increased insulin resistance, reduction of essential synaptic proteins [202],[203]. As a result, the present findings based on the utilization of rapamycin may not be solely dependent on the inhibition of mTORC1 signaling.

Despite the fact that there might be some unwanted effects associated with rapamycin, this dissertation still provides the preliminary data as to the potential linkage between Cr supplementation, cognitive function and mTORC1 signaling. However, one thing worth to mention is that hyperactive mTORC1 signaling due to pathological aging also contributes to the cognitive deficits, as seen in Alzheimer's disease [204]. Whereas the hypoactive mTORC1 signaling also leads to abnormal neurocognition, such as seen in certain pathological conditions. Consequently, a precise regulation of mTORC1 signaling is required to achieve optimal cognitive function and maintain a healthy brain.

Cr supplementation upregulates mTORC1 signaling in neuronal cells

In chapter 1, mTORC1 signaling activity and synaptic proteins were found to be upregulated in dentate gyrus by Cr supplementation. Based on literature, increased synaptic proteins might be a direct effect due to upregulated mTORC1 signaling activity, which could further lead to increased synaptic plasticity [108],[123],[169],[195]. As a result, it was intuitively hypothetical that this upregulated mTORC1 caused by Cr supplementation would occur within neurons. For this reason, the present dissertation conducted immunohistochemistry (IHC) to test this hypothesis. Experimental protocols for conducting animal surgery, Cr supplementation and experimental conditions, groups (LPS condition, non-LPS condition) were kept same as in chapter 1. Protein

phosphorylation of p70S6K (p-p70), a readout for mTORC1 signaling activity, was co-stained with NeuN (neuronal nuclei), a marker for neuronal cells, within dentate gyrus (Figure 4.2 & 4.3).

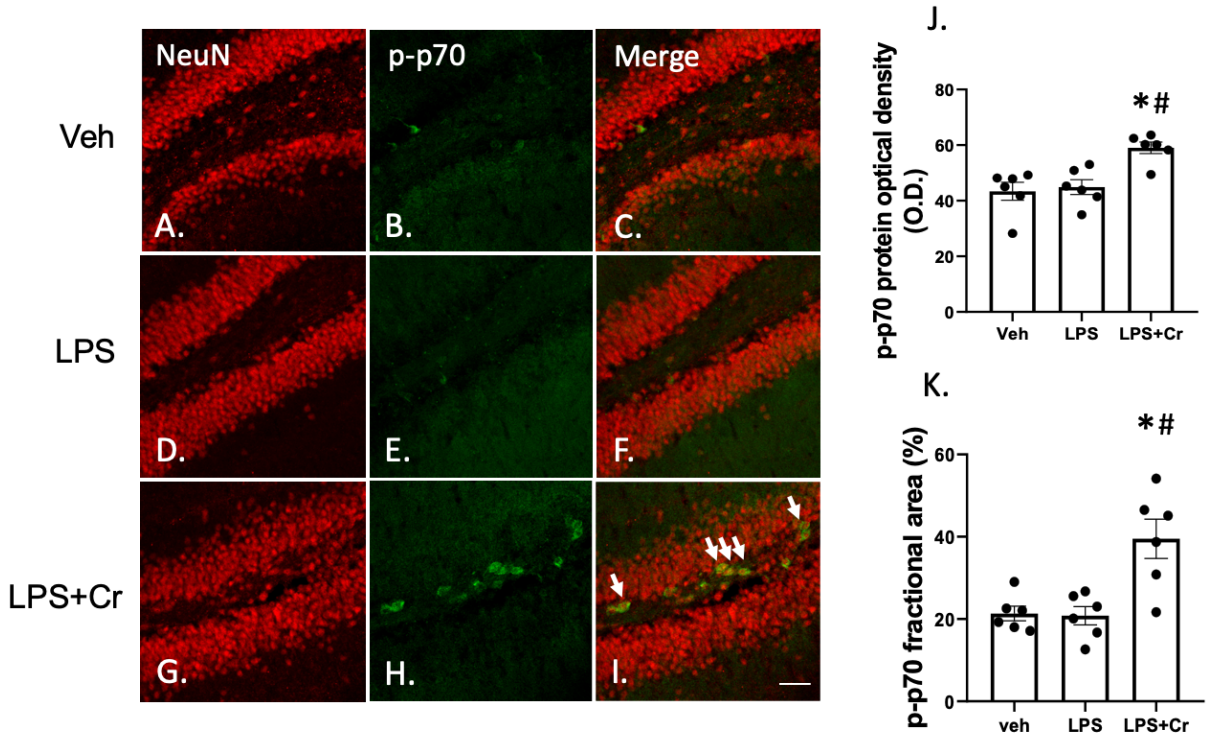

Figure 4.2. Phosphorylation of p70S6K (p-p70) protein within neuronal cells in dentate gyrus of veh, LPS, and LPS+Cr group. The representative photomicrographs depict neurons (NeuN; red; A., D., G.) and p-p70 (green; B., E., H.) in dentate gyrus. C., F., I. Representative image of merged photomicrographs of NeuN and p-p70. The white arrows indicate p-p70-positive neuronal cells. p-p70 protein expression within neurons is expressed as optical density (J.) and fractional area (%) (K.). The significance of differences among groups were analyzed using One-way ANOVA, * significantly different from Veh, $P < 0.05$, # significantly different from LPS, $P < 0.05$. Scale bar: 50 μm.

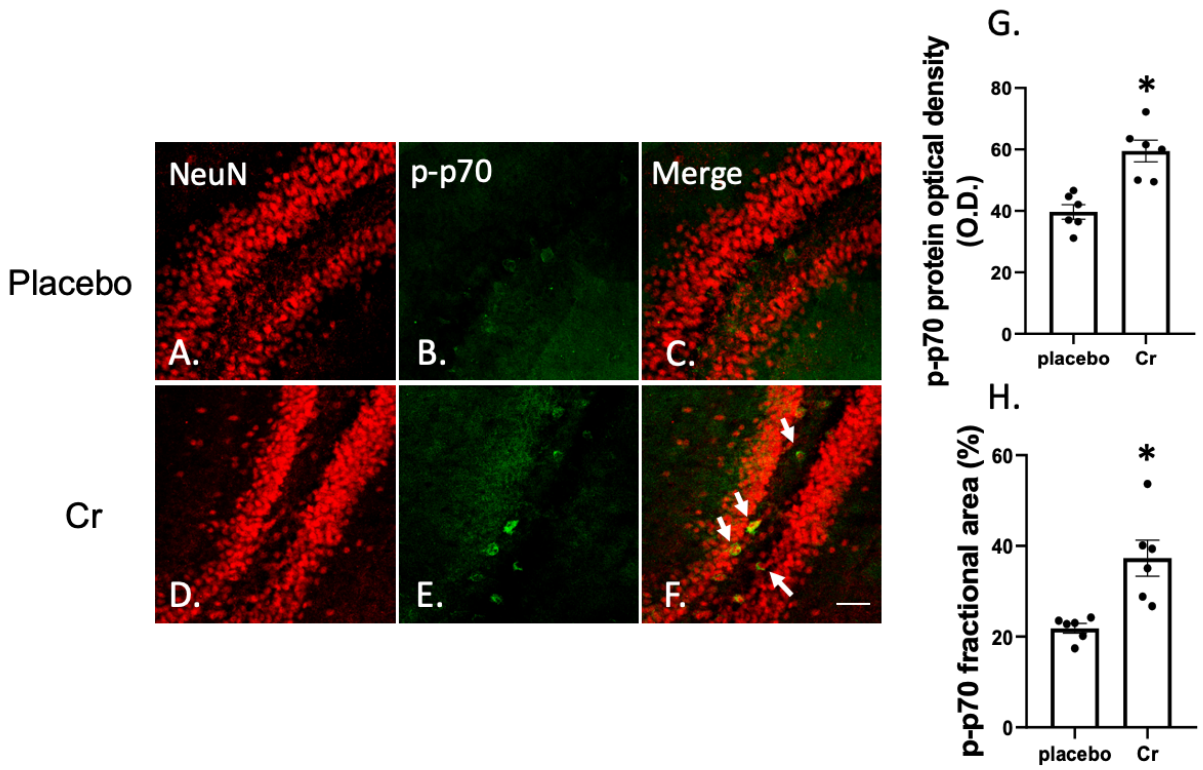

Figure 4.3. Phosphorylation of p70S6K (p-p70) protein within neuronal cells in dentate gyrus of placebo and Cr group. The representative photomicrographs depict neurons (NeuN; red; A., D.) and p-p70 (green; B., E.) in dentate gyrus. C., F. Representative image of merged photomicrographs of NeuN and p-p70. The white arrows indicate p-p70-positive neuronal cells. p-p70 protein expression within neurons is expressed as optical density (G.) and fractional area (%) (H.). The significance of difference between groups was analyzed using Student's *t* test, * significantly different from Veh, $P < 0.05$. Scale bar: 50 μm.

According to IHC analysis, p70S6K protein phosphorylation was found to be upregulated by Cr supplementation, regardless of the presence or absence of LPS, which matches previous western data in chapter 1. Moreover, increased p70S6K phosphorylation was apparently elevated in cells expressing NeuN, informing that the activation of mTORC1 signaling in neurons possibly mediated cognitive effects of Cr supplementation through enhancing translation of synaptic proteins. This result is not unexpected given that numerous literatures suggested [193],[205] that mTORC1 signaling mediates the synaptic events within neuronal cells and, thus, may be beneficial to cognition. Taken together, this finding illustrates novel insight into Cr supplementation and its neuronal effects based on in-vivo experimental model.

The dentate gyrus is constituted by three layers. The molecular layer, which is relatively cell free, the polymorphic layer that are predominately resided by mossy cells, and the principal cell layer called granule layer, which is made up of densely packed granule cells [206]. Interestingly, the spreading of p-p70-positive neurons is seemingly to mainly locate at the boundary area of the granule and polymorphic layers, at which there are pyramidal basket cells [206]. While pyramidal basket cells are inhibitory interneurons, which produce and release inhibitory neurotransmitter, GABA. Large amounts of existing evidence indicate that inhibitory interneurons play a central role of gating learning and memory function, and their malfunction could lead to neurodegenerative diseases [207],[208]. Although It is currently unknown that how mTORC1 signaling modifies the inhibitory interneurons, or pyramidal basket cells, to mediate cognitive processing, the present IHC data implies that enhanced mTORC1 signaling may be mediated through this particular population of neurons to modulate cognitive function, following Cr supplementation. Future studies need to examine more in-depth as to the role of mTORC1 and Cr supplementation in specific neuronal subtypes.

Potential link between Cr supplementation and enhanced mTORC1 signaling

It remained mysterious that how Cr supplementation activate the mTORC1 signaling in the CNS, as the present study did not investigate the potential linkage between Cr and enhanced mTORC1 signaling. However, it is deserved to be hypothesized in order to gain a more comprehensive understanding of Cr supplementation and its underlying neuro-molecular mechanisms. There is no doubt that Cr is an energy buffer, which mediates the ADP/ATP ratio through the creatine kinase reaction [209]. AMP-activated protein kinase (AMPK) is a conserved sensor of cellular energy changes and, AMPK could be activated by increased ADP/ATP and AMP/ATP ratios [210]. Through its detection of cellular energy, AMPK dynamically regulates the ATP-consuming processes, such as molecular signaling that mediates protein synthesis and translation of ribosomal proteins. One of major downstream signaling pathways regulated by AMPK is the mTORC1 signaling and, recent findings suggested that activated AMPK suppresses mTORC1 signaling [211]. As a result, it is possible that, in the present study, Cr supplementation decreased ADP/ATP ratio, which decreased the activation of AMPK and, then in turn, increased mTORC1 signaling. It might be very interesting because, if Cr supplementation changed ADP/ATP ratio, partial cognitive benefits seen from Cr supplementation in the present study could come from increased energy source when energy demand is high during the cognitive behavioral tasks. Another potential mechanism that links Cr supplementation and enhanced mTORC1 signaling could be IGF-1 signaling. It was reported that Cr regulates insulin-like growth factor (IGF-1) secretion, which binds to the IGF-1 receptor and triggers downstream phosphatidylinositol-3-kinase (PI3K)-AKT signaling. Accumulated evidence pointed that activated IGF-1 signaling and AKT signaling are upstream mechanisms that regulate the mTORC1 signaling pathway. As such, it is possible that Cr upregulates mTORC1 signaling could also through stimulating IGF/PI3K/AKT signaling cascades.

Neuroinflammation elicited by LPS is not influenced by Cr supplementation

It is well known that chronic low-grade inflammation can be detrimental physiological systems throughout the whole body. Chronic inflammation caused by aging could lead to damage to the skeletal muscle, bone and cognitive function [212]–[214]. Moreover, chronic inflammation is associated with onset and progression of neurodegenerative diseases, such as MCI.

Early study [215], found that 1-hour after repeated-sprint exercise in human subjects, Cr supplementation significantly reversed sprint exercise elicited elevated plasma pro-inflammatory factors level, implying that Cr may possess an anti-inflammatory property. Along with some other reports, anti-inflammatory effects of Cr supplementation were demonstrated in a variety of models of inflammation, both in-vitro and in-vivo [216]–[220]. While it remains controversial, as some other studies did not demonstrate or observe the anti-inflammatory effects of Cr supplementation.

In chapter 2 and 3, 6 weeks of Cr supplementation was not found to alter the elevated pro-inflammatory level elicited by LPS. Furthermore, markers for reactive astrocytes (GFAP) and microglia (IBA-1) were not changed by Cr supplementation. Though those results conflict with some previous reports, exact molecular mechanisms underlying the anti-inflammatory effects of Cr supplementation were not elucidated by previous studies. Current evidence regarding the effectiveness of Cr supplementation to suppress pro-inflammation might be model and species dependent. Given the mixed models and species used by previous experiments, it is necessary to investigate whether Cr supplementation interfere with the toll-like receptors and canonical NF-κB signaling, of which are activated by LPS to elicit pro-inflammatory effects [145]. The current dissertation did not examine the specific effector receptors and inflammatory pathway relevant to Cr supplementation, as it is beyond the scope of current research aim.

Lastly, the anti-inflammatory effects induced by Cr supplementation observed by previous studies can be assumed to be due to the antioxidant capacity of Cr [221]. Generally, oxidative stress and inflammation interplays with each other to further amplify the effects, while solid evidence existed

demonstrates that Cr supplementation has a direct antioxidant activity [222]. Consequently, it is possible to assume that the anti-inflammatory effects exerted by Cr supplementation is a result of diminished oxidative stress.

Interestingly, although Cr was not found to alter proinflammatory status within targeted brain regions, rapamycin application was shown to suppress the activation of microglia and proinflammatory. It was demonstrated that suppressed mTORC1 signaling within immune cells could downregulate the secretion and production of proinflammatory cytokines, through directly inhibiting phosphorylation of NF-κB p65 subunit and NF-κB signaling activation induced by LPS [223]. Additionally, suppressed mTORC1 signaling induced by rapamycin was reported to enhance microglial autophagy and thus, inhibiting activation of microglia and production of proinflammatory cytokines elicited by LPS in vivo [224]. Although the investigation of microglial activation status and its upstream regulators is beyond the scope of current study, it is possible that rapamycin induced suppression of mTORC1 signaling may serve as an upstream regulator within immune cells to downregulate LPS induced proinflammation. While mTORC1 signaling activation following Cr supplementation may primarily only occur within neuronal cells, without interfering inflammation (Figure 4.2). Collectively, those data implied that mTORC1 signaling may have a cell-specific effect to regulate different downstream signaling pathways and targets. As such, a cell-specific knockdown of mTORC1 signaling may ensure a more comprehensive view regarding its functional role under different scenarios.

Limitations and Future directions

The current dissertation has examined spatial learning and memory, recognition memory, memory retention and cognitive flexibility following Cr supplementation. However, there are also other cognitive domains remained to be tested for a more comprehensive view of benefits of Cr supplementation. Correspondingly, the brain regions involved in cognitive processing, except the dentate gyrus, should be analyzed further to gain a more thorough understanding toward neural

networks forming cognitive benefits of Cr supplementation. Currently, there is a lack of agreement concerning to the optimal dosage of Cr supplementation for brain health, future studies should perform multiple different dosing protocols to compare each effect resulted from different durations and dosages, which certainly will prepare the field to cross-comparing neuronal effects of Cr supplementation.

The principal consideration of not using genetic approach to knockdown mTORC1 signaling was due to the fact that LPS, or inflammation, can negatively affect the transgene expression by adeno-associated virus (AAV) [225]. In this study, the persistent level of AAV vectors in target tissue indicated that the loss of transgene expression might be a direct consequence of transcriptional silencing due to inflammation. However, although different promotors driving transgene transcription may respond to inflammation differently [225], it is highly risky to utilize AAV or genetic approach to knockdown genes in a neuroinflammatory disease model. In contrast to the genetic approach, the use of rapamycin to knockdown mTORC1 signaling in chapter 2 did bring some concerns: 1). Studies have reported that chronic use of rapamycin could lead to side effects and unwanted blockage of mTORC2 signaling in certain tissues, which may result in confounding factors to the data interpretation and experimental observation; 2). The pharmacological approach cannot selectively inhibit mTORC1 signaling in specific cell type, rendering the inconclusive view regarding to the functional role of mTORC1 in mediating cognitive function. As a result, although inflammation may be a limiting factor for viral-expressing transgenes, it is possible to knockdown mTORC1 signaling prior to the induction of inflammation to testing the role of mTORC1 signaling behind Cr supplementation. Furthermore, inducible transgenic animal models may be used to either knockdown mTORC1 signaling or overexpress innate inhibitor for mTORC1 protein complex, such as Deptor and PRAS4 proteins, where they promote the inhibition of mTORC1 complex [226],[227].

It is known from current dissertation work that Cr activates mTORC1 signaling in the central nervous system, while the upstream mechanism that connects Cr to the activation of mTORC1 signaling in the brain remain elusive. One hypothesis remains to be tested is that insulin-like growth factor 1 receptor (IGF-1R) signaling seemingly mediates this coupling that leads to both molecular and functional outcomes found in chapter 2. It is possible that Cr supplementation stimulates insulin-like growth factor (IGF-1) secretion, which then binds and activates IGF-1R to trigger downstream phohphatidylionositol-3-kinase (PI3K)-AKT (protein kinase B) signaling, leading to the phosphorylation of mTOR [228],[229]. Furthermore, resistance training has been found to initiate the activation of IGF-1R signaling and, improve cognitive function [136]. It is normal to combine resistance training and Cr supplementation to enhance the training effects in skeletal muscle, thus it will be also interesting to see that if this combination can also exert accumulated cognitive effects in the central nervous system. Except the specific upstream mechanism, it is equally important to study whether Cr supplementation affects the central nervous system locally or indirectly through peripheral mechanism. In chapter 3, Cr treatment has been shown to directly upregulate the mTORC1 signaling activity in PC12 cells, suggesting that Cr is sufficient to cause effects in neuronal cells. Although it is very preliminary, future study could knockdown or knockout, though inducible transgenic animal model, creatine transporter gene in the blood brain barrier to testing that whether Cr requires to enter central nervous system to elicit cognitive effects.

The current dissertational work provides a basis of neurocognitive and neuro-molecular mechanisms underlying Cr supplementation, which will pave the way for future more in-depth investigations. Although there are limitations and unsolved problems accompanying my experiments, this work sparks the potential study of mTORC1 signaling behind the cognitive effects of Cr supplementation and, provides fundamental support for clinical trials of using Cr supplementation as a therapeutic treatment against cognitive deficiency.

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Appendix

Appendix A: Abstracts from first authored original research manuscripts

Transcriptomic analysis reveals different molecular signaling networks in between selectively bred low voluntary wheel running and wild-type rats after injection of AP-1 inhibitor into nucleus accumbens

Xuansong Mao, Kolter B. Grigsby, Taylor J. Kelty, Nathan R. Kerr, Tom E. Childs, Frank W. Booth

Abstract

Understanding the neuro-molecular mechanisms that mediate the physical activity level is of paramount significance, given the tremendous health benefits associated with physical activity. Here, we examined the effects of intra-nucleus accumbens (NAc) inhibition of activator protein-1 (AP-1), an important transcriptional factor downstream to the cAMP response element binding protein (CREB), on voluntary wheel running behavior in wild-type (WT) and low voluntary running (LVR) female rats, respectively. A followed transcriptome analysis was performed in order to further dissect molecular changes between experimental animals received Veh and AP-1 inhibitor. Within WT rats, AP-1 inhibition caused a significant decrease in overnight running distance in comparison to the Veh-injected WT rats (p = 0.009). Following transcriptomic and bioinformatic analysis have identified gene products that were reported to regulate rewarding process through different signaling mechanisms. In addition, cellular function and network analysis revealed involvement of molecules that regulate cellular proliferation and development, which were cellular processes regulated by AP-1. In contrast, intra-NAc AP-1 inhibition in LVR rats significantly increased nightly running distance in comparison to the Veh-injected LVR rats (p = 0.0008). Further analysis identified gene products that are associated with regulating intracellular Ca^{2+}

homeostasis, calcium ion binding and neuronal excitability. In short, our study aims to gain a comprehensive understanding of transcriptional profile that was due to AP-1 inhibition in NAc, in which it could not only enhance the knowledge regarding molecular regulatory loops within NAc for modulating voluntary running behavior, but also provide further insights into molecular targets for future investigations.

Appendix B: Abstracts from co-authored original research manuscripts

Resistance-exercise training ameliorates LPS-induced cognitive impairment concurrent with molecular signaling changes in the rat dentate gyrus

Taylor J Kelty, Todd R Schachtman, **Xuansong Mao**, Kolter B Grigsby, Thomas E Childs, T Dylan Olver, Paige N Michener, Rachel A Richardson, Christian K Roberts, Frank W. Booth

Journal of Applied Physiology, July 2019, Volume: 127(1): 254-263

Abstract

Effective treatments preventing brain neuroinflammatory diseases are lacking. Resistance-exercise training (RT) ameliorates mild cognitive impairment (MCI), a forerunner to neuroinflammatory diseases. However, few studies have addressed the molecular basis by which RT abates MCI. Thus experiments were performed to identify some molecular changes occurring in response to RT in young, female Wistar rats. To induce MCI, intraventricular lipopolysaccharide (LPS) injections were used to increase dentate gyrus inflammation, reflected by significantly increased TNF-α (~400%) and IL-1β (~1,500%) mRNA ($P < 0.0001$) after 6 wk. Five days after LPS injections, half of LPS-injected rats performed RT by ladder climbing for 6 wk, 3 days/wk, whereas half remained without ladders. RT for 6 wk increased lean body mass percentage ($P < 0.05$), individual muscle masses (gastrocnemius and tibialis anterior) ($P < 0.05$), and maximum lifting capacity ($P < 0.001$). The RT group, compared with sedentary controls, had 1) ameliorated spatial learning deficits ($P < 0.05$), 2) increased dentate gyrus phosphorylation of IGF-1R, protein kinase B, and GSK-3β proteins ($P < 0.05$), components of downstream IGF-1 signaling, and 3) increased dentate gyrus synaptic plasticity marker synapsin protein ($P < 0.05$). Two follow-up experiments (without LPS) characterized dentate gyrus signaling during short-term RT. Twenty-four hours following the third workout in a 1-wk training duration, phosphorylation of ERK1/2 and

GSK-3β proteins, as well as proliferation marker protein, PCNA, were significantly increased ($P < 0.05$). Similar changes did not occur in a separate group of rats following a single RT workout. Taken together, these data indicate that RT ameliorates LPS-induced MCI after RT, possibly mediated by increased IGF-1 signaling pathway components within the dentate gyrus.

Resistance-exercise training attenuates LPS-induced astrocyte remodeling and neuroinflammatory cytokine expression in female Wistar rats

Taylor J. Kelty, **Xuansong Mao**, Nathan R. Kerr, Thomas E. Childs, Gregory N. Ruegsegger, and Frank W. Booth

Journal of Applied Physiology, Jan 2022, Volume: 132(2): 275-580

Abstract

Neuroinflammation is an early detectable marker of mild cognitive impairment, the transition state between normal cognition and dementia. Resistance-exercise training can attenuate the cognitive decline observed in patients with mild cognitive impairment. However, the underlying mechanisms of resistance training effects are largely unknown. To further elucidate mechanisms of the known cognitive health benefits from resistance-exercise training, we tested if resistance-exercise training could ameliorate lipopolysaccharide-induced neuroinflammation. Five-week-old female Wistar rats received intracerebroventricular injections of lipopolysaccharides to induce neuroinflammation and cognitive impairment. Rats then underwent 3 wk of progressive ladder climbing to recapitulate resistance-exercise training in humans. Cognition was assessed toward the end of the training period by novelty object recognition testing. Neuroinflammation was measured one and 24 h after the last resistance-exercise training workout. Resistance-exercise training ameliorated cognitive impairment, diminished lipopolysaccharide-induced neuroinflammatory cytokine expression, and attenuated astrocyte remodeling in the dentate gyrus 24 h post exercise. Here, we provide evidence that the ladder-climbing model of resistance-exercise training in rats can improve cognition as early as 3 wk. In addition, these data support the hypothesis that resistance exercise can reduce lipopolysaccharide-induced neuroinflammation in the dentate gyrus.

Acute wheel-running increases markers of stress and aversion-related signaling in the basolateral amygdala of male rats

Kolter B. Grigsby, Nathan R. Kerr, Taylor J. Kelty, **Xuansong Mao**, Thomas E. Childs, and Frank W. Booth

Abstract

Physical activity (PA) is a non-invasive, cost-effective means of reducing chronic disease. Most US citizens fail to meet PA guidelines, and individuals experiencing chronic stress are less likely to be physically active. To better understand the barriers to maintaining active lifestyles, we sought to determine the extent to which short- versus long-term PA increases stress- and aversion-related markers in wild-type (WT) and low voluntary running (LVR) rats, a unique genetic model of low physical activity motivation. Here, we tested the effects of 1- and 4-weeks of voluntary wheel-running on physiological, behavioral, and molecular measures of stress and Hypothalamic Pituitary Adrenal (HPA)-axis responsiveness (corticosterone levels, adrenal wet weights, and fecal boli counts). We further determined measures of aversion-related signaling (kappa opioid receptor, dynorphin, and corticotropin releasing hormone mRNA expression) in the basolateral amygdala (BLA), brain region well characterized for its role in anxiety and aversion. Compared to sedentary values, 1-, but not 4-weeks of voluntary wheel-running increased adrenal wet weights and plasma corticosterone levels, suggesting that HPA responsiveness normalizes following long-term PA. BLA mRNA expression of Prodynorphin (*Pdyn*) was significantly elevated in WT and LVR rats following 1-wk of wheel-running compared to sedentary levels, suggesting that aversion-related signaling is elevated following short-, but not long-term wheel-running. In all, it appears that the stress effects of acute PA may increase molecular markers associated with aversion in the BLA, and that LVR rats may be more sensitive to these effects, providing a potential neural mechanism for their low PA motivation.

Appendix C: Presented abstracts outside of dissertation topics

Impacts of short-term inhibition of PKA in Nucleus Accumbens on voluntary wheel running

Xuansong Mao, Kolter B. Grigsby, and Frank W. Booth

Objectives: Based upon a Booth lab goal of establishing molecular regulators of physical activity motivation, my current study focuses on the effects of short-term inhibition of protein kinase A (PKA) activity in the nucleus accumbens (NAc). The NAc is a brain region integral to motivated behaviors. Downstream immediate-early gene (IEG) expression from PKA has been shown to exhibit rapid responses to acute stimuli, such as voluntary wheel-running behavior. According to previous work in our lab, long-term NAc overexpression of the endogenous PKA inhibitor, Protein Kinase Inhibitor Alpha (PKIα), increased nightly running distance in rats selectively bred for low voluntary running (LVR) behavior (Mol Neurobiol 2018 Jun 21). However, paradoxically, the same PKIα overexpression failed to increase running distance in wild-type (WT) rats. It is known that chronic manipulation of the NAc PKA pathway produces different molecular (gene expression profiles) and behavioral outcomes from that of acute manipulations. Given the above, the goal of the current work is to determine how short-term inhibition of PKA in the NAc influences its downstream gene networks and the nightly voluntary running behavior in WT rats.

Methods: An ex vivo preparation of the NAc was utilized to determine the effects of Rp-cAMPS, a selective protein kinase A inhibitor, upon its stimulation of dopamine D1-like receptor agonist SKF 38393 on downstream gene expression level in sedentary WT female rats. Further, real-time PCR was implemented to analyze the transcriptional expression of IEGs (Homer-1, Arc, Zif268) following Rp-cAMPS administration.

Results: Data showed that there were no significant difference of mRNA level for Homer-1, Arc or Zif268 among the vehicle, 50uM, 100uM and 200uM Rp-cAMPS treatment groups upon the stimulation of 10uM SKF 38393.

Conclusions: In addition to the PKA, other protein kinases such as Ca++ activated and growth factor activated kinases have both been shown to phosphorylate CREB at Ser133, and thus, lead to activation of gene transcription. Given the above results of the ex vivo experiment, in which NAc slices were treated with multiple dosages of Rp-cAMPS concurrent with the stimulation of SKF 38393, it is possible that other protein kinase pathways could be compensating the effects of short-term inhibition of PKA and, in turn, lead to no difference of IEG expression. Further experiments will need to be performed in order to testify this hypothesis.

Overexpression of Protein Kinase Inhibitor Alpha increases low voluntary running motivation

Xuansong Mao, Kolter B. Grigsby, Frank W. Booth

Significance and hypothesis: Physical inactivity is associated with the risk of 40 chronic diseases and has been identified as the fourth leading risk factor for global mortality. Therefore, efforts to increase physical activity level would be beneficial to health. In order to study the low physical activity, Booth's lab has developed a low voluntary running (LVR) rat line. Therefore, this study investigates the molecular trigger for increasing physical activity motivation. We hypothesize that overexpression of protein kinase inhibitor alpha (PKIa) in nucleus accumbens (NAc) increases voluntary running motivation. **Methods**: Wild Type (WT) and LVR male rats were injected with either adeno-associated virus (AAV) expressing an empty-vector (EV) or AAV driving the overexpression of PKIa within the NAc. Following 3 weeks running observation, a running wheel based operant runway test (ORT) was deployed to determine the voluntary running motivation. Endogenous genes expression were then assayed via qRT-PCR. **Results**: WT and LVR male rats following AAV-PKIa overexpression showed no running distance difference compared with their AAV-EV control during the course of 3 weeks. Yet, LVR male rats following overexpression of PKIa spent significantly less time to approach the running wheel than AAV-EV control. **Conclusions**: Overexpression of PKIa did not increase nightly running distance in both WT and LVR male rats; however, the low motivation of LVR males were significantly increased in approaching the running wheel by the overexpression of PKIa in NAc. It is possible that the larger body weight of male rats prevents them from increasing running distance following the overexpression of PKIa even under the increased voluntary running motivation. Future study should also investigate both voluntary running distance and motivation in female rats following the overexpression of PKIa.

Chronic creatine supplementation and resistance training rescue cognitive deficits in a lipopolysaccharide (LPS) induced mild cognitive impairment rodent model

Xuansong Mao, Taylor J. Kelty, Nathan R. Kerr, Heidi S. Green, Frank W. Booth

Background and rationale: Mild cognitive impairment (MCI) is a mental disorder defined by memory loss and cognitive decline, in which it has a great risk of progressing to dementia, such as Alzheimer's disease. Given the fact that dementia results in more severe cognitive deficits and more substantial negative effects associated with daily life, targeting the MCI phase as a therapeutic window for preventing further progression to a more irreversible condition becomes an ideal approach to maintain the function for daily life. In recent years, creatine (Cr) supplementation has been revealed to benefit, not only the normal brain functions, but also the impairment of brain functions; therefore, Booth lab has determined to investigate the role of Cr in a LPS induced MCI rodent model and its functional mechanisms related to the cognitive deficits. Furthermore, our lab has showed and published that 6 weeks of resistance training (RT) ameliorates the MCI induced by LPS in the same rodent model (Kelty et al., 2019), we have also determined to study that whether RT has an additive effects on MCI when Cr supplementation is being orally administered. **Methods:** Female Wistar rats at 7-8 weeks old were injected with LPS to induce MCI. After one week recovery, LPS-injected rats were randomly chosen to accept oral Cr administration, a dosage determined by conversion of human equivalent doses (HEDs) to animal doses based on body surface area (BSA), or both oral Cr administration and RT (Cr + RT) for 6 weeks. Barnes Maze Test (BMT) was deployed at the end of the study, after which all rats were sacrificed and dentate gyrus were collected. **Results:** 6 weeks of RT significantly increased maximal lifting capacity ($P<0.001$) and individual muscle masses (gastrocnemius, $P<0.001$, plantaris, Tibialis anterior, Extensor digitorum longus, $P<0.05$). Both Cr administration and Cr + RT groups significantly decreased time latency and errors for nose poke ($P<0.05$), step down

(P<0.001), entry into the goal box (P<0.05) in BMT; while there was no difference found between Cr and Cr + RT group.

Vita

Xuansong Mao was born in September of 1992 in Zhenjiang, Jiangsu Province, China. Xuansong Mao graduated from Soochow University with his Bachelor of Science (B.S.) in sports training in May of 2015. Xuansong Mao then was granted Master of Science (M.S.) in exercise science in August of 2017. Xuansong Mao joined Department of Biomedical Sciences at University of Missouri where he began his Ph.D. training under Dr. Frank W. Booth's mentorship. His study and research focused on molecular biology with a particular specialization on neuroscience. After 4.5 years training, Xuansong Mao defended his dissertation thesis and graduated from Dr. Frank Booth lab. Xuansong Mao aims to continue his research by investigating exercise and neurodegenerative diseases for his future career.