IMPACT OF SOCIAL ISOLATION AND SPACE RADIATION ON CCL2 EXPRESSION IN FEMALE WISTAR RATS

Zachary N. M. Luyo[1,2,3], Riley S. Heerbrandt[1,2,3], Alea F. Boden[1,2,3], Frederique E. E. Keumeni[1,2,3], Richard A. Britten[2,4], Laurie L. Wellman[1,2,3], Larry D. Sanford[1,2,3]

[1]Sleep Research Laboratory, [2]Center for Integrative Neuroscience and Inflammatory Diseases, [3]Biomedical and Translational Sciences, [4]Radiation Oncology, Macon & Joan Brock Virginia Health Sciences Eastern Virginia Medical School at Old Dominion University, Norfolk, VA, USA

Abstract

Astronauts conducting long-term space missions will be exposed to spaceflight stressors including social isolation (SI) and space radiation (SR). Both SI and SR exacerbate the body's stress response, which is controlled (in part) by the hippocampus (HPC). SI- and SR-induced stress can induce morphological alterations (i.e., astrocyte morphology) in the neuroinflammatory system of the HPC. Some of these morphological changes may be due to differences in CCL2 expression, a chemokine known to influence astrocyte morphology and blood-brain barrier permeability. However, it is unclear if spaceflight stressors alter CCL2 expression in the HPC. Therefore, this study utilized qPCR to assess CCL2 expression in female Wistar rats exposed to ground-based analogs of SI and SR. CCL2 expression was normalized to a housekeeping gene (GAPDH) and analyzed using one-way analysis of variance (ANOVA) with Treatment as a between factor. ANOVA revealed that SI animals had significantly more CCL2 expression compared to SHAM (control), suggesting that SI may produce a priming effect on the neuroimmune system. Future research should investigate additional neuroinflammatory genes across multiple regions of the brain, to better understand the mechanisms through which spaceflight stressors induce morphological changes in the brain.

Introduction

The proposed Mars missions will expose astronauts to several hazards associated with spaceflight. One of these, social isolation (SI), can induce both physical (e.g., decreased sensorimotor function, impaired immune response)[1-4] and psychological deficits (e.g., emotional bluntness, depression)[5-7] that could impact mission performance and team dynamics. In addition to SI, astronauts will be exposed to larger dosages of space radiation (SR) than ever before. SR exposure can influence several components of bodily function, including thermoregulation, sensorimotor function and executive function[8-11]. In addition, SI and SR have the potential to influence health for years following exposure. SI can result in a higher likelihood of high blood pressure, cardiovascular disease and type II diabetes[12-15] and SR exposure can increase the future risk of cancer and neurodegenerative diseases[1,10,16-22].

Both SI and SR have both been shown to exacerbate the body's stress response (e.g., endocrine function)[23-25], which can lead to neuroinflammation if not properly regulated[1,26,27]. The hippocampus (HPC) is a brain region implicated in a stress-regulatory neurocircuit and is also critical for several components of mission performance (e.g., memory, motor function, emotional regulation)[28,29]. While the HPC can regulate stress, it is also vulnerable to stress. Chronic stress has been shown to decrease hippocampal

connections to other regions in the stress-regulatory neurocircuit[28]. Additionally, stress-induced neuroinflammation can inhibit hippocampal neurogenesis, which can lead to psychiatric disorders such as major depressive disorder[30]. Therefore, it is critical to understand how SI- and SR-induced alterations in the stress response and subsequent neuroinflammation impact the HPC.

Neuroinflammation results in increased blood-brain barrier (BBB) permeability and inflammatory gene modulation to allow for the infiltration of peripheral immune cells[1,26,27]. BBB permeability is partially controlled through CCL2, a chemokine which alters the actin cytoskeleton of astrocytes. Astrocytes are the primary neuroimmune cells that produce CCL2. By altering actin cytoskeletons, CCL2 alters an astrocyte's ability to maintain endothelial tight junctions[31]. Dysregulation of CCL2 is seen in several diseases (e.g., multiple sclerosis, stroke) and can exacerbate pre-existing neurodegenerative diseases[31,32]. Previous work in our lab has shown that astrocytes in the HPC can have an altered morphology or be mostly ablated in response to SI and SR, respectively[1]. However, it is unclear whether these morphological changes in astrocytes are due to changes in gene expression in response to spaceflight stressors. CCL2 may be upregulated when exposed to SI or SR, which would alter the actin cytoskeleton of astrocytes. This would not only lead to altered astrocyte morphology but help also explain why BBB permeability changes in response to spaceflight stressors. Therefore, this study utilized quantitative reverse transcription polymerase chain reaction (RT-qPCR) to investigate alterations in CCL2 expression in the HPC.

Methods

Subjects

Female outbred, retired breeder Wistar rats (8-9 months old upon arrival) from Hilltop Lab Animals, Inc. (Scottdale, PA, USA) served as subjects (n = 2-3 per group). Animals were either individually housed (IH) as a control group (SHAM) or IH with opaque barriers between cages (SI) to prevent visual contact. (SI housing was time-matched with the SR group to begin 35 days post-SR and continued until the end of study.) Rats assigned to the SR treatment group were sent to the NASA Space Radiation Laboratory (NSRL) in Brookhaven National Laboratory (BNL; Long Island, NY, USA) to receive a one-time dose of SR (15cGy simplified 5-ion galactic cosmic radiation (GCRsim)) and were IH.

Animals were housed on a 12:12 light:dark cycle and kept at an ambient temperature of 24.5°C ± 0.5°C. Food and water were available ad libitum and Nylabone® chew toys were provided as enrichment. All experimental procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Experimental Animals and were approved by Eastern Virginia Medical School's Institutional Animal Care and Use Committee (Protocols: 19-018, 22-009, 24-009).

Tissue Collection

All rat euthanization and tissue collection was conducted according to previous methods[1]. In brief, rats were sedated via isoflurane (inhalant: 5%, <5 min) and euthanized via cardiac perfusion with 1x phosphate-buffered saline (PBS). Following euthanization, brains were extracted and sliced along midline. The left hemisphere was not used for this study. The HPC of the right hemisphere was microdissected and stored at -80°C in RNAlater until it was processed.

RNA Isolation

HPC samples stored in RNAlater were thawed, homogenized and processed via Quick-RNA Miniprep Plus Kit according to the included protocol (Zymo Research, Cat.#R1057T). The purity of the isolated RNA samples were assessed using the NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Scientific™, Cat.#ND-ONE-W). Samples with high concentration (>50ng/µL) and good purity (260/280 > 1.8) were used to synthesize cDNA.

cDNA Synthesis

Selected RNA samples were diluted to 50ng/µL using nuclease-free water (based on their initial concentrations) and processed using the iScript cDNA Synthesis kit (BioRad, Cat.#1708890) as follows. 20µL reactions were created by combining 4µL of 5x iScript Reaction Mix, 1µL of iScript Reverse Transcriptase, 5µL of nuclease-free water and 10µL of 50ng/µL RNA sample. Reactions were then processed using a C1000 Touch™ Thermal Cycler (BioRad, Cat.#1851196) according to the following protocol included in the kit: Priming (5 min. at 25°C), Reverse Transcription (20 min. at 46°C) and RT inactivation (1 min. at 95°C). Samples were then collected, allowed time to cool, and stored at -20°C.

qPCR

Synthesized cDNA samples were thawed and diluted to a 1:25 mixture with nuclease-free water and prepared for PCR as follows. Each 20µL reaction was run in duplicate on a 96-well plate. Each well contained 2µL of the 1:25 cDNA sample, 10µL of 2x Universal SYBR Green Fast qPCR Mix (ABclonal, Cat.#RK21203), 7.2µL of nuclease-free water, and 0.8µL of a PCR primer. The primer added was either PrimePCR™ SYBR® Green Assay: GAPDH, Rat #10025636 (BioRad, Cat.#qRnoCID0057018) as a housekeeping gene or PrimePCR™ SYBR® Green Assay: CCL2, Rat #10025636 (BioRad, Cat.#qRnoCED0009272) to assess CCL2 expression in the HPC. The 96-well plate containing the 20µL reaction mixes was loaded onto the CFX Duet Real-Time PCR System (BioRad, Cat.#12016265) and run according to the following protocol: Pre-denaturation (3 min. at 95°C), Cycling (5s at 95°C followed by 30s at 60°C, 40 cycles with a collection step after each cycle) and Melting Curve (instrument default).

Calculations

PCR quantification cycles (Cq) are values that indicate how many DNA amplification cycles were necessary for a sample to cross a pre-set fluorescence threshold. Therefore, a low Cq value indicates that the gene of interest was abundant in the sample, as it required fewer amplification cycles to pass threshold. The Cq values of each well containing sample on the 96-well plate were obtained from the CFX Duet Real-Time PCR System and averaged between duplicates. To assess differences in CCL2 expression, Cq^{CCL2} values were normalized to the housekeeping gene (GAPDH) using the ΔCq equation below.

$$\Delta Cq = Cq^{CCL2} - Cq^{GAPDH} \qquad (1)$$

The ΔCq values from the SHAM group were averaged to make the Control Average (CA). Fold change for each ΔCq was then calculated using the equation below.

$$Fold\ change = 2^{-(\Delta Cq - CA)} \qquad (2)$$

Statistical Analyses

Calculated ΔCq and fold change were analyzed using one-way analysis of variance (ANOVA) with Treatment (SHAM, SI, SR) as a between factor. Holm-Sidak post-hoc were performed when indicated by significant ANOVA.

Results

ANOVA on ΔCq values revealed a significant Treatment effect ($F(2,5)=6.465$, $p<0.05$). Pairwise comparisons revealed that SI had significantly lower ΔCq compared to SHAM (**Fig. 1.** $p<0.05$). There were no significant differences in ΔCq between SR and other treatment groups.

ANOVA on fold change revealed no significant Treatment effect. While there was a trend of SI having higher fold change compared to SHAM ($p=0.104$, **Fig. 2**), there were no significant differences between Treatment groups.

Discussion

These qPCR results show that CCL2 expression is significantly increased in SI animals (indicated by decreased ΔCq) compared to SHAM. This suggests that SI alters the neuroinflammatory environment of the HPC via alterations in CCL2 expression. These findings compliment previous work from our lab showing that SI can induce morphological changes in astrocytes, while SR exposure results in astrocyte ablation and a loss of BBB integrity[1]. One potential explanation for these findings is that SI and SR exposure have different effects on the neuroimmune system. SI may be priming the neuroimmune response through the activation of astrocytes which increases the production of CCL2. Increased CCL2 expression can alter astrocyte morphology[33], which could then then regulate the flow of peripheral immune cells through the BBB. While SI primes the neuroimmune response, SR animals may experience a decrease in their neuroimmune response. Animals exposed to SR experience a widespread ablation of their astrocytes[1]. This limits the brain's ability to produce CCL2, which may explain why animals exposed to SR experience significant damage to BBB integrity: their immune systems are not primed to repair the damage caused by SR.

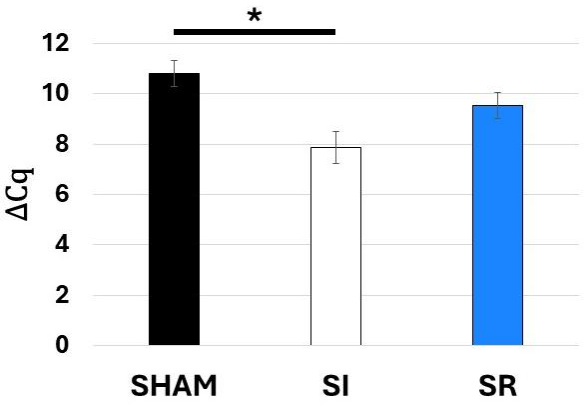

Figure 1. Differences in ΔCq between SHAM, SI and SR. *SI had significantly lower ΔCq compared to SHAM ($p<0.05$)*

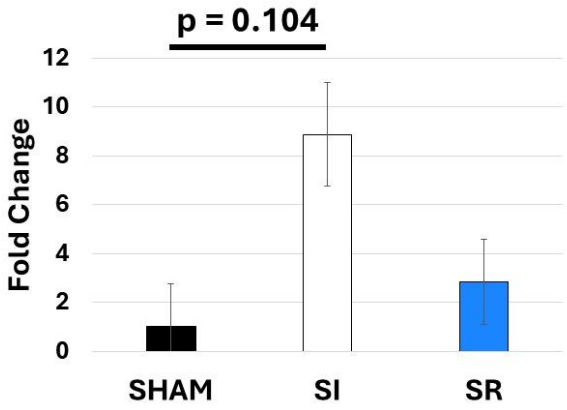

Figure 2. Fold change in CCL2 expression between female SHAM, SI and SR. *There were no significant differences between treatment groups.*

It is important to note that there was a significant difference in ΔCq, but not fold change, between SHAM and SI groups. While both values indicate differences in gene expression, ΔCq shows the difference in quantification cycles (and thus initial CCL2 concentration) between SHAM and SI, while fold change is an exponential transformation of ΔCq. This transformation has the potential to increase variance, which may explain why there is no significant difference seen in fold change. Both ΔCq and fold change are

commonly reported in qPCR analyses, as both are calculations made from initial Cq values; however, previous research has shown that several factors beyond target gene concentration can influence Cq[34]. Therefore, these results should be interpreted cautiously.

Another notable limitation of this study is the low sample size (n=2-3). Prior to statistical analyses, one SR sample was excluded due to having a high Cq value indicative of contamination (Cq>35). This is common practice in qPCR studies[35], as having a Cq>35 can make quantification more difficult[34]. The removal of this SR sample may have prevented us from seeing differences in CCL2 expression in SR animals. Future research should utilize more biological samples (i.e., extracted HPC from more animals) and perform PCR with more technical replicates (i.e., triplicates) to improve quantitation[34].

In summary, these data show that SI can significantly alter the neuroinflammatory response via increases in CCL2 expression. This upregulation manifests as morphological changes, specifically, alterations in astrocyte morphology, which have the potential to prime the neuroimmune system, but can lead to chronic neuroinflammation if not properly regulated. Future research could investigate the potential synergistic effects of SI and SR on CCL2 production, as previous research has shown that these spaceflight hazards together can influence several components of neuroinflammation[1]. Additionally, it is still unclear if the SI-induced priming of the neuroimmune system is consistent or increases over time. If neuroimmune priming does occur throughout the duration of SI exposure, potential ways to mitigate chronic neuroinflammation caused by increased CCL2 expression should be evaluated.

Acknowledgements

This research was supported by the Virginia Space Grant Consortium Graduate Research Fellowship.

Animal tissue samples used for this study were obtained from animals studied in NASA CBS VNSCOR grant 80NSSC19K1582.

The authors would like to thank Amanda Yermal and the Popovics Laboratory (Mariah Jensen-Wachspress, Samantha McGuire, PI: Dr. Petra Popovics) for their assistance in troubleshooting RNA isolation, cDNA synthesis, qPCR processing and data analysis.

References

1 Adkins, A. M. *et al.* Alterations in Blood–Brain Barrier Integrity and Lateral Ventricle Differ in Rats Exposed to Space Radiation and Social Isolation. *Life* **14**, 636 (2024). https://doi.org/10.3390/life14050636

2 Cole, S. W. *et al.* Myeloid differentiation architecture of leukocyte transcriptome dynamics in perceived social isolation. *Proceedings of the National Academy of Sciences* **112**, 15142-15147 (2015). https://doi.org/10.1073/pnas.1514249112

3 Strewe, C. *et al.* Functional changes in neutrophils and psychoneuroendocrine responses during 105 days of confinement. *Journal of Applied Physiology* **118**, 1122-1127 (2015). https://doi.org/10.1152/japplphysiol.00755.2014

4 Adkins, A. *et al.* Differential Impact of Social Isolation and Space Radiation on Behavior and Motor Learning in Rats. *Life* **13**, 826 (2023). https://doi.org/10.3390/life13030826

5 Palinkas, L. A. The psychology of isolated and confined environments: Understanding human behavior in Antarctica. *American Psychologist* **58**, 353-363 (2003). https://doi.org/10.1037/0003-066x.58.5.353

6 Gunderson, E. K. E. Emotional Symptoms in Extremely Isolated Groups. *Archives of General Psychiatry* **9**, 362 (1963). https://doi.org/10.1001/archpsyc.1963.01720160052006

7 Mullin, C. S. SOME PSYCHOLOGICAL ASPECTS OF ISOLATED ANTARCTIC LIVING. *American Journal of Psychiatry* **117**, 323-325 (1960). https://doi.org/10.1176/ajp.117.4.323

8 Acharya, M. M. *et al.* New Concerns for Neurocognitive Function during Deep Space Exposures to Chronic, Low Dose-Rate, Neutron Radiation. *eneuro* **6**, ENEURO.0094-0019. (2019). https://doi.org/10.1523/eneuro.0094-19.2019

9 Lonart, G. *et al.* Executive Function in Rats is Impaired by Low (20 cGy) Doses of 1 GeV/u⁵⁶Fe Particles. *Radiation Research* **178**, 289-294 (2012). https://doi.org/10.1667/rr2862.1

10 Salem, P. P. *et al.* Proton Radiation Therapy: A Systematic Review of Treatment-Related Side Effects and Toxicities. *International Journal of Molecular Sciences* **25**, 10969 (2024). https://doi.org/10.3390/ijms252010969

11 Sanford, L. D. *et al.* Sleep and Core Body Temperature Alterations Induced by Space Radiation in Rats. *Life* **13**, 1002 (2023). https://doi.org/10.3390/life13041002

12 Christiansen, J. *et al.* Loneliness, Social Isolation, and Chronic Disease Outcomes. *Annals of Behavioral Medicine* **55**, 203-215 (2021). https://doi.org/10.1093/abm/kaaa044

13 Xia, N. & Li, H. Loneliness, Social Isolation, and Cardiovascular Health. *Antioxidants & Redox Signaling* **28**, 837-851 (2018). https://doi.org/10.1089/ars.2017.7312

14 Song, Y. *et al.* Social isolation, loneliness, and incident type 2 diabetes mellitus: results from two large prospective cohorts in Europe and East Asia and Mendelian randomization. *eClinicalMedicine* **64**, 102236 (2023). https://doi.org/10.1016/j.eclinm.2023.102236

15 Grant, N., Hamer, M. & Steptoe, A. Social Isolation and Stress-related Cardiovascular, Lipid, and Cortisol Responses. *Annals of Behavioral Medicine* **37**, 29-37 (2009). https://doi.org/10.1007/s12160-009-9081-z

16 Cherry, J. D. *et al.* Galactic Cosmic Radiation Leads to Cognitive Impairment and Increased Aβ Plaque Accumulation in a Mouse Model of Alzheimer's Disease. *PLoS ONE* **7**, e53275 (2012). https://doi.org/10.1371/journal.pone.0053275

17 Rudobeck, E. *et al.* Low-dose proton radiation effects in a transgenic mouse model of Alzheimer's disease – Implications for space travel. *PLOS ONE* **12**, e0186168 (2017). https://doi.org/10.1371/journal.pone.0186168

18 Azizova, T. V., Bannikova, M. V., Grigoryeva, E. S., Rybkina, V. L. & Hamada, N. Occupational exposure to chronic ionizing radiation increases risk of Parkinson's disease incidence in Russian Mayak workers. *International Journal of Epidemiology* **49**, 435-447

(2020). https://doi.org/10.1093/ije/dyz230

19 Miller, K. B. *et al.* Ionizing radiation, cerebrovascular disease, and consequent dementia: A review and proposed framework relevant to space radiation exposure. *Frontiers in Physiology* **13** (2022). https://doi.org/10.3389/fphys.2022.1008640

20 Cucinotta, F. A. & Durante, M. Cancer risk from exposure to galactic cosmic rays: implications for space exploration by human beings. *The Lancet Oncology* **7**, 431-435 (2006). https://doi.org/10.1016/s1470-2045(06)70695-7

21 Verma, S. D. *et al.* Astrocytes regulate vascular endothelial responses to simulated deep space radiation in a human organ-on-a-chip model. *Frontiers in Immunology* **13** (2022). https://doi.org/10.3389/fimmu.2022.864923

22 Seidler, R. D., Mao, X. W., Tays, G. D., Wang, T. & Zu Eulenburg, P. Effects of spaceflight on the brain. *The Lancet Neurology* **23**, 826-835 (2024). https://doi.org/10.1016/s1474-4422(24)00224-2

23 Choukèr, A. *et al.* Effects of confinement (110 and 240 days) on neuroendocrine stress response and changes of immune cells in men. *Journal of Applied Physiology* **92**, 1619-1627 (2002). https://doi.org/10.1152/japplphysiol.00732.2001

24 Vitale, E. M. & Smith, A. S. Neurobiology of Loneliness, Isolation, and Loss: Integrating Human and Animal Perspectives. *Frontiers in Behavioral Neuroscience* **16** (2022). https://doi.org/10.3389/fnbeh.2022.846315

25 Burke, M. *et al.* Sexual dimorphism during integrative endocrine and immune responses to ionizing radiation in mice. *Scientific Reports* **14** (2024). https://doi.org/10.1038/s41598-023-33629-7

26 Adkins, A. M., Colby, E. M., Kim, W. K., Wellman, L. L. & Sanford, L. D. Stressor control and regional inflammatory responses in the brain: regulation by the basolateral amygdala. *J Neuroinflammation* **20**, 128 (2023). https://doi.org/10.1186/s12974-023-02813-x

27 Barnum, C. J., Pace, T. W., Hu, F., Neigh, G. N. & Tansey, M. G. Psychological stress in adolescent and adult mice increases neuroinflammation and attenuates the response to LPS challenge. *Journal of Neuroinflammation* **9**, 9 (2012). https://doi.org/10.1186/1742-2094-9-9

28 Dhikav, V. & Anand, K. Hippocampus in health and disease: An overview. *Annals of Indian Academy of Neurology* **15**, 239 (2012). https://doi.org/10.4103/0972-2327.104323

29 McEwen, B. S. & Gianaros, P. J. Central role of the brain in stress and adaptation: Links to socioeconomic status, health, and disease. *Annals of the New York Academy of Sciences* **1186**, 190-222 (2010). https://doi.org/10.1111/j.1749-6632.2009.05331.x

30 Wu, A. & Zhang, J. Neuroinflammation, memory, and depression: new approaches to hippocampal neurogenesis. *Journal of Neuroinflammation* **20** (2023). https://doi.org/10.1186/s12974-023-02964-x

31 Semple, B. D., Kossmann, T. & Morganti-Kossmann, M. C. Role of

Chemokines in CNS Health and Pathology: A Focus on the CCL2/CCR2 and CXCL8/CXCR2 Networks. *Journal of Cerebral Blood Flow & Metabolism* **30**, 459-473 (2010). https://doi.org/10.1038/jcbfm.2009.240

32 Arfaei, R. *et al.* Decoding the role of the CCL2/CCR2 axis in Alzheimer's disease and innovating therapeutic approaches: Keeping All options open. *International Immunopharmacology* **135**, 112328 (2024). https://doi.org/10.1016/j.intimp.2024.112328

33 Ye, Q. *et al.* Astrocytic Slc4a4 regulates blood-brain barrier integrity in healthy and stroke brains via a CCL2-CCR2 pathway and NO dysregulation. *Cell Reports* **43**, 114193 (2024). https://doi.org/10.1016/j.celrep.2024.114193

34 Ruiz-Villalba, A., Ruijter, J. M. & Van Den Hoff, M. J. B. Use and Misuse of Cq in qPCR Data Analysis and Reporting. *Life* **11**, 496 (2021). https://doi.org/10.3390/life11060496

35 Grzybkowska, A. *et al.* Identification of Optimal Reference Genes for qRT-PCR Normalization for Physical Activity Intervention and Omega-3 Fatty Acids Supplementation in Humans. *International Journal of Molecular Sciences* **24**, 6734 (2023). https://doi.org/10.3390/ijms24076734