CRISPR-Cas9 Mediated Whole-Body C3 Knockout Mice Demonstrate Resilience to Acute Stress Response: Single-Cell Brain Transcriptomics Highlights the Importance of Systems Therapy

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Abstract

Introduction: Acute stress response (ASR) is a precursor to many long-term psychological disorders, including post-traumatic stress disorder (PTSD). Despite its significance, there is no robust intervention to mitigate ASR. Studies have linked complement component C3 to neurological disorders and behavioral deficiencies associated with ASR. Functional analyses suggest that C3-triggered proinflammation plays a critical role in neurogenesis and neurodegeneration. C3-knockout (C3-KO) mice exhibit increased learned fear and resilience to chronic stress caused by social interactions with intruders [1,2]. Additionally, our human PTSD study revealed a strong association between blood C3 levels and PTSD, a major consequence of ASR 03]. To explore the therapeutic potential of C3 modulation, we conducted a CRISPR-Cas9-mediated whole-body C3 knockout at the germline and evaluated its effects on ASR using the Aggressor-Exposed Social Stress (Agg-E SS) model.

Materials and Methods: In this model, naïve mice (Sham or C3-KO) were exposed to conspecific aggressor mice for 10 consecutive days, 6 hours per day, in a cage-in-cage arrangement. Periodically, the barrier was removed to allow direct interaction [5]. After 10 days of Agg-E SS, the stressed mice were placed in the aggressor's home cage with a fenestrated barrier physically separating them, and their behaviors were measured in the presence of contextual threat. Mice were euthanized and postmortem brain tissues were processed for single-cell RNA sequencing (scRNA-seq).

Results: C3-KO mice demonstrated significant resilience to Agg-E SS compared to wild-type mice. Brain scRNA-seq data revealed five major cell types—astrocytes, microglia, oligodendrocytes, ependymal cells, and neural progenitors—with distinct gene clusters differentially enriched by C3-KO under Agg-E SS conditions. Functional analysis showed cell type-specific diversity. For example, C3-KO prevented Agg-E SS-induced collagen degradation in ependymal cells but not in astrocytes. Similarly, long-term depression caused by Agg-E SS was inhibited by C3-KO in ependymal cells but not in oligodendrocytes or microglia.

Conclusion: C3-KO mice showed promising results in ameliorating ASR, as evidenced by behavioral analysis. The scRNA-seq data revealed significant diversity among brain cell types, emphasizing the need for customized therapeutic approaches. These findings suggest that targeting C3 could be a potential strategy for mitigating ASR and its associated neurological impacts.

C3 Gene Selection

Figure 1.

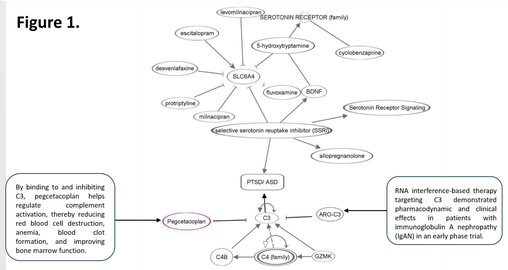

Figure 1. C3-mediated protein networks toward ASR: Molecular pathway showing Selective serotonin reuptake inhibitor (SSRI), sole FDA approved drug to treat PTSD and C3/ complement family work in independent routes to mitigate PTSD/ASR. Hence pertinent drugs can work in complementary fashions. Nevertheless, cross-drug toxicity needs to take in account.

Figure 2A.

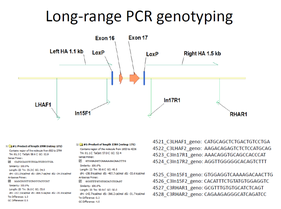

Figure 2B.

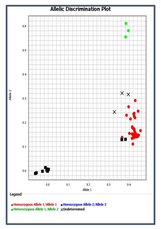

Figure 2. Generation and validation of a germline whole-body C3 knockout model. **(A)** The targeted spatial region of the C3 gene used to generate the germline whole-body C3 knockout is shown. **(B)** Allelic discrimination plots following genotyping demonstrate successful homozygous knockout. Early results indicate detection of the expected mutation resulting in a homozygous genotype. The same methods were applied to control mice, and analysis is ongoing. Comparison of PCR product sizes and allelic discrimination plots between experimental and control samples confirmed successful C3 knockout.

Study Design

Figure 3.

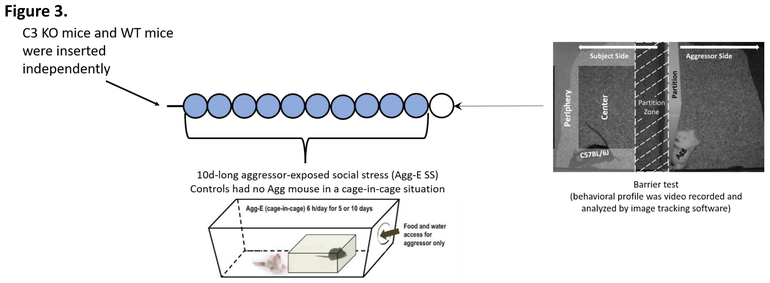

Figure 3 [5]. Study timeline of Aggressor-exposed social stress (Agg-E SS). Typical aggressor male mouse strain, namely SJL was co-housed in a box-in-box paradigm with the naïve mouse (WT or C3KO C57BL/6j male) for 6h daily for 10 consecutive days. On 11th day, the naïve mice were placed for a barrier test, where the home cage of SJL mouse was separated by a fenestrated barrier. The behavior of the naïve mouse was video taped and analyzed to find how a stressed mice (WT and C3KO) behave to the cues of past traumatized experience.

Results

Figure 4A.

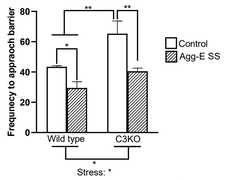

Figure 4B.

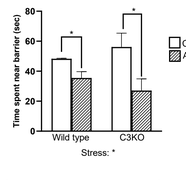

Figure 4C.

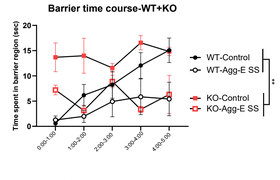

Figure 4. Ethogram analysis of the naïve mice near barrier separating it from the aggressor mouse: Longer time spent near barrier is a sign of high curiosity (natural trait of rodents) overpowering the fear. C3KO Agg-E SS mice spent less time near barrier **(A-B)**. However, the time course analysis **(C)** found that C3KO control mice showed no sign of fear from the beginning of barrier test; while C3 KO-Agg-E SS mice showed a different pattern than the WT Agg-E SS mice. KO treatment emerged as the major factor in differentiating the behavioral profile.

Figure 5A.

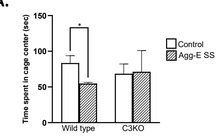

Figure 5B.

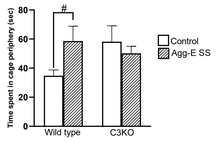

Figure 5. Ethogram analysis of the naïve mice spending time away from the aggressor mouse: Time spent inside the cage center, rather an open space is a sign of less fear, and the results showed that C3 KO caused reducing the fear among Agg-E SS mice (A). Time spent near periphery is a sign of anxiety and a trait underlining the withdrawal behavior from cues related to fearful experience. The result showed that wild type Agg-E SS mice spent long time near periphery, but C3 KO reduced their time spending in the peripheral region (B).

Figure 6.

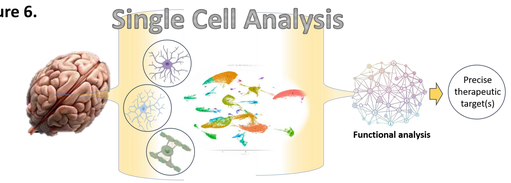

Figure 6. Single cell analysis of the brain harvested from WT and C3KO mice with and without ASR. Nuclei from frozen mouse brain were isolated with 10x Genomics kit, debris removed, and 7AAD-positive nuclei FACS-sorted. Sorted nuclei were counted and loaded (≤17,000/sample) on Chromium Next GEM for 3′ v3.1 library prep. Libraries were sequenced on NovaSeq X Plus and processed with Cellranger 7.2.0 using mm10 reference.

Diagnostic Panel

Figure 7.

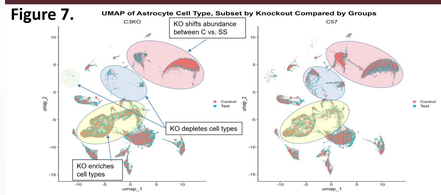

Figure 7. Identification and cluster annotation of <u>astrocytes</u> by UMAP. The left panel shows astrocyte clusters from C3-KO mice without and with ASR (C vs. SS), and the right panel shows astrocyte clusters from WT mice without and with ASR (C vs. SS). Highlighted areas indicate clusters in which astrocyte populations are shifted.

Figure 8.

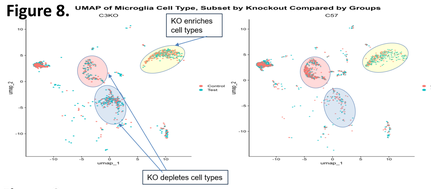

Figure 7. Identification and cluster annotation of <u>microglia</u> by UMAP. The left panel shows astrocyte clusters from C3-KO mice without and with ASR (C vs. SS), and the right panel shows astrocyte clusters from WT mice without and with ASR (C vs. SS). Highlighted areas indicate clusters in which microglia populations are shifted.

Figure 9.

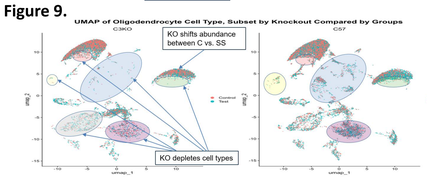

Figure 7. Identification and cluster annotation of <u>oligodensdrocytes</u> by UMAP. The left panel shows astrocyte clusters from C3-KO mice without and with ASR (C vs. SS), and the right panel shows astrocyte clusters from WT mice without and with ASR (C vs. SS). Highlighted areas indicate clusters in which oligodensdrocyte populations are shifted.

Figure 10.

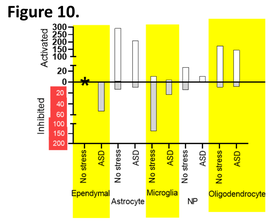

Figure 10. Bar plot showing the brain cell-specific networks. Y-axis presents the number of activated and inhibited (highlighted in red) networks in different brain cells. A variable profile was observed across the brain cell lines.

Conclusions

- C3 is a potential therapeutic target for ASR, as the C3-mediated network can regulate ASR/PTSD through an SSRI-independent pathway.
- A whole-body C3 knockout mouse model was generated without observable mortality or morbidity.
- C3-KO mice exhibited beneficial behavioral phenotypes in mitigating ASR, consistent with prior studies.
- Single-cell brain RNA-seq revealed cell type–specific effects of ASR and C3-KO.
- Ongoing work focuses on cell-specific mechanistic insights to guide development of novel therapies to ameliorate ASR.

References:

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The experiments reported herein were conducted in compliance with the Animal Welfare Act and per the principles set forth in the "Guide for Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 2011.