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INVESTIGATION OF NEUROPROTECTION BY NAD+ IN RESPONSE TO

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by

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INVESTIGATION OF NEUROPROTECTION BY NAD+ IN RESPONSE TO DEEP SPACE RADIATION

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ABSTRACT

Background: As space programs such as NASA prepare for interplanetary missions to Mars, the potential hazards of long-term space travel are being increasingly considered. Of these hazards, astronaut exposure to galactic cosmic radiation (GCR) is of specific concern. Despite increased efforts to study the consequences of deep space radiation exposure, studies have been inconsistent in their dosage and types of radiation used. This study marks the first to use a simulated form of GCR, accurately representing the dosage and full spectrum of ions astronauts would encounter on an extended journey to Mars. The disruption of cognition and behavior by radiation has been a hallmark of previous investigations, however, it is unknown whether accurate full-spectrum GCR causes similar impairment. Additionally, reports exploring the efficacy of radioprotectant drugs are lacking despite their potential utility in deep space travel. Nicotinamide Mononucleate, an NAD+ precursor, shows promise for radioprotection, as its roles in promoting DNA repair and longevity pathways in mice are well established.

Objective: To define behavioral responses to accurate full-spectrum Galactic Cosmic Radiation (GCR) in mice and test the neuroprotective potential of an NAD+ booster, nicotinamide mononucleotide (NMN).

Methods: Male C57BL/6 mice (n=48) began NMN treatment (600 mg/kg daily) and GCR radiation exposure at six months of age. Mice (n=24) were exposed to GCR for

24 total days for a total dose of ~49.92 cGy before being shipped to Harvard Medical School for analysis. Between nine and 11.5 months of age, all mice underwent a series of behavioral assays. Learning and memory behaviors were assessed using the Barnes Maze test and measured by comparing time spent at the target hole and target quadrant. Anxiety and motility were assessed using the Open Field test and measured by comparing the % time spent in the center of the maze and total distances traveled. Dominance and aggression were assessed using the Tube Dominance test and measured in total number of bouts won by each mouse.

Results: Mice from all four groups showed no difference in percentage of time spent at the target hole or in percentage of time spent within the target quadrant, indicating that mouse learning and memory were unaffected by chronic GCR and NMN treatment. Mice from all four groups showed no difference in percentage of time spent in the center of the maze, or in total distance traveled, suggesting that mouse anxiety and motility were unaffected by chronic GCR and NMN treatment. Irradiated mice were found to be significantly more dominant than non-irradiated mice as determined by the Tube Dominance test. When comparing irradiated mice +/- NMN, it was found that NMN treated mice scored 90.9% lower in dominance.

Conclusions: Behavioral results indicate very limited potential for neurological impairment by accurate, full-spectrum GCR in mice, however, further research is needed to confirm this. Mouse dominance behavior was found to be affected by GCR, suggesting future behavioral studies should include dominance and aggression analyses. NMN shows potential as a novel radioprotective agent that should be further investigated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DSB.. Double strand break

ECG...Electrocardiogram

GCR .... Galactic Cosmic Radiation

HZE.. High Charge & Energy

ISS.. International Space Station

LEO... Low Earth Orbit

ROS... Reactive oxygen species

NAD...Nicotinamide Adenine Dinucleotide

NMN ....Nicotinamide Mononucleate

NR..Nicotinamide Riboside

ROS... Reactive oxygen species

1. INTRODUCTION

Biomedical studies exploring the health risks associated with deep space travel are a fundamental part of preparing for future missions to Mars. Of the potential dangers facing would-be space travelers, radiation is perhaps the most daunting; as its potential to cause biochemical harm is well established (Darby et al., 2005; Hayashi et al., 2003; Mossman, 2012). Biological life is protected from galactic cosmic radiation (GCR) by Earth's magnetic field, however, beyond low Earth orbit (LEO), humans are exposed to GCR levels that are nearly 10 times higher than on the international space station (ISS) (Nelson, 2016). This form of ionizing radiation originates from outside our solar system and is composed primarily of high energy protons (~87%), and helium ions (~12%). GCR is unique in its inclusion of heavier elements, commonly referred to as high charge and energy (HZE) nuclei, making up the final 1-2% (G.D. Badhwar, P.M.O'Neill 1994).

1.1 Mechanism of Damage and Danger to Humans

Ionizing radiation has many useful applications, particularly in a clinical setting where X-rays, radiotherapy, and fluoroscopy are instrumental in diagnosing and treating disease (Andrew J. Einstein, 2009). While GCR differs from these clinically relevant forms of radiation, they are alike in their ability to ionize atoms and therefore share a similar potential for harm. Figure 1, a nuclear emulsion plate worn on the ankle of Neil

Armstrong during the Apollo 11 lunar landing mission, illustrates how these high energy
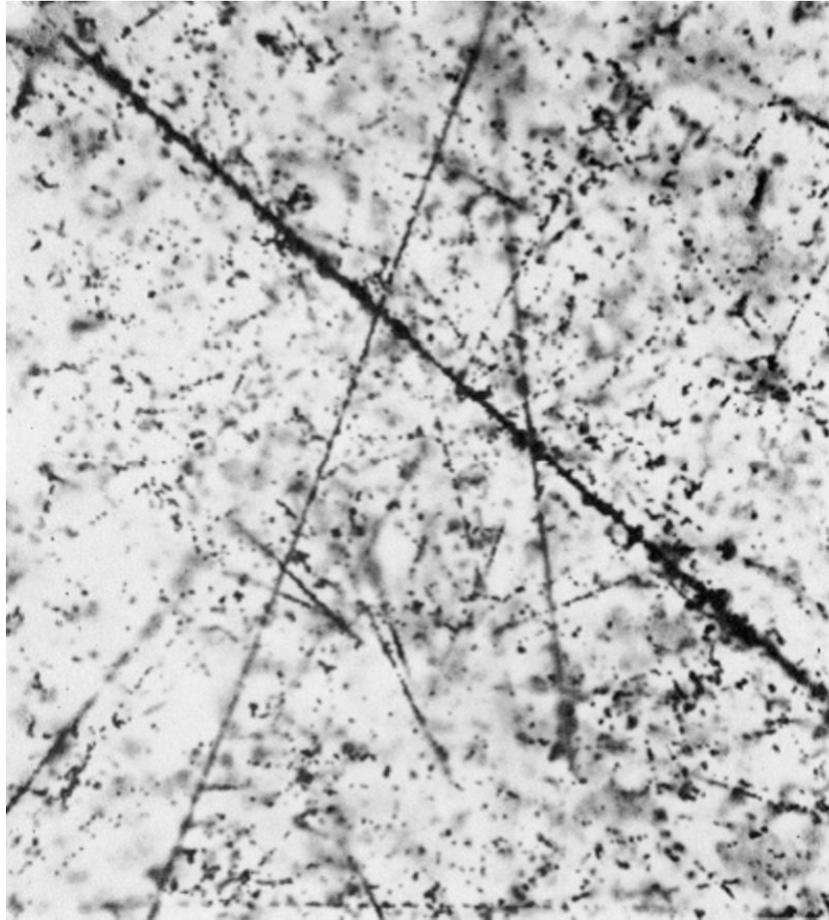

Figure 1: An illustration of the ambient radiation exposure in deep space as a microscopic field of view (140 x 165 μm) of an Ilford G.5 nuclear emulsion that was worn on the ankle of Neil Armstrong during the Apollo 11 lunar landing mission in July, 1969. Individual silver grains due to gamma rays and electrons form a background against which numerous proton tracks can be seen, along with one track of a heavy ion with an estimated Z of 10–12. Fig. 1 of Schaefer et al. 1972.

ions may disrupt their immediate environment (Schaefer et al., 1972). The largest streak

in the emulsion was traced by an ion with an estimated Z of 10-12, further demonstrating

how heavier ions associated with GCR are of particular concern (Schaefer et al., 1972).

HZE ions are able to penetrate several inches of material/tissue (spacecraft aluminum for

example) and create a cascade of ionizing damage within the materials they are passing;

producing a penumbra of secondary particles capable of similar damage (Straume et al., 2017). It has been estimated that while in the deep space environment, each of an astronaut's cells will be traversed by a proton once every three days, and by a heavy ion (Z>2) once every few months (Norbury et al., 2016; NASA, 2015). On a cellular level, this can disrupt sensitive organelles, enzymatic function, and perhaps most critically, DNA structure (Stephen P. Jackson, 2002). Over time, cell dysfunction can lead to damaged tissues and functional changes in entire organ systems.

An astronaut on a three year trip to Mars is expected to encounter an absorbed dose of radiation between ~0.3 and ~0.6 Gy (Cucinotta et al., 2014). For context, this is approximately 50 to 100 years' worth of normal (Earth) background radiation; although biological effects vary widely between individuals and with different types of radiation (United States Nuclear Regulatory Commission, 2019). Acute radiation sickness is typical for doses above 0.7 Gy, with symptoms emerging at doses as low as 0.3 Gy when delivered as a single dose (CDC, 2018). Considering this, significant acute effects from GCR would not be expected, however, in a three year mission to Mars, exposure may still present itself before completion.

In the context of space travel, the potential disruption of an astronaut's CNS and cognition poses a particular threat. Previous investigations have shown significant cognitive performance deficits and brain structure differences in astronauts following space flight to the ISS (Pattyn et al., 2007), and after long-term ISS missions (Roberts et al., 2019). While these results may only reflect the low Earth orbit environment in which

the ISS is located, exposure to GCR beyond LEO may amplify these effects and further research is needed to understand its role in astronaut CNS function.

1.2 Animal Models of GCR

Studying human neural mechanisms in response to GCR is limited by physical and ethical concerns. Most studies involving human responses to radiation are retrospective and non-specific to GCR (Darby et al., 2005; Hayashi et al.). Additionally, reports involving astronauts previously exposed to GCR are limited by individual susceptibility and low sample sizes (Cucinotta, et al., 2008). The use of animal models has shed light on the physiological and behavioral responses associated with different types and doses of radiation. Acute, high-dose ionizing radiation exposure in mice has been shown to induce mucosal damage, stem cell loss, and immune suppression along with long-term instances of cancer and fibrosis in every major organ system (Deckhunt et al., 2005). These findings are well established and often replicated in stem cell and cancer related biology studies (Booth et al., 2005; Hu et al., 2010). In regard to CNS function, acute high-dose radiation has been shown to cause neurotoxicity and neurodegeneration in mice and humans through mechanisms that are poorly understood (Smart, 2017).

Less is known about the effects of low-dose chronic radiation exposure, a category that includes GCR. Studies exposing male and female mice to 400 days (20mGy/day) of gamma radiation, reported a decrease in lifespan, increased neoplasm/cancer incidence rate, chromosome abnormality, and immunodeficiency (Braga-Tanaka et al., 2018). Previously, the challenge of accurately modelling the entire GCR spectrum has been a

major limitation. Because of this, radiation in previous studies was typically delivered in a single acute dose, often simulating GCR with a single form of HZE nuclei ($_{48}$Ti or $_{16}$O for example) (Parihar et al., 2016). Very few investigators have looked at chronic, space relevant-dose radiation in rodents (Sokolova et al., 2015; Acharya et al., 2019; Limoli et al., 2018) and to-date, none have investigated the effects of chronic full-spectrum GCR in mice.

1.3 GCR and Rodent Cognition

The ability of high energy ions to create double stranded DNA breaks, triggering mitotic cellular collapse, is one of the main reasons for increased radio-sensitivity in quickly dividing cell populations (Vignard, Mirey, & Salles, 2013; Eric J. Hall & James D.Cox, 2010). Previously it was thought that this aspect of radiation left the CNS (with limited mitotic potential) resistant to ionizing radiation. However, recent evidence has shown that other subcellular mechanisms have the ability to cause CNS dysfunction following exposure to ionizing radiation (Smart, 2017). Some of these changes include oxidative stress, dendritic, axonal and synaptic degeneration, microglial activation, and neuronal death (Smart, 2017; Giusti, 2009; Cekanaviciute, & Costes, 2018).

Although these mechanisms remain somewhat poorly defined, neurocognitive responses to radiation can be tracked in rodent models through non-invasive behavioral testing. Many different tests have been designed and used over the years, but fewer have become widely regarded as standardized measures of behavior in animal models (Van der Staay & Steckler, 2002; Hanell & Marklund, 2014). Reliable behavioral assessments,

such as those used in this study (Barnes Maze and open field) have been previously used

to evaluate cognition in irradiated rodents with fairly consistent results. Some of the

earliest work studying the effects of GCR in rodents by Denisova et al. (2002) and

Shukitt-Hale et al. (2000) reported significant deficits in learning and memory behavioral

tests following exposure to acute, high dose HZE nuclei ($_{56}$Fe). These findings have been

replicated in similar behavioral studies, with varying types and doses of radiation,

summarized in table 1.

Table 1: Chronological summary of studies investigating behavioral responses to HZE ion irradiation.

Author/Year	Particle/Dose	Animal Model	Summary: Cognitive/Behavioral Assay & Outcome
Joseph et al. (1992)	Acute: ($_{56}$Fe) 0.1–1Gy	-Male "Rats" (unspecified background)	Wire suspension task: Irradiated rats displayed compromised motor behavior
Rabin, et al. (1998)	Acute: ($_{56}$Fe) 0.1–1Gy	-Male "Rats" (unspecified background)	Conditioned taste aversion: Exposure to $_{56}$Fe radiation was more effective at establishing aversion to sucrose intake than neutron or $_{60}$Co radiation. Cognitive/behavioral responses to HZE ions differ from responses to other radiation types at similar doses.
Shukitt-Hale et al. (2000)	Acute: ($_{56}$Fe) 1.5 Gy	-Male Sprague-Dawley Rats	Morris water maze: Irradiated rats demonstrated impairment in spatial learning and memory with increased latency to find platform and less time in target platform quadrant
Denisova et al. (2002)	Acute: ($_{56}$Fe) 1.5 Gy	-Male Sprague-Dawley Rats	Radial arm maze spatial memory test: Irradiated rats showed impaired learning and were less successful at solving the maze when spatial memory strategies were rewarded
Shukitt-Hale et al. (2003)	Acute: ($_{56}$Fe) 1 Gy	-Male Sprague-Dawley Rats	Radial arm maze spatial memory test: Irradiation adversely affected maze performance in some but not all measures; irradiated animals made significantly more errors, earlier than controls
Pecaut et al. (2004)	Acute: ($_{56}$Fe) 0.1, 0.5, & 2 Gy	-Female C57BL/6 mice	Open field: no significant effects due to radiation Rotorod test: No significant differences due to radiation Acoustic startle: No significant effects on startle response by radiation
Britten et al. (2011)	Acute: ($_{56}$Fe) 20, 40, & 60 cGy X-rays: 13Gy	-Male Wistar rats	Barnes maze spatial memory test: Learning impairment in both X-ray and HZE exposed mice at all doses
Rabin et al. (2011)	Acute: ($_{12}$C, $_{28}$Si, $_{16}$O, and $_{48}$Ti) 5-100 cGy	-Male Sprague-Dawley Rats	Operant conditioning: Irradiation causes dose-dependent reduction in operant responses on a fixed-ratio schedule. Disruption by the same particle varied proportionally with dose, while less predictable differences were seen between particle types

Cherry et al. (2012)	Acute: ($_{56}$Fe) 100 cGy	-Male C57BL/6 mice -Female APP/PS1 mice	Fear conditioning: Significant decrease in freezing (normal) behavior Novel object recognition: Significant decrease in time with novel object in male and female mice
Haley et al. (2013)	Acute: ($_{56}$Fe) 0.1, 0.2, & 0.5 Gy	-Male and female C57BL/6J mice	Novel object recognition: irradiation impaired novel object recognition in male and female mice with less time exploring the novel object compared to controls Morris water maze & fear conditioning: no significant differences reported for male or female mice
Rabin et al. (2015)	Acute: ($_4$He)0.1-10 cGy or ($_{137}$Cs) source Gamma rays 50-400 cGy	-Male Sprague-Dawley Rats	Elevated plus maze: Both $_4$He particle radiation and gamma rays increased baseline anxiety levels in rats Novel location recognition: $_4$He particle radiation at all doses disrupted spatial memory performance Operant conditioning: $_4$He particle radiation at all doses disrupted motivation and responsiveness
Parihar et al. (2016)	Acute: ($_{16}$O and $_{48}$Ti) 5-30 cGy	-Male Tg(Thy1-EGFP)MJrsJ mice (Jax)	Novel object recognition: 30 cGy irradiation significantly reduced recognition memory Object in place task: 30 cGy irradiation significantly decreased memory retention Temporal order task: All doses of radiation significantly impaired recency memory in mice
Wyrobek & Britten (2016)	Acute: ($_{56}$Fe) 5-20 cGy	-Male Wistar rats	Barnes maze spatial memory test : Dose-dependent decline in learning and spatial memory in irradiated male rats
Krukowski et al. (2018)	Acute: ($_4$He, $_{16}$O, and $_1$H) 15 or 50 cGy	-Male and female C57BL/6J mice	Elevated plus maze: Irradiation increases anxiety-like behavior in only male mice Three chamber social approach: Irradiation diminished social interaction in only male mice Novel object recognition: Irradiation impaired recognition memory in only male mice
Parihar et al. (2018)	Chronic: ($_4$He) 30 cGy	-Male C57BL/6 mice	Fear extinction: Compromised fear extinction in irradiated male mice Water maze: Impaired cognitive flexibility one year post-irradiation in male mice Elevated plus maze & forced swim tests: Elevated anxiety and depression-like behavior in male mice Object in place & temporal order tests: Decreased exploration time and impaired memory following irradiation in male mice
Acharya et al. (2019)	Chronic: 80% $_{252}$Cf Neutrons + 20% photons -18 cGy	- Male C57BL/6 mice	Social interaction: Irradiated mice displayed increased avoidance behavior compared to controls Episodic & spatial memory: Significant decrease in recognition memory (novel object test) and spatial memory (object in place test) in irradiated mice Anxiety & depression: Radiation increased anxiety-like behavior but not depression behavior Fear extinction: All mice learned freezing behavior but irradiation compromised fear extinction 24 hours following training

Most recently, Parihar et al. (2018) and Acharya et al. (2019) demonstrated that both charged and uncharged particles were able to disrupt rodent behavior. Mice exposed to charged helium ion radiation delivered at low, space-relevant doses were found to have altered memory, anxiety, and depression like behavior, along with functional changes in neuronal circuitry persisting a year after irradiation (Parihar et al., 2018). Mice exposed to long-term (6 month), low-dose (0.18 Gy) neutron radiation also showed distress behaviors with decreased performance in learning and memory related tasks (Acharya et al., 2019). These findings have been critical in establishing chronic, low-dose GCR type radiation as a threat to human cognition. Still, investigations involving accurate full-spectrum GCR, defined by a HZE contribution <0.1 Gy in combination with 90% proton & $_4$He ion radiation, are absent.

1.4 Radioprotective Interventions

As previously outlined, ionizing radiation has become a common diagnostic tool in modern healthcare. Despite the increasing occurrence of radiative exposures, the use of radioprotective treatments is uncommon in clinical or other settings (Lin, 2010). Research investigating the effectiveness and potential mechanisms of proposed radioprotective agents is ongoing, but has yet to produce results showing a clear clinical benefit (Smith, Kirkpatrick, et al., 2017). Despite this, many proposed agents (typically antioxidants) have shown to contribute to the amelioration of processes associated with radiation-induced damage; including accumulation of reactive oxygen species (ROS), lipid peroxidation, and tissue inflammation (Kuefner et al., 2015). For example, N-

Acetyl-Cysteine, which has been studied for its antioxidant abilities, was shown in one study to decrease ROS by 4.8 times, improving survival in mice, and maintaining healthy intestinal anatomy in pretreated mice subjected to large doses of acute radiation (Jia et al., 2010). In the context of HZE ion radiation however, Haley et al. previously reported that the antioxidant alpha-lipoic acid had no protective effect on cognition in mice irradiated with $_{56}$Fe nuclei, and did not alter any measures of oxidative damage (Haley et al. 2013).

Near-future plans for space travel have brought with them a renewed interest in radioprotective agents; seeing as GCR is a significant limitation in long-term space tourism. Although anti-oxidants have shown some benefit in animal studies, DNA double strand breaks (DSBs) are one aspect of radiative damage shown to be largely unchanged in antioxidant intervention studies (Smith, Kirkpatrick, et al., 2017). DNA DSBs resulting from exposure to ionizing radiation are critical lesions that can lead to cell death in Eukaryotes (Cannan & Pederson, 2017). Misrepaired DSBs also represent a major threat; causing genetic disruption that may lead to cancer and other diseases (Li et al., 2011). Endogenous eukaryotic mechanisms designed to detect and repair these breaks are an organism's main line of defense, and recent investigation comparing 18 rodent species found DSB repair to be the most highly correlated with lifespan versus all other DNA repair mechanisms (Tian et al., 2019).

Growing evidence suggests that the response to DSBs over time, even when correctly repaired, causes epigenetic noise which can lead to chromatin changes, changes in gene expression and may contribute to premature aging, cognitive decline, and other common pathologies (Hayano, Motoshi et al. 2019). While an ideal radioprotectant

should address the issue of radiative DSB repair, it should also address these post-repair pathways. The importance of epigenetic mechanisms such as DNA and histone modification [including (de)acetylation and (de)methylation], in gene regulation and higher cellular function is now well defined (Berger et al., 2009; Stephens et al., 2012). Early work in yeast led to the theory that epigenetic changes, due to relocalization of chromatin factors in response to DNA damage, may be the main cause of aging, a theory that has successfully been expanded to multicellular organisms over the last twenty years (Sinclair et al., 1997; Smeal et al., 1996; Hannum et al., 2013; Larson et al., 2012).

Many genetic signaling pathways have been studied and implicated with this new theory of aging, including a set of NAD+ dependent genes responsible for maintaining a healthy epigenetic landscape. In the current study, we theorize that NAD+, an endogenous molecule involved in human DSB repair and chromatin/epigenome modification systems, holds great potential as a radioprotective agent to counteract damage by GCR.

1.5 NMN as a Radioprotective Intervention

Nicotinamide adenine dinucleotide (NAD) and its phosphorylated, oxidized, and reduced forms, $NADP_+$, NAD_+, NADH, and NADPH, have critical roles in cellular metabolism and energy production as electron accepting and donating coenzymes (Ansari and Raghava, 2010). In recent history, NAD's role as more than an oxidative cofactor has been increasingly studied, and it is now known to be required for more than 500 different enzymatic reactions in eukaryotic organisms (Rajman et al., 2018).

The bridge between radioprotection and the molecule NAD exists in the discovery of its role in two main pathways; NAD_+ as a cofactor for the family of mammalian sirtuin (SIRT1-7) genes, and mammalian PARP genes (Bitterman et al., 2002; Amé et al., 2004). Both of these protein families hydrolyze NAD_+ as fuel to regulate various cellular functions; including but not limited to cell growth, metabolism, inflammation, and most relevantly, DNA repair and histone modification (Rajman et al., 2018). As previously discussed, DSB repair is of particular importance, and recent literature points to SIRT6 and PARP1 as having the most pivotal roles in sensing DSB and signaling for repair (Tian et al., 2019; Ansari and Raghava, 2010).

Boosting NAD_+ levels in rodent models has been shown to improve and regulate function in almost every major organ system; including (but not limited to) cardioprotection, fatty acid oxidation (by SIRT3), DNA repair (primarily through PARP1), endothelial and vascular function (by SIRT1), and neuronal/ hippocampal function (Kane A. & Sinclair D, 2018; Ahn et al., 2008; Xu et al., 2015; Hou et al., 2018; Park et al., 2016). One popular NAD_+ boosting technique is the use of oral-supplemented precursors to promote increased intracellular levels of NAD_+. Figure 2 outlines one pathway by which NAD_+ is synthesized. NAD_+ may be synthesized de novo from tryptophan, or from precursors such as Nicotinamide Riboside (NR) salvaged from previous cellular mechanisms or from dietary supplementation (Denu John M., 2007).
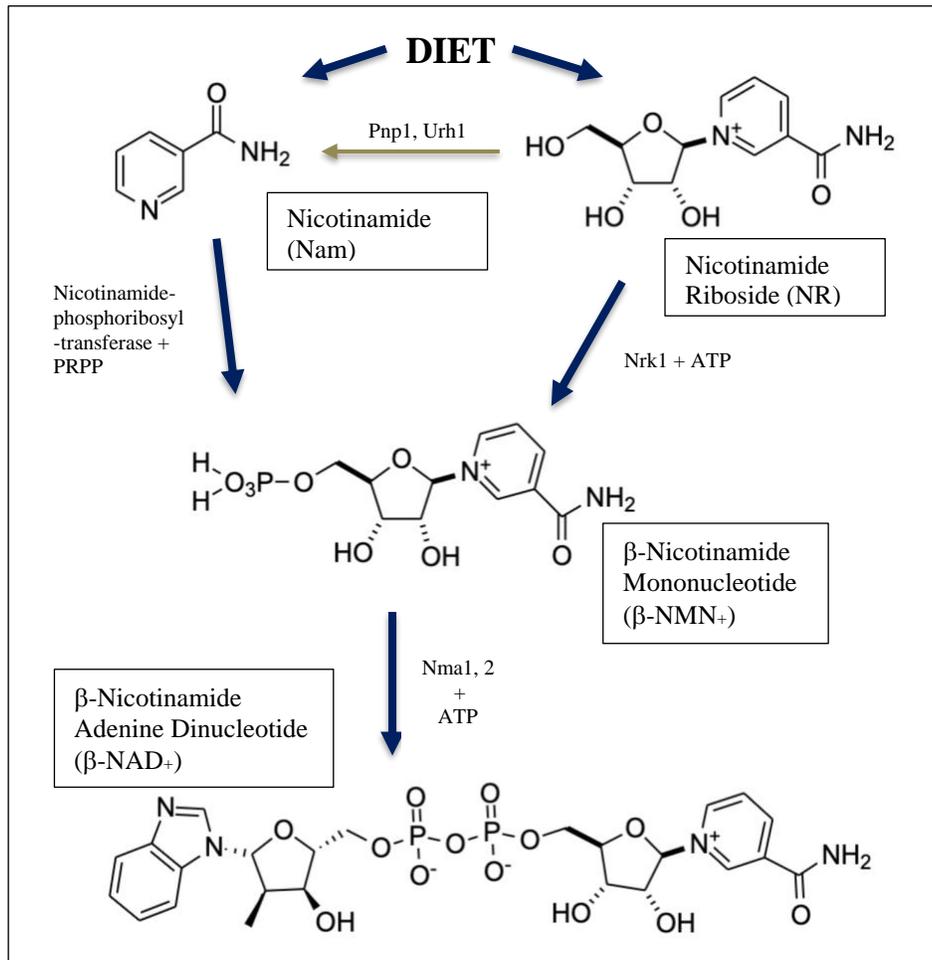
Figure 2: Illustration of several pathways to NAD+ synthesis.

Nicotinamide Mononucleotide (NMN) is one such NAD+ precursor that has been found

to be successfully converted to NAD+ when taken as an oral supplement in mice (Mills et

al., 2016). Importantly, Li et al. previously demonstrated that NMN treated mice suffered

less DNA damage and alterations in immune cell counts in response to gamma radiation

(8 Gy), even when administered post irradiation (Li et al., 2017).

While DNA DSB repair is the main pathway through which we expect NMN to

promote radioprotection, sirtuin mediated deacetylation is another pathway that addresses

post-repair epigenetic changes. Hayano, Motoshi et al. (2019) demonstrated that an

12

accelerated aging phenotype can be achieved in mice through inducible, non-mutagenic DSBs. These mice exhibited impaired (aged) cognitive and motor behaviors, along with robust changes in metabolism and physiology in several major organ systems (Motishi et al. 2019). These changes have been attributed to a shift in the epigenetic landscape following incomplete reestablishment of chromatin and epigenetic status after DNA repair (Kim et al., 2018; O'Hagan et al., 2008). Overexpression of SIRT genes has been shown to increase genome stability and delay transcriptional changes with age (Oberdoerffer et al., 2010).

This provides evidence for the idea that ionizing radiation, while being a less extreme source of DSBs, may cause similar post-repair issues that lead to cognitive impairment in irradiated organisms. In the current study, we theorize that boosting NAD_+ levels via NMN supplementation will protect against GCR induced cognitive damage by promoting endogenous DNA repair and gene regulatory pathways in mice.

2. SPECIFIC AIMS

Previous studies have demonstrated how space-relevant doses of radiation can have detrimental physiological effects in mice. Neurocognitive behavioral deficits following gamma, alpha, and HZE ion type radiations are common, but the effects of full-spectrum GCR have yet to be defined. This thesis seeks to investigate the cognitive effects of accurate, full-spectrum simulated-GCR in mice. In order to test the hypothesis that chronic, Mars-trip mimicking doses of GCR are sufficient to cause changes in memory, anxiety, and aggression, we utilize several controlled behavioral testing methods in male C57BL/6 mice. This thesis will highlight the advantages and limitations of these methods in obtaining reliable data tracking long-term behavioral variation. Additionally, we will address whether NMN, an NAD_+ precursor, protects irradiated mice from any potential cognitive deficits. In doing so, we hope to extend the current understanding of neurocognitive responses to GCR, in preparation for future missions through interplanetary space.

3. METHODS

3.1 Animals and NMN treatment

All animal procedures were carried out in accordance with National Institutes of Health and Institutional Animal Care guidelines and were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine (UCI) and at Harvard Medical School. A cohort of male C57BL/6 mice (n=48) were ordered from The Jackson Laboratory to UCI for irradiation and initial NMN treatment. NMN treatment began one week before irradiation at approximately 6 months of age, at a dose of 600 mg/kg daily in drinking water. NMN was tested for purity with high performance liquid chromatography (HPLC) & an endotoxin assay. Mice traveled from UCI to Harvard Medical School at seven months of age and remained in quarantine for approximately two weeks.

Throughout the study mice were separated into single housing due to fighting. By nine months of age, before behavior protocols were performed, ~40% of the mice were singly housed (control/water n=6, radiation/water n=3, control/NMN n=5, and radiation/NMN n=5).

Between six and seven months of age, 83% of the non-irradiated (control) mice were switched in their treatment +/- NMN (12 NMN mice were switched to water, and eight water treated mice were switched to NMN). NMN treatment in these groups was kept constant following the switch and mice were grouped based on their ongoing treatment. These mice were kept in the study following the observation that there were no behavioral differences between controls that had received NMN and those who had not

(four controls were not switched and received only water treatment through the entire study). The final mouse group numbers following the re-grouping were: control/water n=16, radiation/water n=12, control/NMN n=8, and radiation/NMN n=12.

<u>3.2 Radiation</u>

At six months of age, mice (n=24) were irradiated with 33 beams of varying energies delivered over approximately 80 minutes a day, 6 days a week for a total of 24 days (2.0799 cGy/day = 49.92 cGy total). Table 2 summarizes the spectrum and dosage

Table 2: Full GCR spectrum radiation

Ion	Daily dose (cGy)	Total dose (cGy)	% of total
Protons	1.5314	36.7536	~73.6
Helium	0.3665	8.796	~17.6
Oxygen	0.064	1.536	~3.1
Carbon	0.049	1.176	~2.4
Silicon	0.034	0.816	~1.6
Titanium	0.018	0.432	~0.9
Iron	0.017	0.408	~0.8

of administered radiation. Charged particles were generated and delivered at the NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory at dose rates between 0.015 and 0.94 cGy/min. Dosimetry was performed, and spatial beam

uniformity was confirmed by the NSRL physics staff. During daily irradiation

procedures, mice were moved from their home cages (four mice per cage), and put in

irradiation boxes in groups of 2-3. Every day mice were boxed with the same mice as the

prior day(s). Control mice were separated into cages similarly, and left undisturbed for

the duration of the procedure. Following 80 minutes of irradiation, all mice were moved

back to their home cages, taking approximately two hours from start to finish.

3.3 Behavioral Testing

Barnes Maze: At nine months of age, spatial learning and memory were evaluated

in mice using the Barnes Maze protocol, a relatively non-invasive way of testing memory

and learning behaviors (Sunyer et al., 2007; Pitts, 2018). The Barnes Maze is a white

circular elevated platform containing 20 holes around the outside rim (Figure 3). 19 of

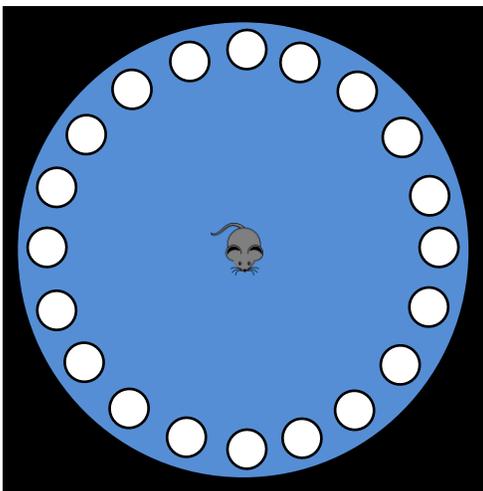these are covered with plastic, whilst one is open and provides an escape for the mouse

Figure 3: Illustration of Barnes Maze

from with plastic, whilst one is open and provides an escape for the mouse from the

exposure of the platform. Mice are trained to seek the hole containing an "escape" box

17

and later tested with the escape box removed to observe behavior. For each group of 16 animals, testing occurred over a 5 day period including four days of training followed by a single testing day. All sessions were viewed and recorded through a camera paired to TopScan tracking software from CleverSys Inc. Analysis was done on recordings from the test day. Mice were habituated to the testing room conditions for an hour before each day's training or testing sessions.

Setup

Day 1: Priming- 1st day trials began with a 90 second roaming period for the mice to explore the maze. After 90 seconds had elapsed, mice were guided to the exit hole and kept in the covered escape box for 2 minutes before being returned to their cage. Each of the 16 mice underwent this trial a single time before any training trials began. Maze and escape boxes were cleaned thoroughly with 70% ethanol after each trial.

Days 1-4: Training- Each test animal was given 3 minutes to explore per trial. If the mouse successfully found and entered the exit hole, it was rewarded by covering the hole and allowing it to remain in the box for 60 seconds before returning to its cage. If the mouse did not find the hole within 3 minutes it would be guided to the location and allowed 60 seconds of covered time in the box similar to successful trials. Each of the 16 mice repeat this trial 4 times a day with a minimum of 15 minutes between any individual mouse's consecutive trials. Time elapsed before the mouse found the hole was recorded for each trial (maximum being 180 seconds for mice who did not find the hole in time). Maze and escape boxes are cleaned thoroughly with 70% ethanol after each trial.

Day 5: Testing- The escape box was removed and the hole was made to match the other 19. Each testing trial was recorded in its entirety and the video saved for future analysis. Mice were allowed 90 seconds of recorded exploration. As before, the maze was cleaned thoroughly with 70% ethanol between trials.

<u>*Open Field*</u>: In order to assess mouse anxiety and motor function at 10.5 months of age, we used the open field maze test. Adapted from the protocol described in Gould et al. (2009), this assay involved four separate, square, enclosed spaces (mazes); enabling 4 mice to run at once. Each maze was surrounded by walls too high for a mouse to escape, with an inner diameter of 20 inches to produce the feeling of openness within the space.
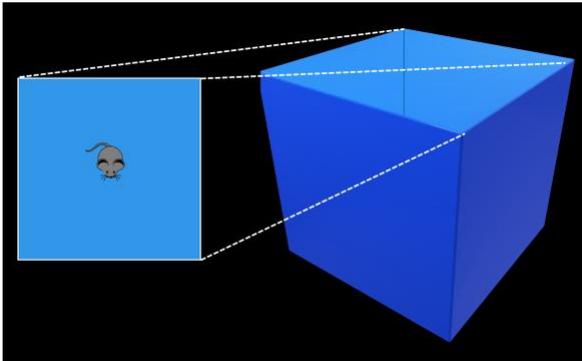

Figure 4: Illustration of open field maze

The four adjacent mazes were recorded using an overhead camera connected to a computer running TopScan tracking software from CleverSys Inc. for video analysis. Measures of total distance traveled and percentage of the total time spent in the center vs. the outside of the maze were recorded.

Each trial began with a group of 4 mice being placed into the enclosed maze spaces (no two mice shared a single maze). As soon as the mice were placed in their corresponding mazes, the video analysis software began recording. Each trial lasted one

hour. During this time, mice were left uninterrupted. At the end of the trial, mice were removed from the mazes, which were then cleaned thoroughly with 70% ethanol.

Tube dominance: In order to determine whether irradiated mice differed from controls in respect to aggression and dominance at 11 months of age, the tube dominance
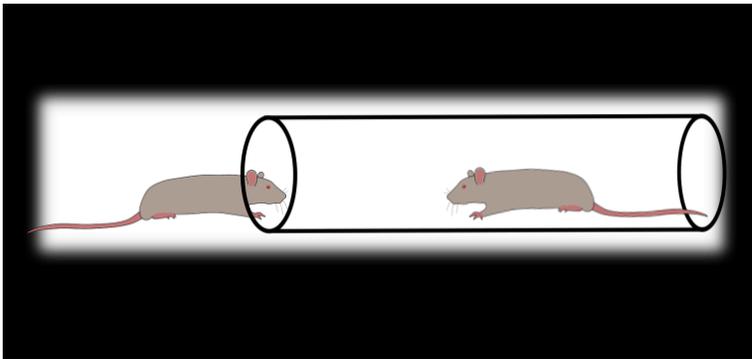

Figure 5: Illustration of tube dominance test

test was performed. This protocol, adapted from Messeri et al. (1975), scores for dominance by pitting mice against each other in a 1-on-1, tube contained, instance. The tube apparatus consisted of a 30 cm long transparent plastic tube. The inner diameter of the tube was 1 3/8"; the size of tube used was chosen to ensure that two mice could not pass each other.

In this test each animal from the group was matched with an opponent from the opposing group, and mouse pairing was limited to not exceed a 15% difference in body weight. Each trial consisted of two mice being released simultaneously at opposite ends of the tube; the trial ended when one animal retreated backwards from the tube, or was forced out by the other animal so all four limbs were outside of the tube (the mouse that was first to leave had lost the trial). Between trials the tube was cleaned with ethanol and

dried completely. Each pairing was scored as a best 2 of 3, where each successful bout

scored a point for the winning mouse (maximum total points per match being 3 with one

mouse earning 2 points and the other earning 1). Thus, two wins in a row awarded the

winning mouse 2 points, while the losing mouse was awarded none, as the trial/pairing

was then finished. With a maximum of one pairing per mouse, per day, each mouse was

paired with every other mouse (round robin style).

First, we compared chronic irradiation mice against controls (both groups treated

with water). Next, all controls +/- NMN were compared. Finally, all chronic irradiation

mice +/- NMN were compared. Total dominance points per mouse were added up and

plotted for comparison.

3.4 Electrocardiogram (ECG)

In order to test whether a cardiac phenotype was present in our irradiated mice,

ECG measurements were collected and analyzed using the ECGenie device from Mouse

Specifics, Inc. and corresponding software (Schuldt et al., 2004). The ECGenie system

allows for ECG recordings to be obtained from awake, non-sedated mice. These methods

avoid common problems in heart rate variability attributed to sedative drug effects.

Each mouse's ECG data was recorded at 10 months of age by placing them on the

platform containing footplate electrodes. They were left untouched during the recording

period, while the output was closely monitored. For each mouse a segment of the

recording data (approximately 5-10 second) without noise was saved for further analysis.

The ECG wave data was scored within the ECGenie software and measures included average heart rate, heart rate variability, and QRS duration.

3.5 Statistical Analysis

Two-way ANOVA, with Tukey post-hoc test, was used to compare data from control and irradiated mice with or without NMN treatment for Barnes Maze, open field, and ECG measurements. When comparing data between irradiated and control mice, or radiated mice with or without NMN treatment for the tube-dominance assay, an unpaired two-tailed t-test was used. All statistical analyses were carried out using Prism (GraphPad) software. All data are represented as mean ± SEM. Values of $p < 0.05$ are considered statistically significant.

4. RESULTS

4.1 Mouse Weight, Food, and Water Intake

To ensure that mice from all groups were eating, drinking, and showing normal body weight, food and water consumption were recorded for one week and body weights were measured. Mice from all groups did not differ significantly in weight (Figure 6A), and consumed approximately the same amount of food and water (Figure 6B-C). Water intake differed between control/water and control/NMN groups, as well as between control/NMN and radiation/NMN, however, this difference was minimal (< 2 grams per mouse daily on average). Similarly, food intake differed between radiation/water and radiation/NMN groups, but by an average of less than 2 grams per mouse daily. Using these averages, control/NMN mice were consuming ~0.0336 grams of NMN per day, and Radiation/NMN mice were consuming ~0.0278 grams of NMN per day. Using group average weights, control/NMN mice were estimated to consume ~949.15 mg/kg NMN daily and radiation/NMN mice were estimated to consume ~847.56 mg/kg NMN daily.
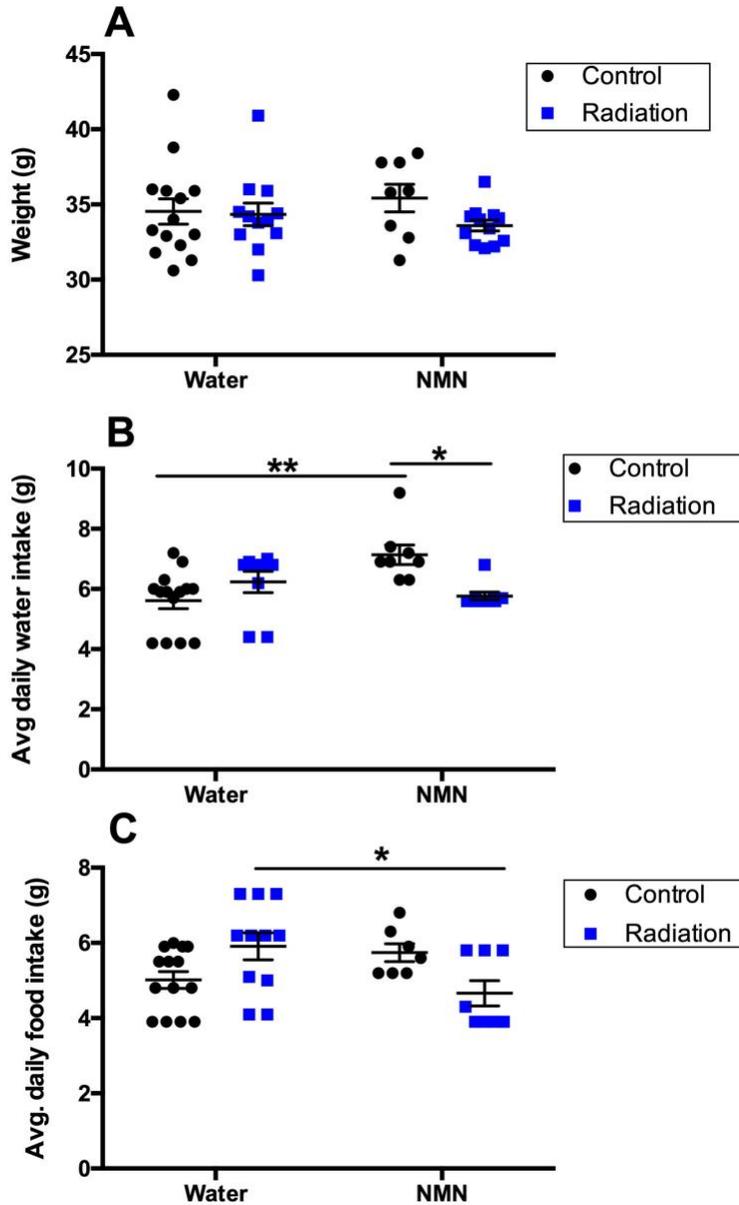
Figure 6: Weight, food, and water intake assessment in male C57BL/6 mice treated with 2.0799 cGy/day GCR for 24 days, +/- oral NMN treatment (600mg/kg per day). **A,** Mice from all four groups did not differ in body weight. **B**, water intake differed significantly between control/water and control/NMN groups, as well as between control/NMN and radiation/NMN. All groups drank, on average, 5-8 grams per mouse daily. **C**, food intake differed significantly between radiation/water and radiation/NMN groups. All groups consumed, on average, 4-6 grams per mouse daily. Error bars indicate SEM. Data compared with two way ANOVA followed by Tukey's multiple comparisons test. *$P<0.05$, **$P<0.005$. Control/water n=14; radiation/water n=11; control/NMN n=8; radiation/NMN n=9.

4.2 Mouse learning and memory unaffected by chronic GCR and NMN treatment

      To explore the effect of radiation and NMN on learning and memory we completed the Barnes Maze assessment for all mice. Mice in all four experimental groups showed a decrease in time spent to find the target hole over the course of the four training days (Figure 7A). This is evidence that the training period was successful. On the testing day, compared to controls, irradiated mice did not differ significantly in the amount of time spent at the target hole or within the target quadrant (7B, 7C). NMN treated mice also did not differ significantly from the other groups in regard to time spent at the target hole or within the target quadrant (7B, 7C). These results suggest that there are no cognitive differences in spatial memory or learning between the different groups.
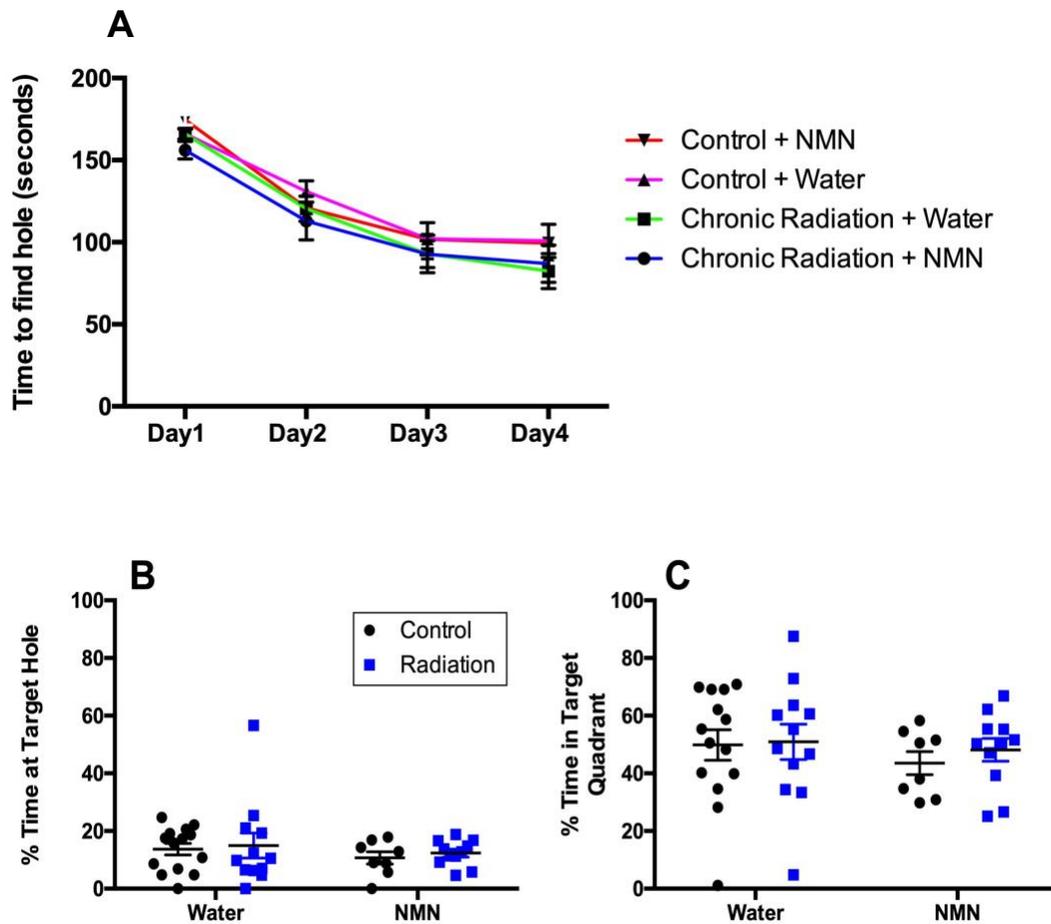
Figure 7: Barnes maze assessment in male C57BL/6 mice treated with 2.0799 cGy/day GCR for 24 days, +/- oral NMN treatment (600mg/kg per day). **A**, All four groups took less time to find the target hole over the four training days, demonstrating normal learning behavior. **B-C.** Mice from all four groups showed no difference in percentage of time spent at the target hole or in percentage of time spent within the target quadrant, indicating that chronic GCR exposure had no effect on spatial memory. Error bars show SEM. Data compared with Two-way ANOVA followed by Tukey's multiple comparison's test. Control/water n=14; radiation/water n=12; control/NMN n=8; radiation/NMN n=11.

4.3 Mouse anxiety and motility are unaffected by chronic GCR and NMN treatment

To investigate the effect of radiation and NMN treatment on mouse activity and anxiety we used the open-field assessment. Compared to controls, irradiated mice did not differ significantly in time spent in the center of the maze or in total distance traveled (Figure 4A-B). Similarly, NMN treated mice did not show significantly different behavior compared to water treated mice (Figure 4A-B). These results suggest no difference in anxiety or motility between the different mouse groups.
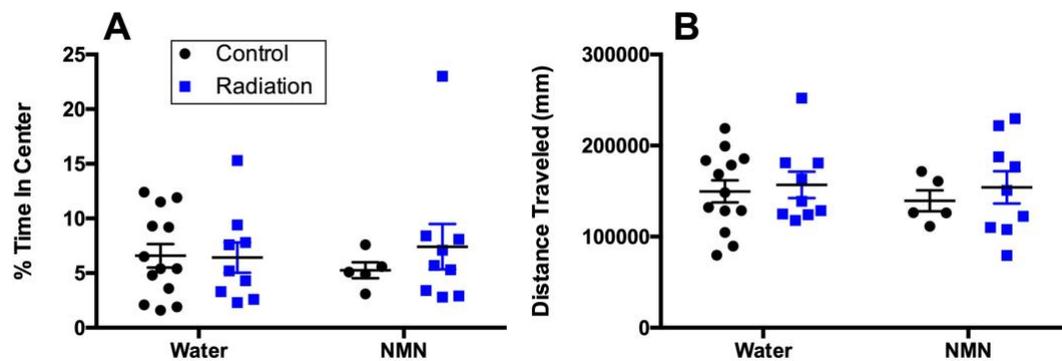

Figure 8: Open field movement and anxiety test in male C57BL/6 mice treated with 2.0799 cGy/day GCR for 24 days, +/- oral NMN treatment (600mg/kg per day). **A, B**, Mice from all four groups showed no difference in percentage of time spent in the center of the maze, or in total distance traveled. These results indicate that chronic GCR exposure had no effect on anxiety or activity levels in the mice. Error bars show SEM. Data compared with Two-way ANOVA followed by Tukey's multiple comparison's test. Control/water n=13; radiation/water n=9; control/NMN n=5; radiation/NMN n=9.

4.4 QRS duration and average heart rate are unaffected by chronic GCR and NMN treatment

To explore a potential cardiac phenotype in the mice, electrocardiogram (ECG) analysis was performed. Irradiated and control mice did not differ significantly in measures of QRS duration or average heart rate (Figure 5A-B). This data suggests that cardiovascular function was unaffected by GCR or NMN treatment.
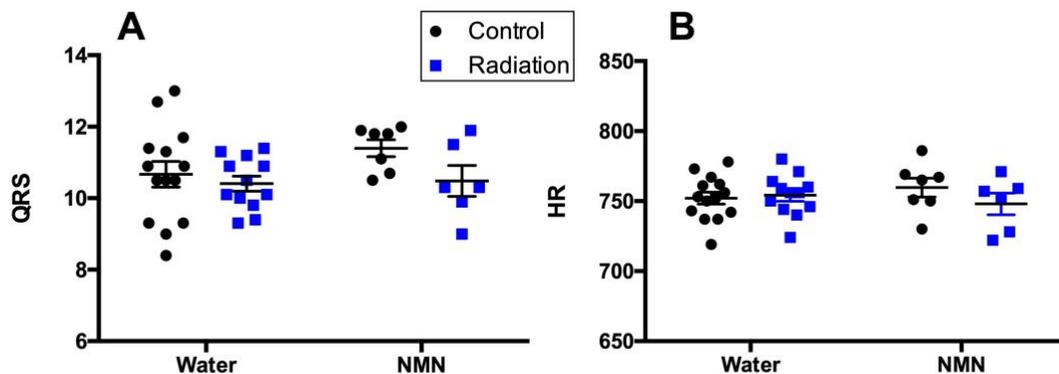

Figure 9: Electrocardiogram analysis in male C57BL/6 mice treated with 2.0799 cGy/day GCR for 24 days, +/- oral NMN treatment (600mg/kg per day). **A, B**, Mice from all four groups showed no difference in average QRS duration (milliseconds) or average HR. Error bars show SEM. Data compared with Two-way ANOVA followed by Tukey's multiple comparison's test. Radiation/water n=12; radiation/NMN n=6; control/water n=14; control/NMN n=7.

4.5 Irradiated mice are more dominant than controls, NMN treatment attenuates this effect

To explore an aggression phenotype in the mice, a tube dominance test was performed. Between irradiated (water treated) and non-irradiated (water treated) groups, a significant difference in dominance/aggression was found (Figure 10). Irradiated mice won 76.9% more bouts than non-irradiated mice (Figure 10A), suggesting an increased

level of dominance/aggression due to irradiation. When pairing irradiated (water treated) mice against irradiated (NMN treated) mice, it was found that the NMN treated mice scored 90.9% lower in dominance/aggression (Figure 10B). Together, these outcomes provided early evidence of GCR as a stimulus for aggression and NMN as a potential therapy.
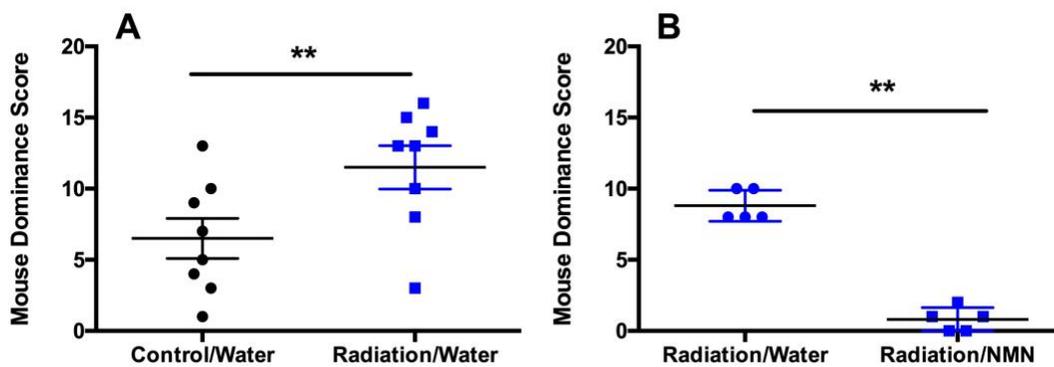

Figure 10: **A,** Irradiated (water treated) mice were found to be significantly more dominant (76.9% more trials won) than non-irradiated (water treated) mice. **B**, When pairing irradiated (water treated) mice against irradiated (NMN treated) mice, NMN treated mice scored 90.9% lower in dominance. Error bars indicate SEM. Data compared with unpaired t-test with equal SD, **P<0.001. **A,** Control/water n=8; radiation/water n=8; **B**, radiation/water n=5; radiation/NMN n=5

5. DISCUSSION

In this thesis we explored, for the first time, the effects of chronic full spectrum, lose-dose GCR exposure on behavior and cognition in mice. We found that irradiated mice show no differences in behavioral assays addressing anxiety (open field), spatial memory, or learning (Barnes maze) compared to control groups. Interestingly we did find however, that irradiated mice scored higher in terms of dominance, as determined by a tube dominance test and that this phenotype was rescued by chronic exposure to NMN.

When considering future interplanetary travel, one of the greatest threats we face is the damaging effect of Galactic Cosmic Radiation. Immune to regular shielding, GCR holds the potential to damage and disrupt every major organ system in our bodies (Chancellor et al., 2014). Previous investigations have shown significant cognitive deficits following irradiative exposure to HZE nuclei in rodent models, however, until recently these studies were limited to dosing regimens simulating radiation levels much higher than one would experience on a six month trip to Mars (Sokolova et al., 2015; Cummings et al., 2007; Acharya et al., 2019; Limoli et al., 2018). Furthermore, studies addressing low, Mars relevant-doses in mice have failed to simulate the entire GCR spectrum, making findings difficult to interpret (Parihar et al., 2019).

Here, we use, for the first time, a true model of GCR and find no difference in cognition, memory or activity in male mice. Our results are surprising for a number of reasons. Previous studies addressing similar behavioral cognitive methods using only Proton and $_4$He ion radiation, reported significant differences between irradiated and

control groups (Cekanaviciute, & Costes, 2018). The full-spectrum GCR used in our study is >90% Proton and 4He ions, present in doses similar to previous studies. The inclusion of HZE in addition to this previously established radiation would only be expected to reinforce those outcomes, rather than diminish them. While our total irradiative dose ~0.49 Gy is less than many previous studies reporting significant cognitive changes, it is still firmly in the range of levels expected to cause noticeable cognitive damage (Smart, 2017; CDC 2018). In short, the majority of our findings relative to behavioral outcomes conflict with the bulk of similar previous literature. This may be due to the unique radiation dosing regimen used in this study. 24 days of low-dose GCR represents a middle ground between the common acute high-dose irradiation methods and the few long-term, low-dose chronic irradiation methods, which have exposed mice to radiation for six months or longer (Rivera et al., 2013; Acharya et al., 2019). CNS architecture is complex, and when paired with the random-targeting nature of radiation, variable results should be expected.

In the current study, we did find that chronic radiation caused a dominance phenotype. We have found no other recent publications addressing dominance and aggression in mice exposed to space radiation. By our search, only one study has paired measures of aggression with irradiative exposure in mice, and this was in the context of isolation-induced aggression, which was found to be slightly diminished by low-dose X-ray exposure (Miyachi et al., 1994). Because our finding is unique, further investigation is needed to confirm its validity. If it were found that GCR repeatedly increases dominance and aggression in mice, this would have major implications for future

astronauts. In humans and mice, aggression is a complex behavior dependent on a variety

of neurotransmitters, endocrines, and brain activity commonly associated with the

amygdala, frontal lobe, and hypothalamus (Matthies et al., 2012; Blanchard and

Blanchard, 2014). The disruption of these systems, affecting CNS function or brain

architecture may come with an increased risk for neurodegenerative disease, long term

cognitive dysfunction, and decreased quality of life (Trojsi et al., 2018; Davis et al.,

2015; Gupta R. & Sen N., 2016). It's likely that less astronauts, organizations, and

funding sources would be enthusiastic about traveling to Mars if these health risks were

known and expected. It is, however, worth noting that aggression is largely influenced by

environmental factors, therefore more research is needed to fully understand the

implications of these findings.

Importantly, we did find that NMN successfully protected against the dominance

phenotype. Also important were the findings that mouse weight, appetite, memory,

anxiety, motility, average heart rate, and QRS duration were largely unaffected by

chronic NMN treatment. It's unclear whether the NMN protected against the dominance

phenotype by having a radioprotective effect or through dominance-specific pathways.

It's known that mammalian internal timekeeping (circadian) systems influence behaviors

of aggression and anger (Hood & Amir, 2018). It's also known that SIRT1, an NAD_+

dependent deacetylase, regulates the gene expression of these circadian clocks through

PER2 deacetylation (Asher et al., 2008). This suggests that NAD_+ boosters such as NMN

may very well have a direct effect on aggression behaviors, independent of irradiation

mechanisms. Similar to other novel findings in this study, more research is needed to

confirm these outcomes, however, it's absolutely worth exploring further as NMN as either a radioprotectant or aggression modulator would have various implications in human cognitive pharmacology.

It's clear that additional studies involving full-spectrum simulated GCR are needed before making any definitive conclusions about the danger of GCR to mammalian CNS, but this investigation provides interesting preliminary data. Several limitations should also be carefully considered when interpreting these findings. 40% of mice in the current study were singly housed. More importantly, the percentage of singly housed mice in each group differed significantly. Radiation/water mice had the lowest percentage of singly housed mice at 25%, while control/NMN had the highest at 62.5% (control/water = 37.5% singly housed and radiation/NMN = 41.7% singly housed). Medendorp et al. (2018) demonstrated that socially isolated mice show altered behavior due to impaired dendrite spine morphology and prefrontal cortex plasticity. It's unclear whether this had a significant effect on our behavioral experiments, however, it's possible that the mix of single housing, radiation, NMN treatment, and individual susceptibility resulted in less consistent behavioral outcomes within groups.

The reliability of our findings is also limited by the use of all male mice. Previous studies have found sexual dimorphism in cognitive behavioral responses to simulated HZE nuclei radiation (Krukowski et al., 2018). They found significant changes in anxiety, social interaction, and memory in male mice, along with microglial activation and hippocampus synaptic loss following a single acute exposure to HZE nuclei (^{4}He, ^{16}O, and ^{1}H). They reported that female mice did not display any of these changes

(Krukowski et al., 2018). In recent years the ratio of male to female astronauts graduating from NASA's astronaut academy has been about one-to-one (Kelly Mars, 2017). Not only does the use of all male mice limit our understanding of how GCR affects 50% of our astronaut population, but it may confound certain behavioral assays such as those looking at dominance and aggression. Both male and female mice express aggressive and territorial behavior, however, aggression is expressed at a higher level in males and operates through different mechanisms in the ventromedial hypothalamus (Hashikawa et al., 2017). For these reasons, future studies should explore the effects of GCR in both male and female mouse populations.

Another limitation lies in the ordering of radiative ion species. Different levels of damage have been reported by switching the order of ion administration ($_{48}$Ti and $_{16}$O specifically) despite keeping dose and dose-rate constant (Cucinotta et al., 2014). While individual susceptibility may be a larger influencer at this level, it's worth considering how the ordering of heavy HZE nuclei in GCR may affect outcomes. With the 33 different beams we used during irradiation procedures in this study, the total amount of unique beam combinations is 8.6833176e+36. Clearly addressing each of these combinations is an unreasonable task, however, future studies could still explore the differences associated with the ordering of ions, especially HZE-type ions, in an effort to optimize modern GCR simulation.

Altogether we've shown, for the first time, how chronic full spectrum lose-dose GCR can be accurately modeled in mice. While mouse memory, learning, anxiety, and motility appeared to be unaffected by chronic GCR exposure and NMN treatment, mouse

dominance was found to be elevated in irradiated mice. NMN treatment protected against this change, but it is unclear whether this was through radioprotection or by other mechanisms. These findings are an exciting step forward in space radiation research. To reach a point of consensus on the dangers of interplanetary space travel, investigators should strive to model GCR in its entirety, as we have done here. It's been said that radiation may be the single biggest obstacle humankind will face beyond Earth's lower orbit and at this time, our understanding remains quite limited. Here we have raised several new questions and concerns regarding how best to approach this issue. Although it will take time to fully understand GCR, the continued investigation of animal model exposure and radioprotectants appears to be the best path forward.

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CURRICULUM VITAE