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PRINCIPAL INVESTIGATOR: Dr. Stephen Lasley

CONTRACTING ORGANIZATION: University of Illinois College of Medicine at Peoria
Peoria, IL 61656-1649

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14. ABSTRACT Chemical exposures are thought to precipitate the chronic symptoms associated with GWI. Chief among these are nerve agents encountered during deployment, treatment with a reversible cholinesterase inhibitor, and the use of pesticides. Cholinesterase inhibition has been proposed as a basis for the symptoms of GWI, and sarin is known to result in neuroinflammation. Physiologic stress experienced in theater also has been proposed as a contributor to GWI, and our results confirm that prior exposure to corticosterone (CORT) can enhance neuroinflammation. In this work we demonstrate that the sarin surrogate diisopropylphosphorofluoridate (DFP) results in neuroinflammation in mouse brain, an effect on multiple proinflammatory cytokines that is markedly enhanced by prior exposure to CORT and suggests the pathophysiological basis of GWI. GWI is characteristic of the "sickness" behavior typical of an inflammatory response to infection or injury, and a protracted condition is consistent with symptoms observed in ill veterans. We also have demonstrated that episodic exposure to CORT over an 180-day post-DFP period reveals a markedly enhanced priming effect that persists indefinitely. Finally, we found that an anti-inflammatory response, suggesting the use of non-steroidal anti-inflammatory drugs in the chronic treatment of GWI.					
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1. INTRODUCTION:

Several conditions and exposures have been hypothesized to serve as instigating events that precipitated the recurring/chronic symptoms associated with Gulf War Illness (GWI) (Binns et al., 2008; 2014). Chief among these are nerve agent exposure resulting from detonations at Khamisiyah and other Iraqi weapons facilities (Binns et al., 2008; 2014), prophylactic treatment with the reversible cholinesterase inhibitor, pyridostigmine bromide (PB), and the use of pesticides/insect repellants (e.g., DEET) in theater. Inhibition of cholinesterase due to sarin and PB exposures has been put forth as a basis for the symptoms of GWI (Golomb et al., 2008) and, at least with respect to sarin, such exposures can result in neuroinflammation in a rat model (Spradling et al., 2011a; 2011b). Whether reversible inhibition of acetylcholinesterase (AChE) with PB or exposure to insecticides or insect repellants produces a neuroinflammatory response remains to be examined. Physiological stress experienced in theater also has been proposed as a potential contributor to GWI (Sapolsky 1998) and, consistent with this view, are our observations that prior exposure to the rodent stress hormone, corticosterone (CORT), can enhance neuroinflammation associated with neurotoxicity (Kelly et al., 2012). In this CDMRP-supported work we demonstrate that the sarin surrogate diisopropyl fluorophosphate (DFP) results in neuroinflammation across the mouse brain, an effect markedly enhanced by prior exposure to the stress hormone CORT, findings that we propose may serve as the pathophysiological basis of GWI in an animal model. A markedly enhanced expression of multiple proinflammatory cytokines in multiple brain areas was engendered by DFP alone but especially when preceded by exposure to CORT in the drinking water. These findings mimic, in an animal model, the features of sickness behavior. GWI is a multi-symptom disorder with features characteristic of “sickness” behavior (Dantzer and Kelley, 2007; Dantzer et al., 2008), e.g., cognitive impairment, fatigue, depression, sleep disruption, muscle and joint pain, and gastrointestinal and dermatological problems (Fukuda et al., 1998; Steele, 2000; Haley, 2001; Golomb, 2008). Typically, sickness behavior is the normal manifestation of an inflammatory response to infection or injury, and one that resolves with restoration of homeostasis (Pavlov et al., 2003). In GWI, the sickness behavior symptoms persist, findings suggestive of a heightened or chronic neuroimmune/neuroinflammatory reaction, the etiology for which remains unknown. Thus, we believe that our animal model of GWI is consistent with symptoms observed in ill GW veterans and that they represent a protracted sickness behavior phenotype over the past 23 years since the 1991 Gulf War.

Importantly, the heightened “primed” state for mounting an enhanced neuroinflammatory response to a known inflammogen, lipopolysaccharide (LPS), persisted to a small degree out to 180 days post-dosing, i.e. the equivalent in the mouse of 20 years. In experiments performed outside those specified in the grant, we now know that episodic exposure to CORT over the 180-day post-DFP dosing period reveals a markedly enhanced priming to challenge with an inflammogen, such as LPS, that persists indefinitely. We take these findings to mean that day

to day living conditions for a veteran suffering from GWI is sufficient to retain the “primed” neuroinflammatory response to inflammogens in the environment/living space. Finally, our finding that the atypical tetracycline-like anti-inflammatory drug, minocycline (MINO), dampens the inflammatory response in our model, argues for the use of such non-steroidal anti-inflammatory drugs in the chronic treatment of GWI. Future studies will benefit from a better understanding of the molecular underpinnings of the immune “priming” in order to prevent it from recurring and persisting over years.

2. **KEYWORDS:**

Gulf War Illness, chronic neuroinflammation, diisopropyl fluorophosphate, physiological stress, minocycline, cytokines, hippocampus

3. **OVERALL PROJECT SUMMARY:**

Work Objective

The project involved the completion of 3 specific aims using mice as research subjects. Specific Aim 1 - evaluate molecular, cellular and functional indices of neuroinflammation in mice exposed to GWI-relevant agents and conditions; specifically, PB, DEET, and DFP. Specific Aim 2 - evaluate the contribution of high physiological stress to measures of neuroinflammation in this model by exposing mice to high levels of the stress hormone, CORT. Specific Aim 3 - evaluate an FDA-approved atypical non-steroidal anti-inflammatory agent, minocycline, as a potential therapeutic drug for GWI symptoms related to neuroinflammation.

Detailed Statement of Work

The results achieved relative to the specific aims of the project are described below, organized according to the original Statement of Work Tasks.

Task 1. Protocol Approval (Meets Aims 1-3, will take ~ 3 months in Year 1) Upon award receipt, the PI, Dr. O’Callaghan and Dr. Lasley, completed and submitted for approval applications for animal use in the proposed project at their respective institutions. ***Outcome:*** *Two approved protocols.*

Task 2. Dose Ranging Studies (Meets Aims 1-3, will take ~ 2 months in Year 1) These studies determined the doses of DEET and PB that can be administered alone and in combination with DFP, LPS and CORT without producing lethality or excessive morbidity.
Outcome: *Selection of doses to be used in full study.*

Task 3. Determine Duration of the Neuroinflammation Caused by CORT & LPS and CORT & DFP (Meets Aim 1, will take ~ 8-9 months across Years 1, 2) Mice were dosed to obtain tissue at days 15-17, 90-92, and 180-182 for molecular assessment of 6 brain areas and at day 15 for cellular/histological assessment – preliminary data had indicated neuroinflammation to be present at day 15. **Outcome:** *Evaluation of tissue for the presence and duration of neuroinflammation and/or neural injury associated with CORT & LPS, CORT & DFP.*

DFP causes neuroinflammation in multiple brain regions. Administration of the sarin surrogate, DFP, caused a brain-wide neuroinflammatory response (frontal cortex, hippocampus, striatum, olfactory bulb, hypothalamus and cerebellum; Figure 2). The expression of multiple cytokine and chemokine mRNA's was enhanced in multiple brain areas after a single dose of DFP. Not all areas were affected equally but all brain areas sampled showed various degrees of enhanced expression of a large number of proinflammatory mediators.

Increased expression of $\text{TNF-}\alpha$, CCL2, LIF, IL-6, IL-1 β , and OSM were seen at 2 hours post-DFP, were increased further by 6 hrs in most cases, with a resolution toward control levels by 12 hrs post-DFP (Figure 3). These data are in general agreement with the effects noted for administration of sarin to the rat (Spradling et al., 2011a, 2011b) and together stand in contrast to the known anti-inflammatory responses expected for enhanced cholinergic agonism.

Immunohistochemical staining for IBA1 (microglia), GFAP (astrocytes), silver and fluoro-jade B (neurodegeneration) was examined 24 hours after DFP exposure. Slight increases in microglial and astrocyte activation can be seen in the CA1 region of the hippocampus (Figure 4). Neither IBA1 nor GFAP showed obvious increases in any other brain region examined. No evidence of neurodegeneration (either by silver or fluoro-jade B stain) is apparent in the hippocampus (Figure 4) or other brain regions assessed.

LPS causes neuroinflammation across multiple brain regions. Systemic LPS (2 mg/kg, s.c.) exposure caused a generally robust increase in neuroinflammatory cytokines and chemokines in cortex, hippocampus, striatum, hypothalamus, olfactory bulb and cerebellum (Figure 5).

The increased neuroinflammation was seen as early as 2 hours and lasted to 12 hours after LPS exposure in multiple brain regions (Figure 6).

Figure 6: Acute time course of LPS neuroinflammation in cortex and hippocampus.

CORT enhances DFP neuroinflammation in multiple brain regions. Enhanced physiological stressors, e.g., heat, exercise and war stress were encountered in theater during the 1991 Gulf War and may have contributed to the development of GWI. To emulate this possibility in our mouse model, we administered the stress hormone CORT in the drinking water prior to administration of DFP. While an anti-inflammatory effect might be expected with a classic anti-inflammatory glucocorticoid such as CORT, we found a marked exacerbation of the neuroinflammatory effect of DFP in all brain areas examined (Figure 7). Again, as with DFP alone, the degree of the CORT-enhanced neuroinflammation varied by brain region, but was observed in all areas sampled.

While neuroinflammation induced by DFP alone was resolving by 12 hours after exposure, CORT pretreatment caused a prolonged neuroinflammatory response in IL-1 β (Figure 8).

Chronic CORT pretreatment exacerbated the DFP-induced increase in activation of astrocytes specific to the CA1 region of the hippocampus (Figure 9). This increase was not enough to increase the GFAP protein concentration in the hippocampus as a whole (data not shown), which showed no change in any treatment group. There was no evidence of neurodegeneration in the hippocampus (Figure 9) or any other brain region observed.

CORT also enhances the neuroinflammatory response to the known systemic inflammogen, LPS. The neuroinflammation occurred in multiple brain regions at 6 hours after LPS exposure (Figure 10).

CORT exposure enhanced the LPS-induced neuroinflammation, as responses were more severe and longer lasting as indexed by the lack of resolution at 12 hours after inflammogen exposure (Figure 11).

One week of CORT in the drinking water was sufficient to exacerbate the neuroinflammatory response to LPS, even 30 days after the cessation of CORT exposure (Figure 12). This priming effect had resolved by 90 days after cessation of CORT exposure (Figure 13).

To model the impact of multiple instances of high physiological stress as would be seen in true theater scenarios, mice were treated with CORT in the drinking water bi-weekly for 3 (Figure 13) or 6 (Figure 14) months. When the mice were exposed to LPS on day 90 (Figure 13) or 180 (Figure 14) the neuroinflammatory response was greatly exacerbated over that of LPS alone.

The response was also intensified over that seen in mice exposed to LPS directly after CORT exposure, in some cases 10-fold (see Figure 10 values on D15 of the regimen).

Task 4. Determine if MINO Protects Against LPS- or DFP-Induced Neuroinflammation in Chronic CORT-Treated Mice (Meets Aim 3, will take ~ 1.5 months in Year 1) Mice were dosed to obtain tissue at days 15-17 (see Figure 1) for molecular assessment of 6 brain areas and at day 15 for cellular/histological assessment. ***Outcome:** Evaluation of tissue to determine if MINO is neuroprotective in CORT& LPS- or CORT & DFP-induced neuroinflammation.*

MINO pretreatment suppresses neuroinflammation resulting from DFP and CORT + DFP. MINO is a tetracycline class antibiotic with anti-inflammatory properties. Previously we showed that the neuroinflammatory response to MPTP and methamphetamine could be suppressed by prior treatment with MINO (Sriram et al., 2006), raising the possibility that MINO could affect the neuroinflammatory response to DFP with or without prior treatment with CORT. In both hippocampus and frontal cortex MINO pretreatment for 2 weeks suppressed the CORT- enhanced neuroinflammatory response to DFP (Figure 15).

Minocycline pretreatment also reduced the CORT-induced exacerbated neuroinflammatory response to LPS exposure (Figure 16).

Task 5. Determine if PB & DEET followed by LPS or DFP Challenge Induces Immediate or Delayed Neuroinflammation (Meets Aim 1, will take ~ 8-9 months across Years 1 & 2)

Mice were dosed to obtain tissue at days 15-17, 90 – 92 and 180-182 (see Figure 1) for molecular assessment of 6 brain areas and at day 15 for cellular/histology assessment.

Outcome: Evaluation of tissue to determine if GWI-relevant agents are able to cause immediate, delayed or persistent neuroinflammation and/or neural injury.

Pretreatment with 14 days of PB and DEET did not affect LPS-induced neuroinflammation at any time point or in any brain region studied, and therefore data from those experiments have been omitted from the rest of this report.

PB +DEET do not enhance DFP-induced neuroinflammation. Among the many potential exposures encountered by troops deployed to the 1991 Gulf War was prophylactic administration of PB to counteract nerve agent exposure and DEET to ward off insects.

Therefore, we administered both of these compounds for two weeks and evaluated the subsequent proinflammatory response to DFP.

Across multiple brain regions and neuroinflammatory endpoints, PB + DEET did not result in a neuroinflammatory response. When PB+DEET-treated mice were administered DFP, further enhancement of DFP-induced expression of proinflammatory cytokines and chemokines was not observed. Indeed, for some proinflammatory mediators prior treatment with PB + DEET suppressed the DFP-related neuroinflammation at either the 2 or 6 hr post-DFP dosing time point (Figure 17).

Although it appears as though PB+DEET pretreatment may have caused a mild activation of astrocytes (Figure 18), there was no quantitative change in GFAP protein concentration in the hippocampus (data not shown). No evidence of neurodegeneration was found in the hippocampus (Figure 18) or in any other brain region examined.

Task 6. Determine if Chronic CORT Influences the Impact of LPS or DFP Challenge in PB & DEET-Treated Mice (Meets Aim 2, will take ~ 8-9 months across Years 2 & 3) Mice were dosed to obtain tissue at days 15-17, 90 – 92, 180 – 182 (see Figure 1) for molecular assessment of 6 brain areas and at day 15 for cellular/histological assessment. **Outcome:** *Evaluation of tissue to determine if high levels of the stress hormone CORT exacerbate the neuroinflammatory or neural injury effects of GWI- relevant agents.*

PB+DEET do not enhance DFP-induced neuroinflammation with or without prior CORT. Administration of PB+DEET for 14 days combined with CORT in the drinking water during days 7-14 caused a similar exacerbation of DFP-induced neuroinflammation (Figure 19) as seen in mice pretreated with CORT alone before DFP (Figure 7).

PB+DEET combined with CORT pretreatment did not obviously alter astrocyte activation (Figure 20) from that seen with CORT alone prior to DFP (Figure 9). Significant neurodegeneration was not seen in any brain region observed (Figure 20).

Task 7. Determine if MINO Protects Against LPS- or DFP-Induced Neuroinflammation in Mice Given PB+DEET and in Chronic CORT -Treated Mice Given PB+DEET (Meets Aim 3, will take ~ 3 months in Year 3) Mice were dosed to obtain tissue at days 15 - 17 (see Figure 1) for molecular assessment of 6 brain areas and at day 15 for cellular/histological assessment. **Outcome:** *Evaluation of tissue to determine if MINO will prevent neuroinflammation and/or neural injury caused by GWI- relevant agents.*

MINO is a tetracycline class antibiotic with anti-inflammatory properties. Previously we showed that the neuroinflammatory response to known neurotoxicants could be suppressed by prior treatment with MINO (Sriram et al., 2006), raising the possibility that MINO could affect the neuroinflammatory response to DFP with or without prior treatment with CORT or PB+DEET. In both hippocampus and frontal cortex MINO pretreatment for 2 weeks suppressed the neuroinflammatory response to DFP (with combined exposure to PB + DEET) and also suppressed the CORT-enhanced expression of neuroinflammation after DFP.

The combination of the pretreatment with anti-inflammatory MINO and PB+DEET had the potential to reduce the ability for either drug(s) to protect against DFP-induced neuroinflammation. While MINO pretreatment reduced the CORT-mediated exacerbation of DFP neuroinflammation, the combination with PB+DEET diminished this effect (Figure 21).

Task 8. Determine the functional consequences of exposure to GWI-Relevant Agents (Meets Aims 1-3, will take ~ 16 months across years 2 & 3) While the cellular and molecular responses to the Gulf War-relevant exposure regimen are definitively elucidated in the other studies of this project, their significance would be further strengthened if they could be linked to functional measures of CNS activity. A hippocampal slice model could be utilized with tissue obtained from animals exposed to GWI-relevant agents, but the project investigators believed that data more directly relevant to the biochemical findings would be obtained from intact animals. Consequently, hippocampal synaptic transmission was evaluated in anesthetized mice utilizing single pulse as well as more complex patterns of electrical stimulation. This

permitted assessment of both baseline transmission and the capacity for plasticity. All mice in this portion of the project were exposed to the Gulf War-relevant exposure regimens and tested at the UICOM-Peoria performance site.

Only a handful of laboratories have successfully established recording of dentate gyrus field potentials in anesthetized mice by stimulating the entorhinal cortex (Gruart et al, 2006; Jedlicka et al., 2012; Stoenica et al., 2006). The figures below clearly define our ability to quantify hippocampal synaptic transmission with this preparation.

Baseline synaptic transmission was assessed by measuring field potential responses to single pulse stimulation in each mouse – 10 stimulus intensities in the range of 50-1500 uA were applied and excitatory postsynaptic potentials (EPSP) and population spikes (PS) recorded. These data resulted in input/output (I/O) curves spanning the range of minimal to maximal responses (Figure 22 from control animals).

Paired-pulse functions were then determined at stimulus intensities eliciting 20, 50, and 100% of the maximal PS response by varying the interpulse interval (10-1000 msec), so as to assess the integrity of local excitatory and inhibitory connections (Figures 23-24).

Application of paired pulses at short interpulse intervals (10-30 msec) are seen to almost completely inhibit the second pulse, while longer intervals (100-200 msec) result in facilitation of the second pulse amplitude. High frequency train stimulation – 10 burst pairs, 4 biphasic pulses/burst at 400 pulses/sec, 1000 uA pulse intensity, 80 pulses total – when then applied to induce long-term potentiation (LTP) measured as the amplitude of the PS (40-50% increase) and monitored for 60 min post-train (Figure 25). These measures constitute a direct assessment of the capacity for synaptic plasticity, the processes widely thought to underlie cognitive function.

Urethane (1.5-2.0 g/kg, i.p.) was utilized in the mouse preparation due to its long duration of action that insured a constant level of anesthesia. Since biochemical studies had indicated that PB+DEET administration for one week had not altered the priming effect from one week's CORT in the drinking water in enhancing neuroinflammation, these GWI-relevant agents were not used in the neurophysiology studies. So the model consisted of CORT in the drinking water for 7 days followed one day later by challenge with LPS (2 mg/kg, s.c.) or DFP (3-4 mg/kg, i.p.), and field potential recording 12-48 hr later.

The data shown in Figures 22-25 were obtained from a Control group that received only the CORT vehicle in drinking water for 7 days and was challenged with saline. The remainder of the experimental design or for this Task could not be completed – most of the animals in groups receiving CORT died up to 4 hr after urethane and before recording could be initiated or

completed. This occurred even in the presence of saline challenges or when the LPS and DFP challenge doses were reduced or when longer post-challenge intervals up to 48 hr were utilized before instituting recordings. Groups receiving vehicle in the drinking water and challenged with either LPS (N=3) or DFP (N=4) were viable, but provided little information for this project in the absence of the CORT priming effect. We have also tested the alternative CORT protocol of two injected doses (20 mg/kg, s.c.) 7 hrs apart on the day before DFP challenge (3 mg/kg, i.p.) with recordings initiated 16 hrs post-challenge, and all mice survived. We have shown that this alternative protocol also elicits a CORT priming effect in expression of inflammatory mediators, but otherwise the relationship of the two CORT administration regimens remains undefined. Future studies would have to attempt recording in awake intact animals, which has been achieved in rats, or administer CORT in a manner that does not involve 7-day administration in drinking water.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that the sarin surrogate compound, DFP, causes brain wide neuroinflammation, findings consistent with a clinical presentation of sickness behavior symptoms seen in GWI.
- Demonstration that prior administration of high physiological levels of the stress hormone, CORT, augments the neuroinflammatory effect of the sarin surrogate, DFP, out to 30 days after cessation of CORT treatment.
- Demonstration that multiple exposures of high physiological stress levels of CORT over time can have a cumulative effect on the CNS neuroinflammatory response out to 180 days, a time course in the mouse equivalent to 20 years in man and consistent with the persistence of symptoms of GWI.
- Demonstration that none of the neuroinflammatory effects of DFP, with or without CORT, PB, or DEET, results in neurohistological evidence of brain damage.
- Demonstration that PB+DEET do not cause neuroinflammation but reduce the neuroinflammation due to DFP.
- Demonstration that the FDA-approved atypical anti-inflammatory compound, MINO, can suppress the neuroinflammation seen after DFP and/or prior treatment with CORT.

5. CONCLUSION:

Our findings are suggestive of a possible critical and yet unrecognized link between the stressful environs of the 1991 GW theater and agent exposure(s) unique to this war, exposures in which the CNS is primed to amplify future exposure to stressors, pathogens, injury or toxicity. Thus, our observed neuroinflammatory effects in an GWI. The suppression of the neuroinflammatory response in this model by the FDA-approved anti-inflammatory compound,

MINO, is suggestive of a therapeutic approach that can be evaluated in ill veterans with this or similar pharmacotherapy.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

- a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

1. Lay Press:

2. Peer-Reviewed Scientific Journals:

3. Invited Articles:

4. Abstracts:

1. Kelly KA, Miller DB, Lasley SM, O'Callaghan JP. Chronic exposure to glucocorticoids, PB and DEET primes the neuroinflammatory response to the nerve agent DFP in a potential model for Gulf War Illness. Program No. 676.01. Neuroscience 2011 Abstracts. Washinton, DC: Society for Neuroscience, 2011. Online.
2. Kelly KA, Miller DB, Lasley SM, O'Callaghan JP (2013) Glucocorticoid exposure primes the neuroinflammatory response to the nerve agent DFP in amodel of Gulf War Illness. Toxicologist 132(1):74 #350. San Antonio, TX: Society of Toxicology, 2013.
3. Kelly KA, Miller DB, Lasley SM, Fletcher MA, Barnes Z, O'Callaghan JP. The anti-inflammatory glucocorticoid, corticosterone, markedly augments systemic inflammatory responses to the nerve agent DFP in a mouse model of Gulf War Illness. Program No. 755.12. Neuroscience 2013 Abstracts. San Diego, CA: Society for Neuroscience, 2013. Online.

- b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

7. INVENTIONS, PATENTS AND LICENSES: none

8. REPORTABLE OUTCOMES: none

9. OTHER ACHIEVEMENTS: Grant applications resulting from this project are listed below.

- “Stress Hormone Enhancement of DFP-Induced Neuroinflammation: An Unrecognized Phenotype of Nerve Agent Exposure with Implication for Therapeutic Intervention”, NIH (CounterACT), J. P. O’Callaghan(PI), submitted September, 2012.
- “Stress Hormone Enhances Organophosphate-Induced Neuroinflammation: Glial Contributions”, NIH R01, S. M. Lasley/J. P. O’Callaghan/G. Snyder (Multi-PIs), submitted February, 2014.

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11. **APPENDICES:** none