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Stress Hormone Enhancement of OP-Induced Neuroinflammation as an Animal Model of GWI: The Role of Toll-like Receptors and Plasticity

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14. ABSTRACT
Several chemicals and environmental conditions have been implicated in the exposures in theater that caused GWI. We have combined GW agent exposures with corticosterone (CORT) in the mouse to simulate physiological stresses in the war theater. The Morris water maze failed to provide evidence of an impairment in this paradigm. We switched to diisopropylfluorophosphate (DFP) as it was a stronger organophosphate inflammagen, and utilized novel object testing as more compatible with the mouse behavioral repertoire. In addition, we instituted a longer intermittent CORT administration with a single DFP dose, and used lipopolysaccharide (LPS) as an acute neuroimmune reactivator. We found evidence of cognitive impairment in mice in the novel object tests using both CORT administration regimens. These impairments persist until 12-14 days after DFP or LPS. However, the impairments were not present when separate animals were tested 60-63 days after exposures were terminated. Studies of cellular proliferation suggested that mice were in adrenocortical insufficiency at the time of behavioral testing after the 1 week CORT+DFP regimen ended. Preliminary work has indicated changes in gene expression of specific NMDA receptor subunits after CORT+DFP, but additional testing is necessary to understand the cellular basis of the CORT priming phenomenon and the role of neuroinflammation.

15. SUBJECT TERMS
Gulf War Illness, chronic neuroinflammation, diisopropylfluorophosphate, physiological stress, Novel Object paradigms, lipopolysaccharide, neurogenesis

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TABLE OF CONTENTS

INTRODUCTION: ***Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.***

Gulf War Illness (GWI) is a multi-symptom disorder with features similar to "sickness behavior" (e.g., fatigue, joint pain, cognitive impairments, gastrointestinal problems). While these symptoms usually abate over time as physiological homeostasis returns, individuals with GWI experience recurring bouts of severe symptoms. The exposures and conditions in theater that caused GWI remain undefined but several chemicals and environmental conditions have been implicated. We have combined GW agent exposures with corticosterone (CORT) in a mouse model to simulate physiological stresses in the war theater. Previous work has determined that proinflammatory effects of DFP were markedly enhanced by pretreatment with the anti-inflammatory stress hormone CORT. The neuroinflammatory effects observed after CORT+DFP exposure consisted of increased elaboration of proinflammatory cytokine and chemokine gene expression, providing the underlying molecular basis for "sickness behavior". Also, a greater understanding of the basis of the CORT "priming" effect is needed to identify targets for therapeutic intervention. Efforts were undertaken to explore the cortical expression of plasticity-related genes and better understand the bases of the priming. In addition, experiments examined the extended duration of the synaptic and behavioral effects resulting from CORT+organophosphate(OP)-induced neuroinflammation and suggested potential means to diminish this condition by specific treatment approaches to affect neurogenesis and plasticity. The impact of elucidating a role for these mechanisms in this GWI model is invaluable to the understanding of the molecular basis of GWI and the development of targeted therapeutic interventions.

1. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Gulf War Illness, chronic neuroinflammation, diisopropylfluorophosphate, physiological stress, novel object paradigms, lipopolysaccharide, neurogenesis, hippocampus, CORT priming

2. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

All studies at the University of Illinois College of Medicine Peoria were conducted under Specific Aim 3, which purported to define the effects of CORT-induced priming on cognitive function at the molecular and behavioral levels. The pertinent Major Tasks/milestones are listed below with the associated degrees of completion at the current time.

1) Establish the role of CORT + DFP on neurogenesis at two times after DFP administration – 50% completed
2) Define the effect of CORT + DFP on stimulated gene expression in mice monitored for paired-pulse responses and LTP magnitude – approximately 25% completed
3) Confirm the effects of GW agent exposure on behavioral performance in parallel with measures of neurogenesis, gene expression, and synaptic transmission – behavioral testing 100% complete

4) Confirm the effectiveness of therapeutic approaches using trophic agents in ameliorating the CNS signs of CORT + DFP. Establish the basis for novel combination therapy to treat GWI: administration of a non-conventional anti-inflammatory agent with a CNS trophic drug – not initiated.

The progress of the investigation in pursuit of these goals is described in more detail in the following section.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

A full description of the accomplishments, conclusions, and contingencies is provided at the end of this report form.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The lab staff member recruited to this project, who was responsible for much of the data collection and analysis, was mentored and trained in the performance of these duties by a senior lab manager. This was his first experience in an extramurally-supported research lab and his first exposure to behavioral paradigms using laboratory animals. This young man, Jacob R. Jones, has an undergraduate degree in Biology as a pre-med major and will enter medical school in the fall of 2020 with a renewed perspective on biomedical research.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The behavioral results from the 1-week CORT+DFP regimen were presented in March 2019 at the Society of Toxicology annual meeting:

Kelly, K.A., Michalovicz, L.T., Fornal, C.A., Miller, D.B., O'Callaghan, J.P. and **Lasley, S.M.** Behavioral and histological evidence of a neuroimmune basis for Gulf War Illness. The Toxicologist, 168, 1346, 2019.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

This is the final report of the project. The award period has ended.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The Impact section is provided at the end of the Accomplishments discussion at the end of this form.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

The revisions to the experimental design have been manipulations of treatment parameters and also involved inclusion of additional test paradigms. These changes include setup and incorporation of testing with the Morris water maze and Novel Object paradigms, integration of the 5-week intermittent CORT administration exposure protocol into the project, substitution of DFP as the organophosphate in place of chlorpyrifos, and the use of LPS as an acute inflammagen.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

While there was previous experience with the longer CORT administration protocol and LPS dosing in Dr. O'Callaghan's laboratory, the gene expression studies did not involve assessments beyond 1-3 days post-treatment. In contrast, the behavioral studies of necessity had to lower LPS doses and extend DFP/LPS dosing intervals to avoid testing mice experiencing "sickness behavior" from the neuroinflammation. The rationale for the changes in the preceding section is provided in an earlier annual report on the project. The negative results in year 1 and the use of the longer duration CORT+DFP exposure protocol, including piloting of LPS dosing and treatment intervals, have required substantial periods of time and have significantly slowed progress on the project. Animal group sizes have also been higher than anticipated to gain statistical power.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Because of the delays cited above and the inability to complete all studies within the award period, fewer animals were expended in the project. Since some planned experimentation could not be conducted due to time constraints, consumable supplies were also somewhat less than budgeted.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

N/A

All project revisions that involved animals were not enacted until local IACUC and ACURO approval had been obtained. The slow pace of ACURO review of protocol amendments was a bit of a hindrance throughout the project.

Significant changes in use of biohazards and/or select agents

At the dose utilized (0.25 mg/kg, i.p. on consecutive days) LPS is not considered a hazardous chemical and has not posed a danger of lethality to the animals.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Michalovicz, L.T., Locker, A.R., Kelly, K.A., Mille,r J.V., Barnes, Z., Fletcher, M.A., Miller, D.B., Klimas, N.G., Morris, M., **Lasley, S.M.**, O'Callaghan, J.P. Corticosterone and pyridostigmine/DEET exposure attenuate peripheral cytokine expression: Supporting a dominant role for neuroinflammation in a mouse model of Gulf War Illness. *NeuroToxicology*, 70, 26-32, 2019.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of*

publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Kelly, K.A., Michalovicz, L.T., Fornal, C.A., Miller, D.B., O'Callaghan, J.P. and **Lasley, S.M.** Behavioral and histological evidence of a neuroimmune basis for Gulf War Illness. *The Toxicologist*, 168, 1346, 2019.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Casimir A. Fornal, Ph.D., Laboratory Manager
Worked full-time throughout the project at 100% effort conducting the behavioral studies and training and mentoring Mr. Jones during the last year of the project in execution of the behavioral and neurogenesis testing
Dr. Fornal was supported by this GWIRP Award

Jacob R. Jones, B.S., Laboratory Technician (undergraduate degree in Biology)
Provided 30% of his time on this project during its last year
Mr. Jones assisted in the behavioral and neurogenesis data collection/analysis
He was supported by a subcontract from an independent GWIRP Award (S. Chatterjee, Ph.D., PI)

Raghava Sriramaneni, B.S., Laboratory Technician
Worked part-time (60% effort) for a three-month period in year 1 of the project
Mr. Sriramaneni assisted in the Morris water maze testing

Catherine McCarthy, Laboratory Technician
Worked part-time (45% effort) as an undergraduate pre-med major during the summer of year 1
Ms. McCarthy assisted in the Morris water maze testing

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

The following organization was involved in this project as a partner organization with independent funding as part of this Research Expansion Award.

Organization Name: Centers for Disease Control and Prevention
Location of Organization: Morgantown, West Virginia
Partner's Contribution to the Project:
Collaboration – collaboration through Dr. James P. O'Callaghan and his research group

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

A collaborative award has been made to Dr. O'Callaghan for this Research Expansion Award, and an independent annual report is being submitted from his research group.

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*
N/A

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

These data were collected early in the study, but did not uncover CORT+OP exposure effects, and thus were not among the primary findings of the project. They are presented here to provide a complete presentation of the work performed in the project.

The Morris water maze was the initial behavioral test of spatial learning employed instead of the Barnes maze (also a spatial learning paradigm) specified in Major Task 7. There is a wealth of information in the biomedical literature concerning execution and scoring of this test with rodents – much more than for the Barnes maze – and we had previous experience with this paradigm. In this test a mouse must learn the location of an escape platform by swimming in a pool of water at room temperature and using cues in the test environment. The platform sits just below the water surface, and is not visible because the water is made opaque with powdered milk. Acquisition training was conducted over 6 days with four trials per mouse per day with trials separated by 30-40 minutes. After every 8 trials (i.e., every other day) mice are given a probe trial in which the hidden platform is removed from the pool. A video camera records each trial and animal performance is scored by commercial software that measures latency to find the platform, time spent in the platform quadrant of the pool, and the mean distance to the platform.

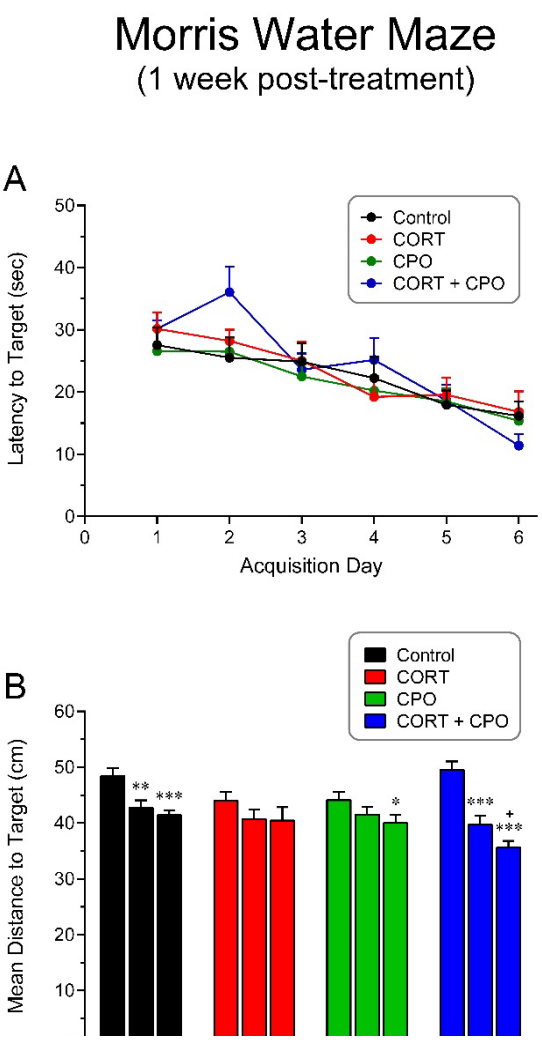

Figure 11. Water maze test results conducted 1 week after CORT + CPO treatment. Latencies (A) and Mean Distance to Target (B) decrease as mice learn location of escape platform (N = 12-14/group). No between group differences were found. $^{**}p < 0.01$; $^{***}p < 0.001$ vs. corresponding mean for 8 trials. $^{+}p < 0.05$ vs. mean for 16 trials.

Mice were administered CORT in their drinking water at 0.4 mg/ml for 7 days and on day 8 chlorpyrifos oxon (CPO) was administered at 2 mg/kg, i.p. The study included four experimental groups: a vehicle Control group, groups receiving only CORT or only CPO, and a group treated with both CORT plus CPO (N = 12-14 mice/group). Testing in the water maze began one week later. The results are shown in **Figure 11A** for the latency measure, which clearly decreased across acquisition trials as the animals learned the hidden platform location (the Trials $F = 18.57$, $p < 0.0001$). We have found the mean distance to target to be the most reliable indicator of learning, and **Figure 11B** demonstrates that learning took place in all groups except for the CORT only group (Treatment $F = 47.10$, $p < 0.0001$; Treatment × Trials interaction $F = 4.96$, $p < 0.0002$). These data indicate that the CORT+CPO regimen did not impair learning in the water maze.

In consideration of the possibility that a cognitive impairment may take longer than one week to develop after CORT+CPO exposure, a small cohort of mice (N = 6/group) were administered the same exposure regimen described above, but 86 days were allowed to transpire between CPO administration and initiation of acquisition training for the water maze. The results of testing are shown in **Figure 12**. Again, there is a decrease in the latency to find the platform across acquisition days in all groups in **Figure 12A** (Trials $F = 24.94$, $p < 0.0001$), and performance on the probe tests

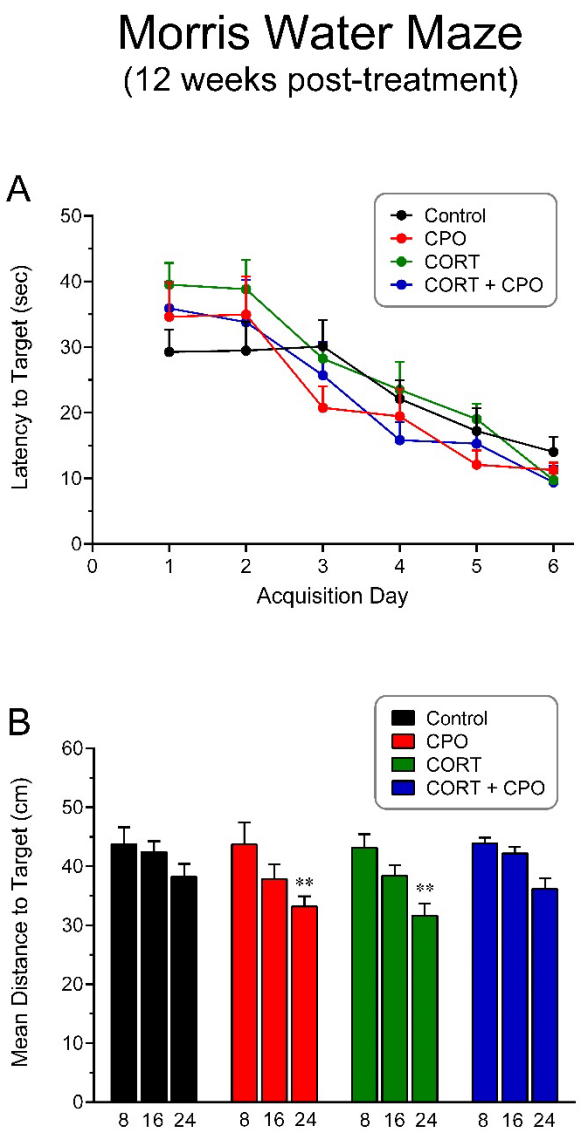

Figure 12. Water maze test results conducted 12 weeks after CORT + CPO treatment. Latencies (A) and Mean Distance to Target (B) decrease as mice learn location of the escape platform (N = 6-8/group). No between group differences were found. $^{**}p < 0.01$ vs. corresponding mean for 8 trials.

in **Figure 12B** demonstrate that spatial learning would likely have occurred in all groups had a full cohort (N = 6-8/group) been tested (Trials $F = 16.11$, $p < 0.0001$). Again, there was no evidence that the CORT+CPO treatment produced an impairment of learning in the water maze.

It was therefore apparent that the priming of the immune response from one week of CORT administration plus the neuroinflammation produced by CPO one day later was not sufficient to affect learning and memory assessed in the water maze 1 or 12 weeks post-administration.

REFERENCES

1. Sparkman, N.L., Kohman, R.A., Scott, V.J. and Boehm, G.W. Bacterial endotoxin-induced behavioral alterations in two variations of the Morris water maze. *Physiol. Behav.* 86, 244-251 (2005).
2. Sparkman, N.L., Martin, L.A., Calvert, W.S. and Boehm, G.W. Effects of intraperitoneal lipopolysaccharide on Morris water maze performance in year-old and 2-month old female C57Bl/6J mice. *Behav. Brain Res.* 159, 145-151 (2005).

3. Lee, J.W., Lee, Y.K., Yuk, D.Y., Choi, D.Y., Ban, S.B., Oh, K.W. and Hong, J.T. Neuroinflammation induced by lipopolysaccharide causes cognitive impairment through enhancement of beta-amyloid generation. *J. Neuroinflammation* 5, 37 (2008). doi:10.1186/1742-2094-5-37
4. Whoolery, C.W., Walker, A.K., Richardson, D.R., Lucero, M.J., Reynolds, R.P., Beddow, D.H., Clark, K.L., Shih, H.Y., LeBlanc, J.A., Cole, M.G., Amaral, W.Z., Mukherjee, S., Zhang, S., Ahn, F., Bulin, S.E., DeCarolis, N.A., Rivera, P.D., Chen, B.P.C., Yun, S., and Eisch, A.J. Whole-body exposure to ^{28}Si-radiation dose-dependently disrupts dentate gyrus neurogenesis and proliferation in the short term and new neuron survival and contextual fear conditioning in the long term. *Radiat Res*. 188, 532-551 (2017).
5. Wong, E.Y.H., and Herbert, J. Roles of mineralocorticoid and glucocorticoid receptors in the regulation of progenitor proliferation in the adult hippocampus. *Eur. J. Neurosci.* 22, 785-792 (2005).
6. Spanswick, S.C., Epp, J.R., and Sutherland, R.J. Time-course of hippocampal granule cell degeneration and changes in adult neurogenesis after adrenalectomy in rats. *Neuroscience* 190, 166-176 (2011).
7. Cameron, H.A., and McKay, R.D.G. Restoring production of hippocampal neurons in old age. *Nature Neurosci.* 2, 894-897 (1999).
8. Wong, E.Y.H., and Herbert, J. The corticoid environment: A determining factor for neuroprogenitors' survival in the adult hippocampus. *Eur. J. Neurosci.* 20, 2491-2498 (2004).

ACCOMPLISHMENTS

Drs. O'Callaghan and Lasley concluded at the beginning of the project that the behavioral studies (Statement of Work (SOW) Major Task 7) should be conducted before the neurophysiological (SOW Major Task 6) and biochemical (SOW Major Tasks 5-6) work to establish a CORT+OP exposure regimen that produced a defined functional effect. This approach was important since behavioral experiments preceding this project being funded had not been successful in this pursuit. Once established, this treatment regimen would then be the basis of the remaining studies conducted on the project at the University of Illinois at Chicago.

The Morris water maze was the initial behavioral test of spatial learning employed. From the data collected it was apparent that the priming of the immune response from one week of CORT administration plus the neuroinflammation produced by chlorpyrifos oxon (CPO) one day later was not sufficient to affect learning and memory assessed in the water maze 1 or 12 weeks post-administration (see **Appendix**). Consequently, changes were made in the experimental design of the GW agent exposure protocol. DFP replaced CPO as the former is a more potent OP and greater inflammagen, and because DFP had been utilized to gather much of the initial data on this GWI model. Also, two other test paradigms have been established in our group – the novel object recognition (NOR) and novel object location (NOL) tests to assess mouse cognition. In these tests the animals are given habituation sessions in the test chamber with two similarly sized objects present. On test days one of the two familiar objects is replaced with a novel one (NOR test) or one of the familiar objects is moved to a distinctly new location in the chamber (NOL test). A video camera records the animals' movements in the test chamber during the tests, and animals are scored on the time they spend exploring the novel object or the familiar object in a new location.

Significant progress was made toward achieving Major Task 7 by identifying a behavioral paradigm that uncovered cognitive impairments in the GWI mouse model. In the primary GW agent exposure regimen mice are administered CORT in their drinking water at 0.4 mg/ml for 7 days and on day 8 an OP is administered (e.g., DFP at 4 mg/kg, i.p.). The study includes four experimental groups: a vehicle Control group, groups receiving only CORT or only DFP, and a group treated with both CORT plus DFP. Novel object testing began three days after DFP (**Figure 1**). It was also reasoned that to achieve sufficient neuroinflammation to induce behavioral changes it may be necessary to: 1) enhance CORT priming in the animals by administration for longer than one week; 2) administer a standard immune stimulus at some time point after DFP to induce a renewed neuroinflammation. Thus, in consultation with Dr. Jim O'Callaghan at the Centers for Disease Control and Prevention we developed a 5-week intermittent CORT administration regimen in which CORT was administered in the drinking water during weeks 1, 3, and 5. DFP would be administered after week 1 of CORT as in the shorter regimen, and then a low dose of LPS (0.25 mg/kg, i.p.) would be injected twice five and six days after the end of week 5 with behavioral testing beginning three days after the last LPS dose (**Figure 1**).

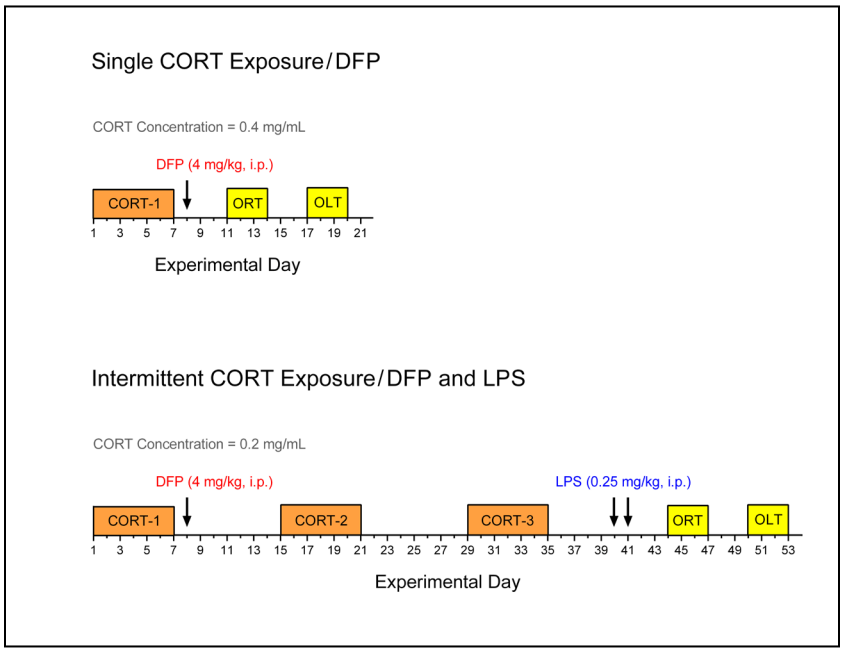

Figure 1. Timelines of CORT, DFP, and LPS dosing and behavioral testing in the two CORT administration regimens utilized in the current project. Multi-day intervals after DFP or LPS dosing before novel object testing were to avoid testing acutely affected animals. ORT, novel object recognition test; OLT, novel object location test.

Several aspects of these exposure parameters had to be tested in pilot experiments, changes were made, and then the parameters were retested. The time intervals after DFP and LPS administration before behavioral testing began were inserted to ensure that animals were not assessed when they did not feel well. We tested higher doses of LPS (0.5 and 1.0 mg/kg), but found them to be too harsh on the animals. A dose of 0.1 mg/kg LPS alone has been reported to not consistently affect behavior (1-2); a dose of 0.25 mg/kg LPS has affected cognitive behavior when tested a few hours later (1-3), but we believed this would not be the case if the post-dose interval was three days. There also is evidence that when this latter dose of LPS is administered once daily for 3-5 days, a potentiated inflammatory response results on the second and subsequent days. This is the basis for administering the 0.25 mg/kg LPS dose on consecutive days as part of the 5-week intermittent CORT exposure protocol. We also inserted a 5-day interval between the end of CORT administration in this regimen and LPS injection to avoid the possibility of inducing neuroinflammation in animals in a state of partial adrenal insufficiency. Establishing these exposure parameters to observe a reliable functional effect was deemed critically important to the project, and as a result this testing consumed most of the second year of the project.

As shown in **Figure 2** for animals given the 1-week CORT administration regimen, the Control, DFP only, and CORT only groups successfully discriminated the familiar and novel objects in the 10 min Novel Object Recognition test using Exploration Time of the objects as the metric. But the CORT+DFP combination group did not (Familiarity factor $F = 24.9$, $p < 0.0001$; Familiarity × Exposure interaction $F = 4.0$, $p = 0.059$) make this distinction. **Figure 3** exhibits a similar effect using Distance Traveled in proximity to the objects as the measure in the same groups of animals (Familiarity $F = 12.8$, $p < 0.0001$; Familiarity × Exposure interaction $F = 4.7$, $p = 0.024$). In this Figure the CORT only group did not make a significant discrimination of

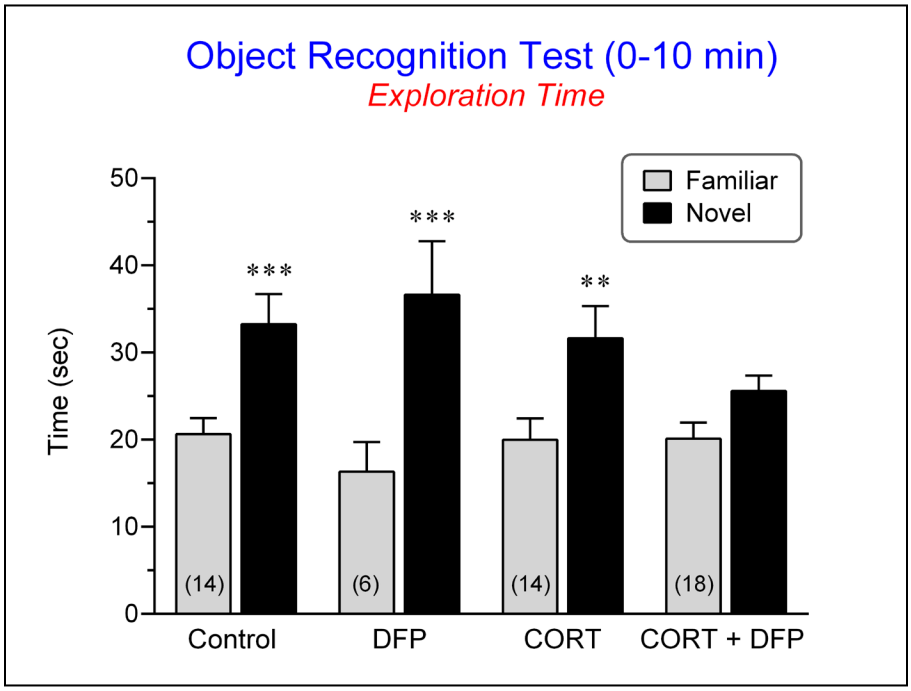

Figure 2. Group performance in the Novel Object Recognition test as a function of time exploring the novel and familiar objects. All groups distinguished the novel objects except for the CORT+DFP group. Values are mean ± SEM with group sizes indicated at the bottom of the Familiar bars for each group. ***$p < 0.001$; **$p < 0.01$ vs the Familiar object by Sidak's multiple comparison test.

novelty, while the distance around the novel object in the CORT+DFP group was significantly less than the corresponding value for the Control group. These latter observations resulted in the statistically significant interaction effect.

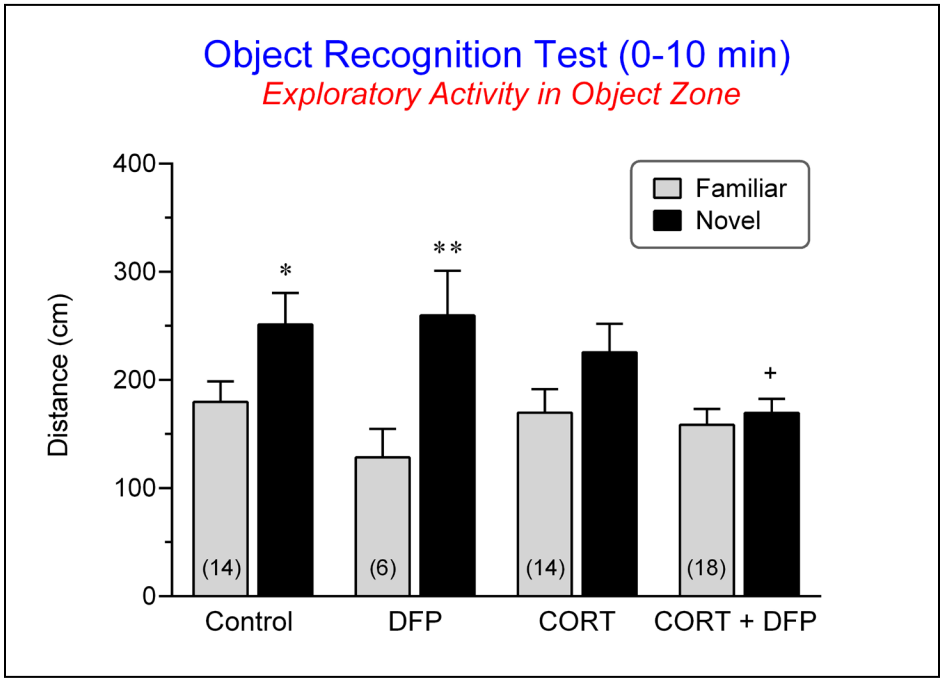

Figure 3. Group performance in the Novel Object Recognition test as a function of distance traveled in the object zones. The CORT+DFP group did not distinguish the novel object in this test, and the activity proximal to the novel object was less than that of the Control group. Values are mean ± SEM with group sizes indicated at the bottom of the Familiar bars for each group. **$p < 0.01$; *$p < 0.05$ vs the respective Familiar object; +$p < 0.05$ vs the Control novel object value by Sidak's multiple comparison test.

No group changes were uncovered in the Novel Object Location data with these experimental groups (data not shown). Thus, the persistence of the neuroinflammation effect on cognitive function extended to 6 days after DFP administration after the 1 week of CORT.

Figure 4 displays the results from the first 20 seconds of Exploration Time in the Novel Object Location test in animals administered the 5-week intermittent CORT exposure regimen described earlier. The value of isolating the first 20 seconds of exploration is that it identifies the period of greatest novelty to the displaced object; the novelty component declines as the test session time extends to 10 minutes. In the Figure both the Control and CORT only groups successfully made the distinction between the familiar and displaced objects, while the group receiving CORT+DFP+LPS did not. Exploration Time's on both the novel and displaced objects in the latter group were significantly different from the corresponding values for the CORT only group (Familiarity $F = 45.4$, $p < 0.0001$; Familiarity × Exposure interaction $F = 13.8$, $p = 0.004$), and the CORT only means were significantly different from Control means. These comparisons result in the statistically significant interaction effect.

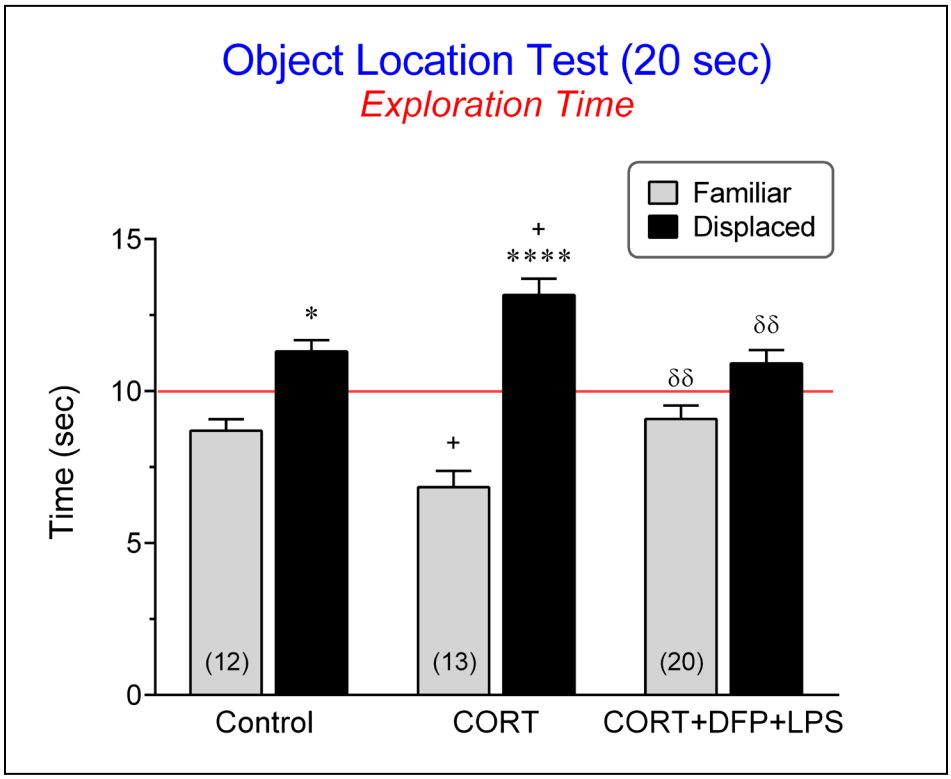

Figure 4. Group performance in the Novel Object Location test as a function of time exploring the novel and displaced objects during the first 20 sec of exploration. The Control and CORT only groups distinguished the displaced object, but the CORT+DFP+LPS group did not. Values are mean ± SEM with group sizes indicated at the bottom of the Familiar bars for each group. ****$p < 0.0001$; *$p < 0.05$ vs the respective Familiar object; $^{+}p < 0.05$ vs. corresponding Control value; $^{\delta\delta}p < 0.01$ vs corresponding CORT value by Sidak's multiple comparisons test.

Figure 5 exhibits a similar effect using Distance Traveled in proximity to the objects in the first 20 sec of exploration as the measure in the same groups of animals (Familiarity $F = 42.2$, $p < 0.0001$; Familiarity × Exposure interaction $F = 9.2$, $p = 0.004$). All groups made a significant discrimination of the displaced object on this measure, but the distance around the displaced object in the CORT+DFP+LPS group was significantly less than the distance around the relocated object in the CORT only group. These latter observations resulted in the statistically significant interaction effect. No group changes were uncovered in the Novel Object Recognition data with these experimental groups (data not shown). Thus, the persistence of the neuroinflammation effect on cognitive function extended to 12 days after the last LPS administration in the 5-week intermittent CORT exposure regimen (see **Figure 1**).

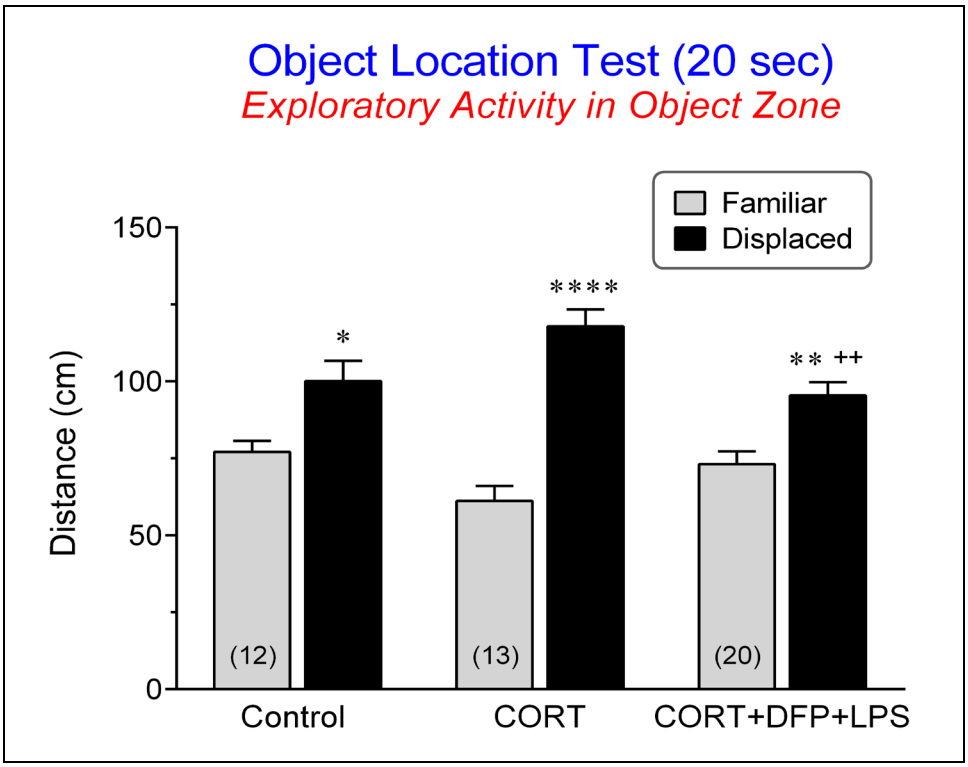

Figure 5. Group performance in the Novel Object Location test as a function of distance traveled in the object zones during the first 20 sec of exploration. All groups distinguished the displaced object, but the distance traveled proximal to the displaced object by the CORT+DFP+LPS group was less than the corresponding CORT only group. Values are mean ± SEM with group sizes indicated at the bottom of the Familiar bars for each group. ****$p < 0.0001$; **$p < 0.01$; *$p < 0.05$ vs the respective Familiar object; $^{++}p < 0.05$ vs. CORT only displaced value by Sidak's multiple comparisons test.

A key objective in the project was to demonstrate the durability of the behavioral impairment over a longer period of time after exposure to CORT and DFP had ended. This was an effort to display the validity of this mouse model in simulating the cognitive problems widely reported by GWI veterans. Accordingly, we next assessed the persistence of the cognitive impairment observed in the Novel Object Recognition test at 5-6 days post-exposure (see **Figures 2** and **3**) by testing separate groups of animals at about two months after CORT+DFP treatment. **Figure 6** compares the group performances at the two post-exposure intervals on the same experimental measures. The left hand panels display exposure effects very similar to those shown in **Figures 2-3**, while the right hand panels present group performance at 62-63 days after exposure was terminated. At the longer interval ANOVAs failed to find significant effects with either the Exploration Time (Familiarity $F = 9.40$, $p < 0.0001$; Familiarity × Exposure interaction $F = 0.69$, $p > 0.05$) or Distance Traveled (Familiarity $F = 5.25$, $p = 0.0003$; Familiarity × Exposure interaction $F = 0.47$, $p > 0.05$). Thus, the CORT+DFP-induced cognitive impairment in Novel Object Recognition testing did not persist until two months after exposure. Animals were also tested in the Novel Object Location paradigm at the long post-exposure interval, but no treatment-related effects were uncovered (data not shown).

We then undertook a neurogenesis experiment to assess hippocampal cellular proliferation using the 1-week CORT+DFP exposure regimen. Mice were perfused at the same age and post-exposure interval that produced the behavioral effects shown in **Figures 2** and **3**, so that the findings could perhaps be related to the cognitive measures. Two 5-bromo-2'-deoxyuridine (BrdU) antibodies were tested using a protocol that previously had yielded good staining, but the antibodies did not perform well under these experimental conditions. Modifications were

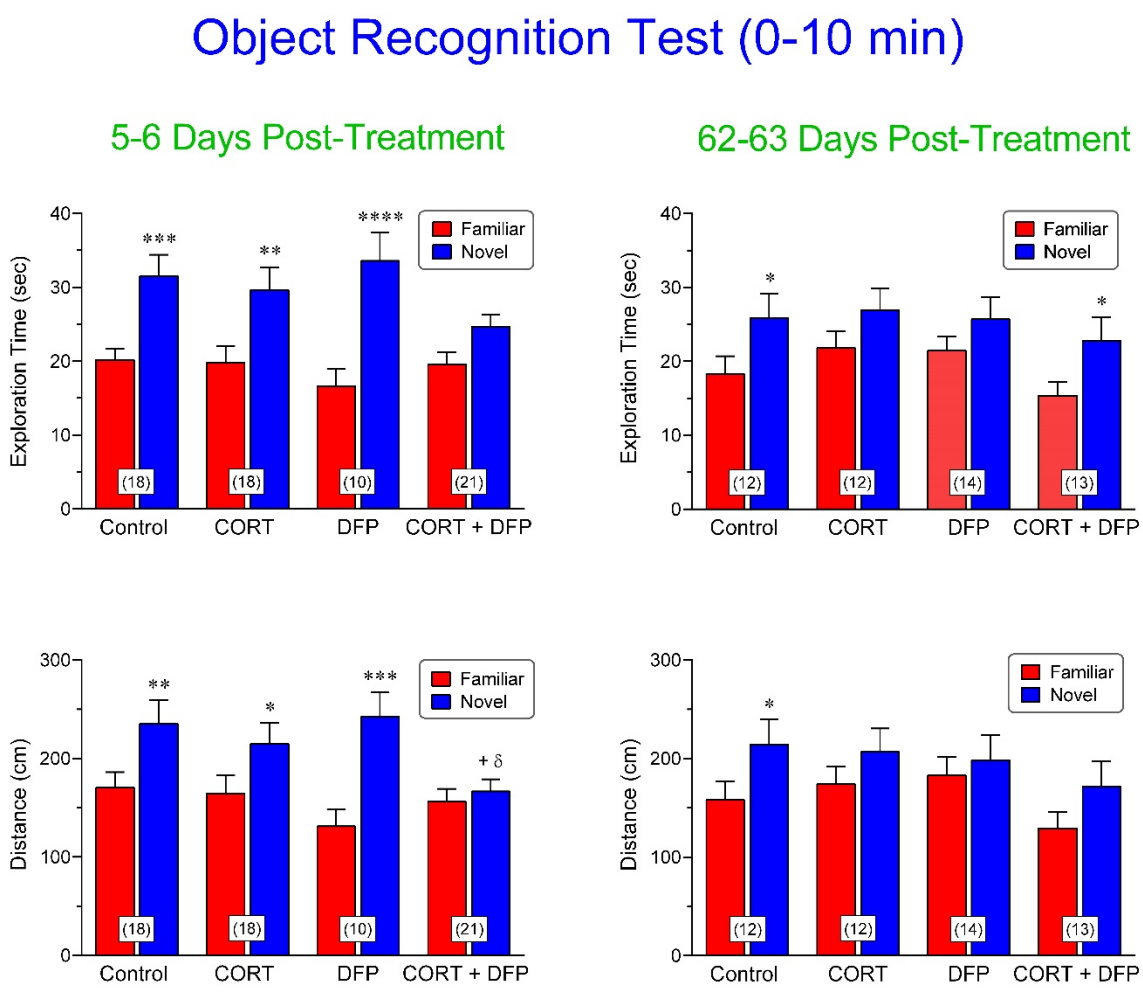

Figure 6. Comparison of group performance in the Novel Object Recognition test at short and long intervals in independent animal cohorts after termination of CORT+DFP exposure using the same measures as in **Figures 2** and **3**. Note that effects in the CORT+DFP group seen after the shorter interval post-exposure do not persist over the longer interval of approximately two months as shown in the right hand graphs. Values are mean ± SEM with group sizes are indicated at the bottom of the Familiar bars for each group. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ vs the associated Familiar object; $^{+}p < 0.05$ vs. CORT only displaced value; $^{\delta}p < 0.05$ vs the Novel value in Control group by Sidak's multiple comparisons test.

subsequently made to the assay and labeling was obtained, but at the expense of excessive tissue damage due to the harsher treatments employed. Therefore, we decided to label proliferating cells in the hippocampal dentate gyrus by staining for the endogenous nuclear protein Ki67. In some respect Ki67 may be a better proliferation marker than BrdU. Unlike the thymidine analog BrdU, which is incorporated into dividing cells only during the DNA synthesis phase of the mitotic process, Ki67 is expressed in proliferating cells during all phases of mitosis and therefore provides a longer time scale assessment of proliferative activity. Also, tissue staining of Ki67 and BrdU is correlated to a high degree (5). Another advantage of Ki67 over BrdU is that changes in BrdU labeling may be due to alterations in BrdU bioavailability

(e.g., systemic absorption or uptake into the brain) rather than actual changes in proliferation, since it is not an endogenous marker like Ki67.

Animals were perfused 5 days post-exposure with 4% paraformaldehyde in phosphate buffer within the same three-hour period of the day. Frozen coronal sections (35-μm thick) were cut throughout the entire hippocampus and a 1-in-8 series of tissue was then processed for Ki67, using a slide-mounted immunoperoxidase technique (4). Sections were counterstained with cresyl violet, dehydrated and coverslipped. All slides were analyzed blind with respect to treatment by a single trained scorer using an Olympus BX-41 light microscope. In every 8th section, Ki67-positive cells were counted bilaterally in the dentate gyrus at 400× magnification. The dentate gyrus included the granule cell layer (GCL), subgranular zone (SGZ), and the hilus. The cell counts for each animal were summed across all sections and then multiplied by 8 to obtain an estimate of the total labeled cell number within the dentate gyrus. In addition, labeled cells were also counted separately in the SGZ/GCL layer and in the hilus. Cells located within two cell-body widths (~ 20 μm) from the border of the GCL were considered to be in the SGZ; cells located more distally were considered to be in the hilus. Proliferation was assessed in both the SGZ and hilus since it is generally believed that proliferating cells in the SGZ and in the hilus give rise primarily to neurons and glia, respectively. Typically, there were 10 sections/mouse containing the dentate gyrus.

The results are shown in **Figures 7** and **8**. The total number of cells labeled with Ki67 in all portions of the hippocampal dentate gyrus are displayed in **Figure 7**. The data demonstrate a striking 60-65% equivalent increase in cellular proliferation in the groups receiving the 1-week administration of CORT in the drinking water with or without DFP (ANOVA $F = 10.03$, $p = 0.0009$). **Figure 8** tells the same story counting only cells in the SGZ/GCL (ANOVA $F = 10.85$, $p = 0.0006$). The increased proliferation in the CORT+DFP group is unexpected, since increased cellular proliferation typically supports enhanced cognitive function. That is, the CORT treatment is at least partially attenuating whatever other CORT+DFP-induced cellular mechanism(s) that is causing the behavioral impairment.

Exogenous CORT administration is known to produce a significant decrease in the number of dividing cells, while conversely removal of glucocorticoids has been shown to dramatically increase cell proliferation (6-8). Our findings can be reconciled to this literature if a state of transient adrenocortical insufficiency results once the 1-week CORT exposure is terminated, and the behavioral testing and animal perfusion are performed before adrenocortical homeostasis is restored. The glucocorticoid deficiency during this transient period results in the elevated Ki67 staining and cellular proliferation that is shown in **Figures 7-8**. A longer post-exposure interval before behavioral testing is begun could be inserted into the protocol, but would have to be balanced against the expected durability of the overall cognitive impairment.

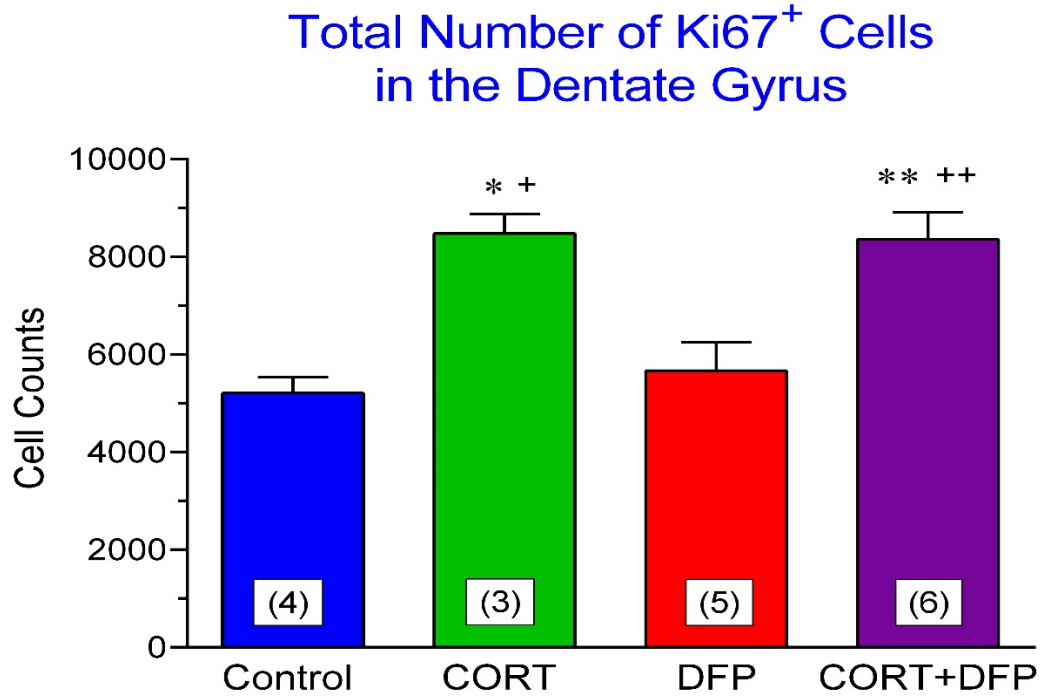

Figure 7. Total counts of Ki67-labeled proliferating cells in all regions of the hippocampal dentate gyrus in groups, ages, and exposure protocol that parallels the treatment of mice in **Figures 2-3**. Groups receiving CORT exhibited 60-65% increases in counts. Values are mean ± SEM with group sizes indicated within each bar. $^{**}p$ <0.01; $^{*}p$ <0.05 vs Control mean; ^{++}p <0.01; ^{+}p <0.05 vs the DFP mean.

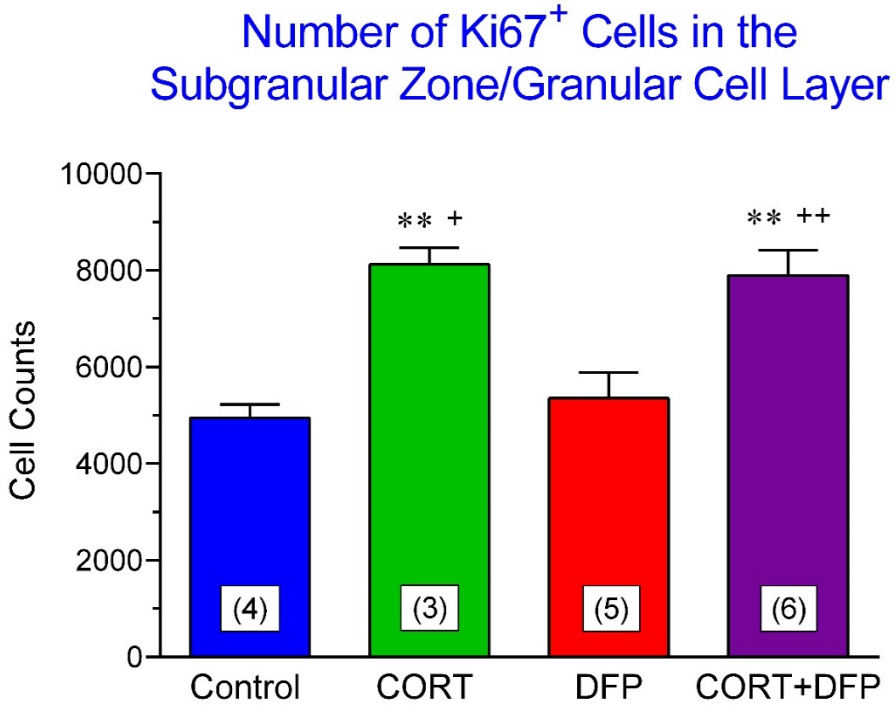

Figure 8. Total counts of Ki67-labeled proliferating cells in the SGZ/GCL of the hippocampal dentate gyrus in groups, ages, and exposure protocol that parallels the treatment of mice in **Figures 2-3**. Groups receiving CORT exhibited 60-65% increases in counts. Values are mean ± SEM with group sizes indicated within each bar. $^{**}p$ <0.01 vs Control mean; ^{++}p <0.01; ^{+}p <0.05 vs the DFP mean.

In separate efforts an initial survey of cortical plasticity-related gene expression was performed for both the 1-week CORT+DFP and the 5-week intermittent CORT+DFP+LPS exposure

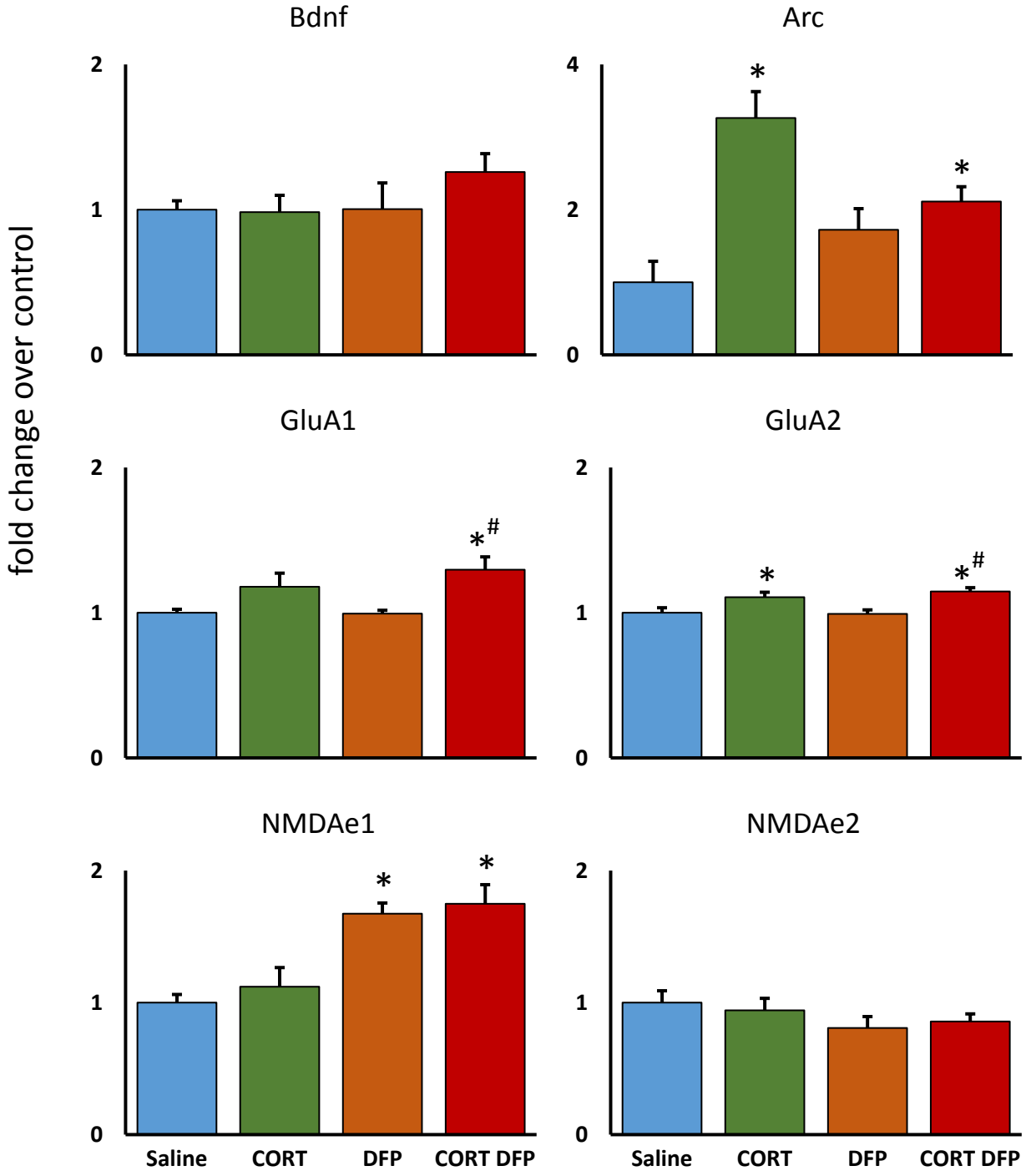

Figure 9. Evaluation of plasticity markers in GWI RNAseq dataset. The cortex from animals exposed to the 1-week CORT+DFP exposure paradigm was used for genome-wide RNAseq expression analysis. From this existing dataset, the expression of Bdnf, Arc, GluA1, GluA2, NMDAe1, and NMDAe2 were extracted and evaluated for statistical significance.

regimens. These data were collected by the O'Callaghan research group and are included in their annual report, but are also presented here as the work was described in Major Task 6 in the SOW. The expression of plasticity-related genes were evaluated in the two exposure regimens to include *Bdnf, Arc, GluA1, GluA2, NMDAε1*, and *NMDAε2* in RNA sequencing datasets. These preliminary data (**Figure 10**) give promising results for more in-depth analysis of these targets. In particular, the differential results with the NMDA receptor subunits are interesting in that *NMDAε1* showed a DFP-induced response in the 1-week CORT+DFP protocol that is maintained in the 5-week intermittent CORT+DFP+LPS dataset and *NMDAε2* responded only in the 5-week dataset.

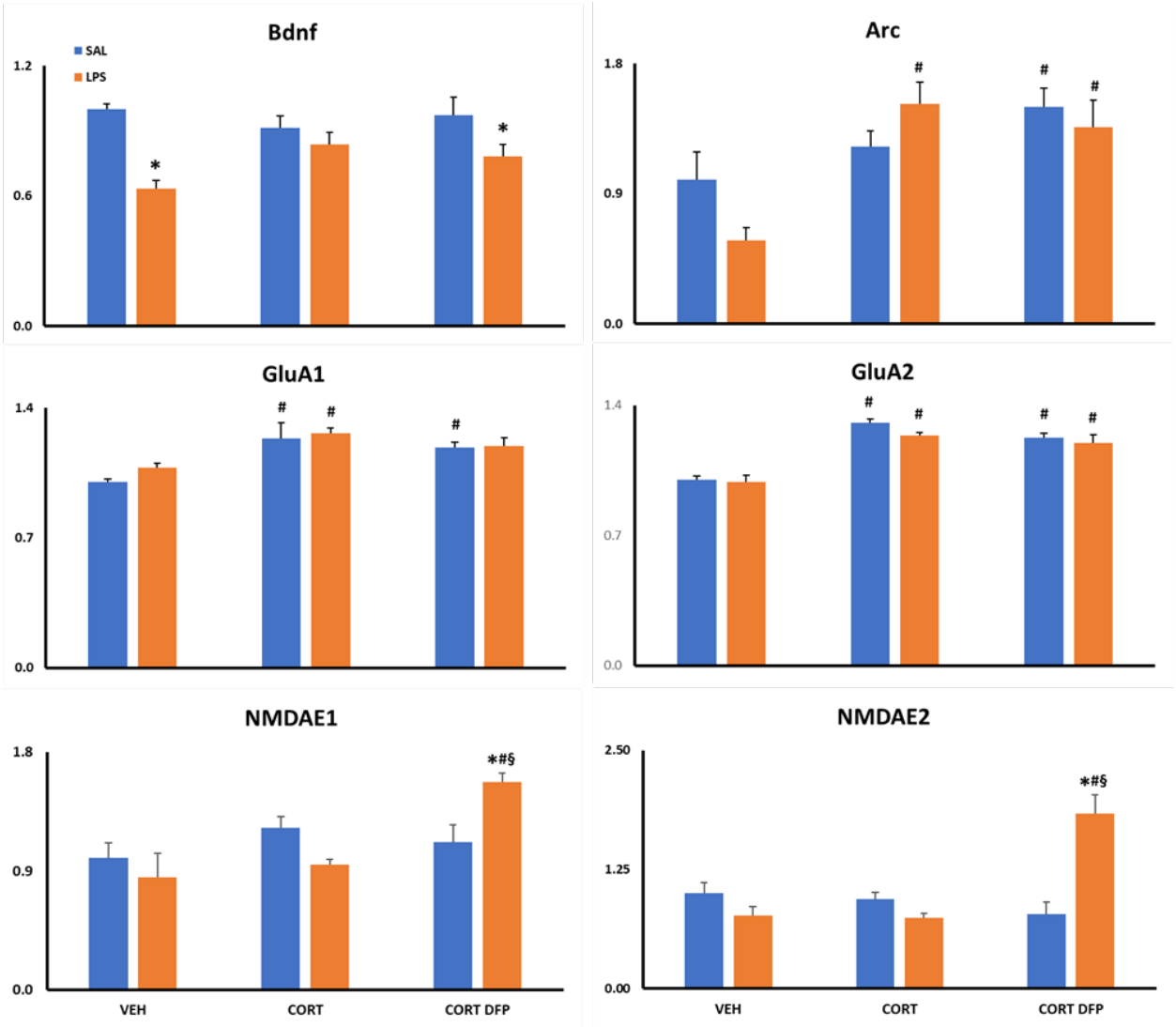

Figure 10. Evaluation of plasticity markers in GWI RNAseq dataset. The cortex from animals exposed to the 5-week intermittent CORT+DFP+LPS exposure paradigm was used for genome-wide RNAseq expression analysis. From this existing dataset, the expression of Bdnf, Arc, GluA1, GluA2, NMDAe1, and NMDAe2 were extracted and evaluated for statistical significance.

IMPACT

The primary impact of the results reported herein is that they are the first demonstration that the enhanced gene expression of proinflammatory cytokines/chemokines produced by the CORT+DFP exposure protocols exerts a demonstrable effect on mouse behavior when tested up to two weeks after DFP or LPS dosing. This finding constitutes a short term confirmation of our hypothesis that the potentiated neuroimmune response to CORT+DFP can affect plasticity and behavior. It is worth noting that the two novel object paradigms involve different types of responding to demonstrate learning, and that the Novel Object Location test constitutes a more difficult task. This latter test requires a form of spatial learning and is thought to be dependent on hippocampal function, while Novel Object Recognition responding is more globally represented in brain activity.

However, an impairment of behavior could not be discriminated when a separate cohort of animals were tested two months after termination of the 1-week CORT+DFP regimen. Both exposure protocols have been quite productive in identifying cellular mechanisms underlying CORT/DFP/LPS administration, but even the Novel Object paradigms have had limited ability to distinguish exposure-related changes. It is possible that the cognitive capacity of mice and their variable responding may not be appropriate for the behavioral task discriminations they were asked to make and that rats may constitute better subjects for these treatment protocols.

The results with the cellular proliferation data were surprising in that they show no association with the observed behavioral impairment in Novel Object Recognition testing, and that they appear to define a state of adrenocortical insufficiency occurring in the period after CORT administration ends. The enhanced proliferation is apparently attenuating whatever other cellular mechanisms are responsible for the disruption in cognitive function. This observation also implies that behavioral testing should be delayed more than three days after CORT+DFP exposure has ended.

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January 24, 2020

Brett Chaney
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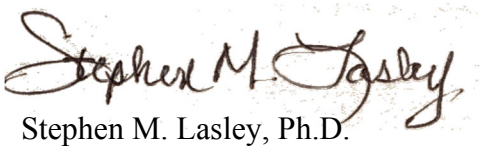
Dear Mr. Chaney:

Attached please find the final report for W81XWH-16-1-0556, "Stress Hormone Enhancement of OP-Induced Neuroinflammation as an Animal Model of GWI: The Role of Toll-like Receptors and Plasticity". It has been completed according to the instructions provided on the https://ebrap.org web site under "Technical Reporting Requirements" and using the provided reporting forms in Word format.

Please do not hesitate to contact me if you need additional information or require further documentation.

Yours,



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