Screening for novel central nervous system biomarkers in veterans with Gulf War Illness


Abstract

Gulf War Illness (GWI) is primarily diagnosed by symptom report; objective biomarkers are needed that distinguish those with GWI. Prior chemical exposures during deployment have been associated in epidemiologic studies with altered central nervous system functioning in veterans with GWI. Previous studies from our group have demonstrated the presence of autoantibodies to essential neuronal and glial proteins in patients with brain injury and autoantibodies have been identified as candidate objective markers that may distinguish GWI. Here, we screened the serum of 20 veterans with GWI and 10 non-veteran symptomatic (low back pain) controls for the presence of such autoantibodies using Western blot analysis against the following proteins: neurofilament triplet proteins (NFP), tubulin, microtubule associated tau proteins (Tau), microtubule associated protein-2 (MAP-2), myelin basic protein (MBP), myelin associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP), calcium-calmodulin kinase II (CaMKII) and glial S-100B protein. Serum reactivity was measured as arbitrary chemiluminescence units. As a group, veterans with GWI had statistically significantly higher levels of autoantibody reactivity in all proteins examined except S-100B. Fold increase of the cases relative to controls in descending order were: CaMKII 9.27, GFAP 6.60, Tau 4.83, Tubulin 4.41, MAG 3.60, MBP 2.50, NFP 2.45, MAP-2 2.30, S-100B 1.03. These results confirm the continuing presence of neuronal injury/gliosis in these veterans and are in agreement with the recent reports indicating that 25 years after the war, the health of veterans with GWI is not improving and may be getting worse. Such serum autoantibodies may prove useful as biomarkers of GWI, upon validation of the findings using larger cohorts.

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Keywords:
Gulf War Illness
Brain injury
Autoantibodies
Cytoskeletal proteins
Serum biomarkers

1. Introduction

Approximately one third of the 697,000 US military personnel who served in the Gulf War (GW) from August 1990 to June 1991, have reported persistent symptoms for many years after the war (RAC, 2008; IOM, 2012; RAC, 2016; White et al., 2016). This complex of symptoms, known as Gulf War Illness (GWI), include memory and attention problems, profound fatigue, chronic muscle and joint pain, severe headaches, persistent diarrhea, respiratory difficulties and skin rashes. GWI is primarily diagnosed by symptom report and no validated objective diagnostic biomarkers currently exist that fully segregate cases from controls. This study was designed to identify objective central nervous system (CNS) biomarkers of GWI using clues from prior clinical studies with GW veterans and from animal studies that modeled chemical exposures experienced by GW veterans.

Clinical studies have reported impaired cognitive functioning and reduced MRI volume and altered white matter microstructural integrity in organophosphate (OP) pesticide, sarin nerve agent and pyridostigmine bromide (PB) anti-nerve gas pill-exposed GW veteran cohorts (White et al., 2016; Sullivan et al., 2013; Chao et al., 2010;...
Heaton et al., 2007; Proctor et al., 2006; Sullivan et al., 2003). Animal studies demonstrated that exposure to higher doses of the prophylaxis pill pyridostigmine bromide (PB), the insect repellent, DEET, and the insecticide permethrin and/or chlorpyrifos led to significant brain damage in animal models of GWI (Abou-Donia et al., 1996a,b). Further studies using 60 days of subchronic dermal exposure to DEET and permethrin, alone or in combination, at dose levels approximately equivalent to the exposures that occurred during the Gulf War in a rat-model of GWI, caused the following: (1) a diffuse neuronal cell death in the motor cortex, the different subfields of the hippocampal formation, and the Purkinje cell layer of the cerebellum, accompanied by sensorimotor deficits; (2) significant reduction of MAP-2–positive immunoreactive structures indicating atypical expression of MAP-2 in dendrites of surviving neurons, within the cerebral cortex and the hippocampus that was characterized by a beaded, disrupted, or wavy appearance; (3) a significant upregulation of GFAP–positive expression in structures in the CA3 subfield of the hippocampus, the motor cortex and the dentate gyrus (Abdel-Rahman et al., 2001, 2002a,b, 2004a,b; Abou-Donia et al., 2000, 2001, 2002, 2004; Terry et al., 2003). Similar results were exhibited in animals treated with sarin alone or accompanied by cited-above chemicals, with and without stress (Abdel-Rahman et al., 2004a).

The cytoarchitecture of the CNS is maintained by a complex cellular milieu that involves neuronal and glial cells that must maintain proper communication in order to function properly (Abou-Donia and Lapadula, 1990; McMurray, 2000). CAMKII phosphorylates cytoskeletal proteins, such as MAP-2, tau and tubulin. CAMKII accounts for 12% of all proteins in the brain. CAMKII has the ability to coordinate and transduce upstream Ca and reactive oxygen species (ROS) signals into physiological and pathophysiological downstream responses in the nervous system and cardiovascular biology and disease (Abou-Donia, 1995; Erickson et al., 2011). Tubulin, the major component of microtubules, is responsible for axonal migration and longitudinal growth and is involved in axonal transport. Although tubulin is present in virtually all eukaryotic cells, the most abundant source is the vertebrate brain, where it consists of approximately 10–20% of its total soluble protein (McMurray, 2000). Microtubule–Associated Protein-2 (MAP-2) is found in dendritic compartments of neurons. A loss of MAP-2, is a reliable indication of irreversible neuropathology and is a sensitive marker of seizure-related brain damage (Ballough et al., 1995). Tau Protein, a normal axonal protein, is involved in stabilization and assembly of axonal microtubules. Levels of tau proteins are elevated in the cerebrospinal fluid (CSF) and serum following TBI (Liliang et al., 2011) and has been used for diagnosis of Alzheimer’s disease. Myelin basic protein (MBP) is an abundant myelin membrane proteolipid produced by oligodendroglia in the CNS and Schwann cells in PNS and may confirm the clinical assessment of neurodegenerative disorders such as multiple sclerosis and stroke (Jauch et al., 2006). Myelin Associated Glycoprotein (MAG) is selectively localized in periaxonal Schwann cell and oligodendroglial membranes of myelinated sheaths, suggesting that it functions in glia–axon interactions in both the PNS and CNS (Schachner and Bartsch, 2000). Giall fibrillary acidic protein (GFAP) is expressed almost exclusively in astrocytes, where it is induced by neural injury and released upon disintegration of the astrocyte cytoskeleton (Rempe and Nedergaard, 2010). GFAP plays an essential role in maintaining shape and motility of astrocytic processes and contribute to white matter architecture, myelination and blood brain barrier (BBB) integrity (O’Callaghan et al., 2015). After traumatic brain injury (TBI), GFAP’s serum concentration peaks at 2–6 h and has a half-life of ~2 days (Diaz-Arrastia et al., 2014). S-100B exerts both detrimental and neurotrophic effects, depending on its concentration in brain tissues (Adami et al., 2001). After release, S-100B acts as a trophic factor for serotoninergic neurons, and plays a role in axonal growth and synaptogenesis during development. Thus, traumatic acute injury results in great destruction of astrocytes leading to massive release (50 to 100 fold) of S-100B into plasma, whereas S-100B levels in psychiatric disorders were only about 3 times higher in patients compared to controls (Uda et al., 1998; Arolt et al., 2003), correlating well with its neuroprotective action. Specifically, S-100B stabilizes tau and MAP-2. Its half-life in the serum is 2 h (Zurek and Fedora, 2012).

A recent study of airline pilots and other flight crew members chronically exposed to organophosphates through combustion of engine oil and hydraulic fluid that contain organophosphate esters resulted in symptoms similar to those reported by GW veterans (fatigue, headaches, confusion and memory problems). Interestingly, these crew members showed significantly elevated numbers of autoantibodies in their blood serum of CNS damage markers including those associated with axonal transport (microtubule associated protein–2 (MAP-2), tubulin, neurofilament triplet proteins (NFL) and microtubule associated protein–tau (tau protein)) and those exclusively associated with CNS glial activation and neuroinflammation (myelin basic protein (MBP), and glial fibrillary acidic protein (GFAP) (Abou-Donia et al., 2013). A follow-up histopathology autopsy study was performed on a deceased pilot with organophosphate exposure that confirmed CNS damage and demyelination (Abou-Donia et al., 2014). Specifically, the histopathological results showed axonal degeneration and demyelination and the post-mortem and pathological examination of the nervous system confirmed the autoantibody biomarker results.

Recent studies with GW veterans have shown persistent signs and symptoms characteristic of CNS injury including brain imaging and cognitive studies (White et al., 2016; Chao et al., 2010, 2011, 2014, 2016; Heaton et al., 2007; Sullivan et al., 2003). There are, however, no validated objective diagnostic tests to identify acute or chronic sequelae of brain injury in this veteran group. Diagnosis of brain injury using cranial computed tomography (CT) scan and magnetic resonance imaging (MRI) techniques such as diffusion tensor imaging (DTI), have not been able to clinically diagnose veterans with GWI because there have been no proven cutoff values for volumetric or other imaging parameters that have been able to provide the required near 100% accuracy in terms of sensitivity/specificity at the individual level to distinguish cases from controls needed for a diagnostic test. Imaging studies have been able to show differences and altered CNS functioning between veterans with GWI and healthy controls but have not yet been able to identify the groups diagnostically because of the significant overlap between the groups (Chao et al., 2010, 2011, 2014, 2016; Heaton et al., 2007). Hence, it is important to develop clinically available, simple and inexpensive biomarkers for detection of neuronal and glial injury essential in the diagnosis and understanding of the temporal progression of CNS damage in GWI. Recently, serum biomarkers such as cytoskeletal proteins, resulting from axonal degeneration, have been used in diagnosing brain injury (particularly traumatic brain injury). The use of these biomarkers is usually measured in serum shortly after brain injury, because they have short half-lives (Zurek and Fedora, 2011; Diaz-Arrastia et al., 2014).

However, many years have elapsed since the time that GW veterans returned from deployment and became ill therefore, this particular approach cannot apply to GWI. Based on results from both chronic and acute injury, we used our novel autoantibody biomarker panel described above for brain injury to test for the indication of CNS damage in veterans with chronic GWI (Abou-Donia et al., 2013, 2014). One prior study compared autoantibodies of myelin basic protein (MBP) and striated muscle antibodies in GW veterans and reported higher MBP and muscle antibodies in veterans with GWI (Vojdani and Thrasher, 2004). Autoantibodies have previously been recognized as potential objective biomarkers of GWI (Golomb, 2012). Therefore, we hypothesized that chemical exposure to pesticides, anti-nerve gas pills and/or sarin nerve gas during deployment in veterans with GWI caused an excitotoxic cascade (through potential glutamatergic, oxidative stress and proinflammatory cytokine signaling) resulting in neurodegeneration and apoptotic loss of brain cells, leading to blood brain barrier leakage of specific neuronal and glial proteins into circulation, with subsequent formation of autoantibodies (AB) against these...
proteins (Abou-Donia et al., 2013; Banks and Lein, 2012; Golomb, 2008; Terry, 2012; Binukumar and Gill, 2010; Soltaninejad and Abbodlali, 2009). In this study, we determined circulating IgG-class autoantibodies in serum from 20 GWI cases and 10 symptomatic (low back pain) controls against the following 9 brain proteins: neurofilament triplet proteins (NFL), tubulin, microtubule associated protein-tau (tau proteins), microtubule associated protein-2 (MAP-2), calcium/calmodulin kinase II (CaMKII), myelin basic protein (MBP), myelin associated glycoprotein (MAG), glial fibrillary acidic protein (GFAP) and S-100B.

2. Materials and methods

2.1. Materials

The sources of proteins were: NFP (bovine spinal cord), tau protein (human), MAP-2 (bovine serum), tubulin (bovine brain), and MBP (human brain), from Sigma-Aldrich (Saint Louis, Missouri); CaMKII (Human) recombinant Protein and MAG recombinant Protein from Novus Biologicals, Littleton, CO, GFAP (human) from Biotrend (Human) recombinant Protein and MAG recombinant Protein from Novus Biologicals, Littleton, CO, GFAP (human) from American Qualex International, Inc. (San Clemente, California). Horseradish peroxidase-conjugated goat anti-human IgG, and enhanced chemiluminescence reagent were obtained from Amersham Pharmacia Biotech (Piscataway, New Jersey). SDS gels, 2–20% gradient (8 × 8), and tris-glycine 15 mM were obtained from Invitrogen (Carlsbad, California). All other materials were purchased from Amersham.

2.2. Ethics statement

Approval for the use of stored blood samples for this study was obtained from the Duke University Medical Center Institutional Review Board.

2.3. Case and control samples

Serum samples from 20 GWI cases with GWI and 10 non-veteran symptomatic controls with lower back pain were tested in this pilot study. GWI veteran serum samples were collected from a study of acupuncture treatment in veterans with GWI from 2010 to 2012 (Conboy et al., 2012). Control serum samples were derived from a separate study of non-veteran patients with chronic lower back pain who served as ‘symptomatic low back pain’ controls from 2011 to 2013 (Jacobson et al., 2015). Veterans with GWI will be referred to as ‘cases’ and low-back pain symptomatic controls will be referred to as ‘controls’.

2.4. Description of the patient cohorts

2.4.1. GWI-case cohort

“The Effectiveness of Acupuncture in the Treatment of Gulf War Illness” PI: Conboy, (8/21/2010–12/26/2012) N = 104; Study Site: New England School of Acupuncture (NESA). Cases were recruited through the Defense Manpower Data Base (DMDC) personnel listings and advertisements. Cases were screened for GWI symptoms and were required to meet the CDC diagnostic criteria for chronic multi-symptom illness (CMI) in order for inclusion in the parent study and in the current study (Conboy et al., 2012; Fukuda et al., 1998). Inclusion in the current study also required that veterans were deployed to the 1990–1991 Gulf War. CMI is characterized by one or more symptoms of at least 6 months duration from at least two of three symptom categories: 1) fatigue; 2) mood-cognition; 3) musculoskeletal pain.

Symptoms were not necessarily required to have started during or after the Gulf War deployment. Exclusionary criteria included that the veteran was 1) currently enrolled in another clinical trial 2) Had another disease that likely could account for the symptoms, as determined by the Medical Monitor 3) Severe psychiatric illness (in the last 2 years psychiatric hospitalization, suicidal attempt, alcohol or substance abuse, use of antipsychotic medication) 4) Unable to complete the protocol based on the evaluation of the Medical Monitor.

2.4.2. cLBP-cohort

“Structural Integration for chronic low back pain” PI: Jacobson (3/4/2011–6/21/2013) N = 46. Study Site: Spaulding Rehabilitation Hospital (SRH). In this cohort, 46 outpatients from the Boston area with chronic nonspecific low back pain were randomized to parallel 20-week long treatment groups of structural integration (SI) plus outpatient rehabilitation (OR) versus OR alone. The details of the study are described in a recent publication (Jacobson et al., 2015). Inclusion criteria for the parent study included: (i) Men and women aged 18–65, (ii) cLBP of ≥6 months duration, not attributed to infection, neoplasm, severe radiculopathy (as indicated by frequent severe pain radiating down a leg), fracture, or inflammatory rheumatic process, (iii) bothersomeness of back pain self-rated on average over the preceding 6 months ≥3 on an 11-point ordinal scale (0 = none, 10 = worst imaginable), (iv) prior arrangement to enter a course of outpatient physical therapy for low back pain at a Boston area rehabilitation clinic, (v) English language fluency and mental capacity sufficient to provide informed consent and participate in the study. Exclusion Criteria for the study included: (i) Impaired hearing, speech, vision, and mobility sufficient to interfere with participation in the study, (ii) current or anticipated receipt of payments from Worker’s Compensation or other insurance for disability attributed to low back pain, (iii) prior treatment with any SI therapy, (iv) plans to initiate additional treatment for back pain during the period of the study other than outpatient rehabilitation care, particularly massage or other manual therapies (e.g., chiropractic or osteopathic manipulation), (v) exclusions for safety: unresolved musculoskeletal pathology of the lower limbs, current pregnancy, any implanted medical device, osteoporosis, any hypercoagulation condition, eczema, skin infection, deep vein thrombosis, burns or other acute trauma including unhealed bone fractures or open wounds, psoriasis, psychiatric illness not well controlled, or current episode of exacerbated major depressive disorder.

2.5. Collection and storage of samples

Samples from the GWI-cohort and the cLBP-cohort were all collected from the Boston area at the same time period at two different sites from 2010 to 2013. All sites followed exactly the same protocol for venipuncture, blood handling, serum separation, aliquoting and storage at −80 °C. The same phlebotomy and sample protocol was distributed in writing to all sites. All samples analyzed were baseline blood samples collected pre-intervention therapy. Samples used for this study have not been previously thawed and are free of hemolysis by visual inspection (Tuck et al., 2009).

2.6. Participant demographics

The participant demographics indicate that a total of 20 veterans with GWI, 18 males and 2 females, compared to 6 females out of 10 cLBP controls participated in the study. The age of the GWI cases ranged from 38 to 61 (mean ± SD 46.0 ± 6.8) compared to 25 to 64 (mean ± SD 50 ± 11.4) years for controls; all study participants were white (Table 1). Seventy percent of veterans with GWI reported taking PB

Table 1

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Cases</th>
<th>Controls</th>
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<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>46 (6.4)</td>
<td>50 (11.4)</td>
</tr>
<tr>
<td>Gender (% female)</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>Race (% Caucasian)</td>
<td>100</td>
<td>100</td>
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</table>

Age range of Cases = 38–61 years and Controls = 25–64 years in 2010–2013 when the blood was collected.

* A total of 20 cases and 10 controls participated in the study.

* Cases were significantly different from controls for gender F < 0.05 but not for age.
pills during the war (n = 14). The groups differed with respect to gender (X² = 8.5; p < 0.05) with significantly more women in the control group but did not differ with respect to age (t-value = −1.3; p > 0.05).

2.7. Western blot assay

To screen for the presence of autoantibodies against a battery of proteins, we applied a Western blot approach as previously reported (Abou-Donia et al., 2013). Each serum sample was analyzed in triplicate. Each protein was loaded as 10 ng/lane except for IgG that was loaded as 100 ng/lane. Proteins were denatured and electrophoresed in SDS-PAGE (4% to 20% gradient) purchased from Invitrogen (Carlsbad, CA). One gel was used for each serum sample. The proteins were transferred into polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech Piscataway, New Jersey). Nonspecific binding sites were blocked with Tris-buffered Saline-Tween (TBST) (40 mM Tris [pH 7.6], 300 mM NaCl, and 0.1% Tween 20) containing 5% non-fat dry milk for 1 h at 22 °C. Membranes were incubated with serum samples at 1:100 dilutions in TBST overnight at 4 °C. After five washes in TBST, the membranes were incubated in a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Amersham Pharmacia Biotech (Piscataway, New Jersey). The dot blots were probed with anti-human IgG (H + L) HRP conjugate antibody (Cat. No. 31410, Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) for 1 h at RT, incubated with ECL reagent (Cat. No. 34096). The membranes were developed by enhanced chemiluminescence using the manufacturer’s (Amersham Pharmacia Biotech) protocol and a Typhoon 8600 variable mode imager. The signal intensity was quantified using Bio-Rad image analysis software (Hercules, California). All tests were performed with the investigators blinded to participant diagnosis.

2.8. Specificity of serum autoantibodies

Previously we checked the specificity of the serum autoantibody by performing peptide/antigen competition assay, in which the serum was spiked with the target protein or peptide (Abou-Donia et al., 2013). The serum from random healthy controls was mixed with or without tau, MAP or MBP. The serum/protein mix was centrifuged at 15,000 rpm to pellet any immune complexes. The supernatants were then carefully removed and used in Western blotting.

2.9. Calculations

The mean value of the optical density measurement from the triplicate testing was used for each serum sample tested and normalized by total IgG. Thus, the results are expressed as mean values of triplicate assays of optical density arbitrary units normalized to total serum IgG.

2.10. Power analysis

A total of 20 GWI cases were available for testing in this convenience sample. Effect size calculations were based on two-sample t-test assuming a common standard deviation between groups. The power analysis assumes that cases and controls are not matched. In a t-test of difference between two independent means, selecting power of 80%, 2-sided alpha 0.05, and size of 20 vs 10, the study was powered to detect an effect only if at least 1.12 SD.

2.11. Statistics

Grouped data are reported as mean ± SD. The values from cases were compared to the control group using t-tests and Pearson correlation analyses (SigmaStat, Systat Software) and p-values were
IgG-class autoantibodies against nine neuronal controls) underwent measurement of the levels of the serum circulating trans with GWI and 10 non-veteran symptomatic low-back-pain controls. Cases were significantly increased levels of autoantibodies against all cytoskeletal proteins except those against S-100B compared to non-veteran symptomatic (low back pain) controls (Table 3). Due to the gender differences between the cases and controls, analyses were also run with just the males in the groups. Although there was only a small number of males (n = 4) in the control group which could be problematic in this type of analysis, results of this comparison showed a very similar pattern of significant differences in all autoantibodies (GFAP p < 0.001; Tau p < 0.001; MAP p < 0.002; MAG p < 0.001; PNF p < 0.006; Tubulin p < 0.003; MBP p < 0.01; S-100B p = 0.31). The majority of GWI serum reacted intensely to neural proteins, while most control serum showed a weak or no reaction. Fig. 4 and b present Western blots results from three representative GWI cases and three controls. The levels of serum autoantibodies in GWI cases and controls to neural-specific proteins expressed as mean values ± SD of triplicate assays of optical density arbitrary units normalized to total serum IgG optical density ranged from 0.30 for S-100B and 4.09 for GFAP for the cases compared to 0.30 and 0.62, respectively for controls are listed in Table 3 and shown in Fig. 2. The percentage of autoantibodies against neural proteins of cases compared to controls (in descending order) were: CaMKII, 927, GFAP 660, Tau 483, Tubulin 441, MAG 360, MBP 250, NFP 245, MAP-2 230, S-100B 103. Fig. 3 presents the mean values ± SD (p < 0.001) of fold increase of autoantibodies against neural proteins for the cases compared with the controls. Serum from controls had no or low levels of circulating autoantibodies to nervous system-specific biomarkers. Autoantibodies against CaMKII were more predominant in the cases’ serum than in controls’ serum (Fig. 3).

Fig. 4 shows that Tubulin and GFAP had the highest values in the GWI cases compared with the controls. Pairwise correlations among the nine autoimmune biomarkers were significant only for the pair Tau and MBP. When comparing the correlation between each pair, only tau and MBP were significantly linearly correlated to each other (Fig. 5). Fig. 5 shows that the control values of those two biomarkers were <1 optical density unit, whereas GWI cases had values strongly linearly correlated with each other such that on average tau was elevated up to 10 times higher than controls in some GWI cases, and MBP was also elevated up to 5 times higher for the same cases vs the controls. Finally, when each biomarker was compared separately between individual cases and controls for potential fold-increase cut-points to discriminate the groups, results indicated that tubulin values had some of the highest-fold increased values in the individual GWI cases compared with the individual control values although only 60% of the individual cases (n = 12) showed that effect (Fig. 6a). However, in 9 (out of the 20) cases tubulin values were elevated by a factor of 3 to 9-fold higher than the controls. In Fig. 6b, GFAP was elevated the most in cases compared to controls. In fact, GFAP was higher in all of the cases compared with all of the controls with 20 out of 20 cases having 2 to 7 fold higher values.

### Table 2

<table>
<thead>
<tr>
<th>Chemical exposures</th>
<th>Exposed %</th>
<th>Environmental and other exposures</th>
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<tbody>
<tr>
<td>Pyridostigmine bromide (PB)</td>
<td>14 70</td>
<td>Khamisiyah notification letter</td>
</tr>
<tr>
<td>(OP)</td>
<td>35</td>
<td>Contaminated food/water</td>
</tr>
<tr>
<td>Carbamates</td>
<td>7 35</td>
<td>Vaccines 18 90</td>
</tr>
<tr>
<td>Pyrethroids</td>
<td>4 20</td>
<td>Malaria 12 60</td>
</tr>
<tr>
<td>DEET</td>
<td>11 55</td>
<td>Sand 18 90</td>
</tr>
<tr>
<td>Sarin</td>
<td>9 45</td>
<td>Tent heater 11 55</td>
</tr>
<tr>
<td>Depleted uranium (DU)</td>
<td>6 30</td>
<td>Jet fuel 14 70</td>
</tr>
<tr>
<td>Solvents</td>
<td>10 50</td>
<td>Oil fires 18 90</td>
</tr>
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</table>

### Table 3

<table>
<thead>
<tr>
<th>Chemicals, environmental and other exposures of cases during the Gulf War.a</th>
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<tbody>
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<td><strong>Chemical exposures</strong></td>
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<tr>
<td>Pyridostigmine bromide (PB)</td>
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### Results

As previously described, we assessed the specificity of the serum autoantibody by performing peptide/antigen competition assay, in which the serum was spiked with the target protein or peptide. The serum bound to tau eliminated the tau band in the Western blot (see Fig. 1) while the band of MAP-2 or MBP was present and not affected. The serum bound to MAP-2 eliminated the MAP-2 band in the Western blot while the bands of tau and MBP were present. These results indicate that each autoantibody in the serum was specifically neutralized by its target protein in serum sample and was no longer available to bind to the epitope present in the protein on the Western blot. This confirmed that the assay used in this study, was specific and accurately determined autoantibodies against tested proteins in serum samples.

To detect autoantibodies in serum, we probed Western blots with individual serum samples. A total of 30 human serum samples (20 veterans with GWI and 10 non-veteran symptomatic low-back-pain controls) underwent measurement of the levels of the serum circulating IgG-class autoantibodies against nine neuronal- and glial-specific proteins. Table 2 lists the number of GWI cases who were exposed to chemical and environmental exposures. It shows that 14 cases (70%) used PB as a prophylaxis against possible exposure to nerve agents and nine cases reported being exposed to the nerve agent sarin. In addition, a total of eight cases reported receiving notification from the Department of Defense (DOD) that they were potentially exposed to sarin and other chemicals due to their proximity to the Khamisiyah, Iraq underground weapons depot where a chemical weapons cache was destroyed in March 1991 (US DOD, 2002). Eight cases reported exposure to depleted uranium. All of the cases reported exposure to one or more insecticides or a mixture of pesticides including organophosphates, carbamates, pyrethroids and organochlorines. Eleven cases used the insect repellant DEET. All cases underwent environmental and other exposures listed in Table 2. Other chemicals that the cases reported exposure to included oil well fires, sand, tent heaters, jet fuel, and solvents. Some veterans reported exposure to malaria and 18 reported being vaccinated. Serum from GWI cases showed significantly increased levels of autoantibodies against all cytoskeletal proteins except those against S-100B compared to non-veteran symptomatic (low back pain) controls (Table 3). Due to the gender differences between the cases and controls, analyses were also run with just the males in the groups. Although there was only a small number of males (n = 4) in the control group which could be problematic in this type of analysis, results of this comparison showed a very similar pattern of significant differences in autoantibodies (GFAP p < 0.001; Tau p < 0.001; MAP p < 0.002; MAG p < 0.001; PNF p < 0.006; Tubulin p < 0.003; MBP p < 0.01; S-100B p = 0.31). The majority of GWI serum reacted intensely to neural proteins, while most control serum showed a weak or no reaction. Fig. 1a and b present Western blots results from three representative GWI cases and three controls. The levels of serum autoantibodies in GWI cases and controls to neural-specific proteins expressed as mean values ± SD of triplicate assays of optical density arbitrary units normalized to total serum IgG optical density ranged from 0.30 for S-100B and 4.09 for GFAP for the cases compared to 0.30 and 0.62, respectively for controls are listed in Table 3 and shown in Fig. 2. The percentage of autoantibodies against neural proteins of cases compared to controls (in descending order) were: CaMKII, 927, GFAP 660, Tau 483, Tubulin 441, MAG 360, MBP 250, NFP 245, MAP-2 230, S-100B 103. Fig. 3 presents the mean values ± SD (p < 0.001) of fold increase of autoantibodies against neural proteins for the cases compared with the controls. Serum from controls had no or low levels of circulating autoantibodies to nervous system-specific biomarkers. Autoantibodies against CaMKII were more predominant in the cases’ serum than in controls’ serum (Fig. 3).

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value than the control mean. Thus GFAP values completely distin-
guished the cases from the controls. GFAP values did not overlap in
cases vs controls in this small sample; however, the separation in
the ranges was small relative to the substantial standard deviations. In
Fig. 6c, tau was higher than controls in 18 cases and 50% of the cases
had double the value of tau compared with the controls. In Fig. 6d,
MAP was higher than the controls in 15 cases and 75% of the cases
had a 0.5 to 11-fold higher value than the controls. In Fig. 6e MAG
was higher than controls in 15 cases and 75% of the cases had up to a 10-
fold higher value than the controls. In Fig. 6f NFP was higher than con-
trols in only 50% of the cases (n = 10) and they showed 0.5 to 11-fold
higher values than controls. CAMKII was higher than controls in 16 cases
and 50% of the cases had a 3 to 30-fold higher value than the controls.
S100B values were not statistically signi-

4. Discussion

This pilot study reports signi-
ficantly elevated levels of autoanti-
bodies against neurotypic- and gliotypic-specific proteins in serum
from a sample of 20 veterans with GWI and 10 non-veteran symptom-
atic (low back pain) controls with musculoskeletal symptoms rather
than CNS symptoms. The increased levels in GWI cases compared to
controls ranged from 9.27 fold for CaMKII to 6.6 fold for GFAP to 2.45
fold for neurofilaments. Autoantibody levels against S-100B were not
different in GWI cases than controls (1.03 fold) consistent with its neu-
ral protective role and in agreement with presence of chronic injury and
absence of acute brain injury in veterans with GWI (Zurek and Fedora,
2011; Diaz-Arrastia et al., 2014; Stalnacke et al., 2006, 2004; Coch and
Leube, 2016). Previous studies, using animal models of GWI, showed
that exposure to the neurotoxicants that were present in the GW envi-
ronment, caused deficits in behavioral outcomes that were accompa-
nied by neuronal and glial degeneration (Abdel-Rahman et al., 2001,
2002a,b, 2004a,b; Abou-Donia et al., 2000, 2001, 2004). Following neu-
rodegeneration, there is accumulation of cellular neurological waste
products or debris such as misfolded or hyper-phosphorylated proteins
that form toxic stable aggregates (Nedergaard, 2013; Edgar et al., 2004).
This extracellular debris send damage signals that cause the CNS im-
mune cells - microglia to become activated and act as profound antigen
presenting cells that secrete pro-inflammatory cytokines (IL-1β, TNF-α
and IL-6) and mediators (reactive oxygen species, ROS) resulting in
the recruitment of T-lymphocytes (Milligan and Watkins, 2009;
Banks and Lein, 2012). Multiple exposures to these waste proteins can cause
microglia and astrocytes to become primed to react more strongly
after each subsequent exposure (Watts and Maier, 2003). This can re-
sult in a persistent neuroimmune response and chronic neuroinflam-
mation contributing to chronic health symptoms, such as those seen
in GW veterans (Johnson et al., 2016; Milligan and Watkins, 2009;
Maier and Watkins, 1998; Watkins and Maier, 2003). These waste
proteins are eventually released into circulation due to defects in the

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**Fig. 2.** Mean autoantibodies against neural proteins from cases and controls expressed in
mean optical density units.

**Fig. 3.** Folds increase of autoantibodies against neural proteins from cases relative to
controls.

**Fig. 4.** The levels of autoantibodies of neural proteins of GW cases and of controls
expressed as optical density units.

**Fig. 5.** Paired correlations of Tau and MBP optical density levels in cases relative to
controls.
brain-blood barrier induced by astrocyte alterations. Waste proteins in the brain ultimately reach the liver through a mechanism known as the "glymphatic system" where they are degraded (Nedergaard, 2013). However, the released proteins that could serve as markers of injury are present in the short-term and cannot be used as biomarkers in the case of chronic GWI (Zurek and Fedora, 2011; Diaz-Arrastia et al., 2014). Thus detection of autoantibodies can serve as surrogate markers for these circulating waste proteins as described in this study.

The highest increase in autoantibodies was against CaMKII which was 9.27 times higher than that of controls followed by GFAP which was 6 times higher than controls. This result is consistent with the veterans' exposure during their deployment to the Gulf War to

Fig. 6. a) Tubulin levels were higher than all controls in 12/20 cases. b) GFAP levels were higher than all controls in 20/20 cases. c) Tau levels were higher than all controls in 17/20 cases. d) MAP levels were higher than all controls in 15/20 cases. e) MBP levels were higher than all controls in 12/20 cases. f) NFP levels were higher than all controls in 10/20 cases. g) MAG levels were higher than all controls in 15/20 cases. h) CAMKII levels were higher than all controls in 16/20 cases. i) S100B levels overlap with cases and controls.
organophosphorus compounds such as pesticides, and the nerve agent sarin that have been shown to increase the activity and mRNA expression of CaMKII (Patton et al., 1983, 1985, 1986; Gupta et al., 1998; Barbier et al., 2009) as well as enhanced CaMKII-induced phosphorylation of NFP, tubulin (Serrano et al., 1986) and tau activity leading to the aggregation, deregulation and accumulation of NFP (Abou-Donia et al., 1993; Norgren et al., 2003) and tubulin in the axon (Abou-Donia, 1993; Jensen et al., 1992, Gupta et al., 2000; Grigoryan and Lockridge, 2009). Aggregated neurofilaments result in slowing of axonal transport as has been illustrated in GW-relevant animal and cell toxicant models (Gupta et al., 1997; Reagan et al., 1994; Terry et al., 2012; Gao et al., 2016; Edgar et al., 2004). GW-relevant exposure models have also been associated with astrocyte activation (Zakirova et al., 2015; Ojo et al., 2014).

Neuronal proteins studied in this pilot analysis represented various anatomical regions of the neuron with distinct functions which can be instructive with regard to the pathobiology of GWI (Lapadula and Abou-Donia, 1992). All of the proteins used are involved in axonal structure and function and are released as products of neural degeneration of various regions of the neuron. MAP-2 is present in the dendrites; CaMKII, tau, tubulin, and neurofilament proteins are located in the axon; myelin basic protein (MBP) and myelin associated glycoprotein (MAG) are an integral part of myelin (McMurray, 2000). Furthermore, the central nervous system-specific glial protein, GFAP and S-100B are secreted by astrocytes after neuronal injury (McMurray, 2000). Following axonal and myelin degeneration, neuronal and glial proteins are released and once in circulation, activated lymphocytes, B and T cells lead to the formation of autoantibodies against these proteins (Schwartz and Shechter, 2010a,b).

Increased autoantibodies against nervous system-specific proteins leads to structural consequences in various regions as follows: increased autoantibodies against neurofilaments proteins, tau, CaMKII and tubulin are indicative of axonal degeneration; increased autoantibodies against MAG and/or MBP suggest demyelination, increased autoantibodies against MAP-2 suggest dendritic degeneration, increased autoantibodies against GFAP suggest astrogliosis, and the low or no-increased levels of autoantibodies against S-100B is consistent with chemical-induced brain injury (Zurek and Fedora, 2011, Diaz-Arrastia et al., 2014; Stalnacke et al., 2006, 2004; Zurek and Fedora, 2011; Diaz-Arrastia et al., 2014; Coch and Leube, 2016). Their lack of increase in this study suggests against acute traumatic brain injury in veterans with GWI.

Important mechanistic clues from animal and cell studies of these GW-relevant toxicants have shown deficits in axonal transport, as well as aberrations in neurofilaments and microtubules, which are the structural railways for axonal transport (Gupta and Abou-Donia, 1995a, b; Gearhart et al., 2007; Grigoryan and Lockridge, 2009; Prendergast et al., 2007, Jiang et al., 2010). Mitochondria are also delivered by axonal transport to provide the energy required to power the biochemical reactions necessary for the functioning of the axon and have shown altered functioning in GW-relevant toxicant models (Middlemore-Risher et al., 2011). GW-relevant chronic low-level organophosphate exposure has also been associated with mitochondrial compromise from oxidative stress induction and with neuroinflammation resulting in cell damage or cell death resulting in debris of waste proteins in the extracellular spaces (Laetz et al., 2009; Kaur et al., 2007; Banks and Lein, 2012). In fact, one hypothesis of GWI suggests that mitochondrial damage and oxidative stress in the brain and the periphery have caused the chronic symptoms of GWI; notably, increased autoantibodies were expressly cited among objective markers and mediators in this model (Golomb et al., 2014; Golomb, 2012; Koslik et al., 2014).

Another hypothesis of GWI suggests that the neurotoxicants acted synergistically to create a self-perpetuating neuroinflammatory state, which in turn has an ongoing negative impact on brain cells including neurons (microtubules, motor proteins, mitochondria) and glia (microglia, astrocytes, oligodendrocytes) and blood-brain barrier function (O’Callaghan and Sriram, 2005). Clinical studies have also found consistent results with GW veteran cohorts who showed impaired cognitive functioning and reduced volume and altered white matter microstructural integrity on MRI in OP pesticide, sarin nerve agent and PB pill exposed cohorts (White et al., 2016; Sullivan et al., 2013; Chao et al., 2010; Heaton et al., 2007; Proctor et al., 2006; Sullivan et al., 2003). These prior results suggest clear CNS alterations in neurotoxin-exposed GW veterans which correlated with behavioral outcomes that are related to neurodegeneration and perhaps with both a chronic neuroinflammatory and Mitochondrial/OS hypothesis.

The only other study that we are aware of that compared CNS autoantibodies in GW veterans compared MBP and striated and smooth muscle antibodies and reported higher MBP and muscle antibodies in veterans with GWI when compared with controls (Vojdani and Thrasher, 2004). The current study validates the prior MBP findings and expands on those findings with a larger panel of 8 additional CNS autoantibody markers. Collectively, these findings suggest that alterations in white matter as evidenced by circulating autoantibodies to MBP appear to be associated with GWI. This finding corresponds with both leading hypotheses for GWI given that white matter alterations can be associated with oxidative stress and neuroinflammation as a result of glial activation and signaling of both proinflammatory cytokines and oxidative stress (Milligan and Watkins, 2009). The additional finding of this study that higher Tau autoantibody levels were significantly linearly correlated with higher MBP autoantibody levels in GWI cases suggests that axonal degeneration may be occurring before demyelination in veterans with GWI and warrants further more conclusive study to distinguish it from the more myelin-specific toxic leukoencephalopathies (Schmahmann et al., 2008; Filley, 2013). These findings also correspond with MRI findings of differences on both white and gray matter brain volumes in neurotoxicant-exposed GW veterans (Heaton et al., 2007; Chao et al., 2010, 2011, 2014, 2016). These findings also clearly suggest that glia and astrocytes in particular should be further studied in GWI given significantly higher levels of GFAP in the GWI cases that correspond with prior animal models of GWI (Abdel-Rahman et al., 2001, 2002a, 2002b, 2004a, 2004b; Abou-Donia et al., 2000, 2001, 2002, 2004; Zakirova et al., 2015; Ojo et al., 2014) and with recent studies illustrating the ability of astrocytes to donate mitochondria to damaged neurons (Hayakawa et al., 2016).
4.1. Limitations and future directions

This study, like all studies has important limitations. Although the present pilot study can serve as a proof-of-concept it has a small sample size and non-matched subject groups for age, gender and for CNS symptoms. This is particularly important as it has also been shown that CNS autoantibodies have been reported to be age-related in animal models (Lal and Forster, 1988). In addition, the convenience comparison group utilized in this study had musculoskeletal symptoms and not CNS symptoms therefore, it remains to be shown that these CNS autoantibody markers can clearly distinguish between GWI cases and additional groups with CNS specific symptoms. However, the strong results including 9-fold higher levels of CAMKII, 6-fold higher levels of GFAP and 4-fold higher levels of tau and tubulin that were presented in this study warrant further research for a blood-based objective marker of GWI in larger, well-characterized veteran cohorts. These results suggest a possible new avenue for further development of an objective biomarker of GWI. The identification of this small panel of neural-specific autoantibody biomarkers in GWI shows promise for further validation in larger study samples that are more carefully matched for subject demographics (particularly age), different types of control groups (i.e. healthy and CNS symptomatic groups) and that classify cases by both the CDC and the more specific Kansas GWI criteria which also specifies the time period of deployment which may be relevant to particular OP and other deployment-related exposures (Steele, 2000; Fukuda et al., 1998). Future directions will be to compare these CNS autoantibody markers with specific behavioral outcomes including cognitive performance and brain imaging of gray and white matter volume and microstructural integrity to further validate these suspected brain-immune-behavioral outcomes.

5. Conclusions

In conclusion, in this pilot study GWI was significantly associated with 2–9 fold increased serum autoantibodies against 8 neuronal- and glial-specific proteins (CaMKII, GFAP, Tau, Tubulin, MAG, MBP, NFP, MAP-2) and not with a marker of more acute damage (SI-100B). The autoantibodies that were found here to be elevated in GWI, targeted proteins/antigens that play critical roles in the structure and function of the neuron including axonal transport and myelination. Many of them are explicit markers for neurodegenerative disorders, consistent with axonal and myelin degeneration of myelinated neurons and with astroglisis, cell signaling and neuroinflammation. These same proteins have been shown to be affected in other clinical groups and animal models with similar organophosphate and carbamate exposures (Abou-Donia et al., 2013, 2014). These results validate prior reports of increased MBP autoantibodies in GWI cases and suggest that oligodendrocyte signaling, glia and white matter alterations should continue to be further studied in GWI and validated with health symptom and behavioral outcomes (Vojdani and Thrasher, 2004). The results also indicate that veterans with GWI may be continuing to show brain neuronal degeneration and glial activation that would be consistent with recent reports of chronically persistent and in some cases worsening health of these veterans (Smith et al., 2013; Ozakinci et al., 2006; Li et al., 2011; Kang et al., 2009; Dursa et al., 2016; White et al., 2016). These results suggest a possible avenue for further development of a panel of objective biomarkers of GWI upon further validation in larger study samples that are more carefully matched for subject demographics.

Conflict of interest statement

The authors report no relationships that could be construed as a conflict of interest.

Acknowledgements

This study was supported in part by DOD Contract No. W81XWH-15-1-0641 and W81XWH-15-1-0640, W81XWH-09-0064 and the National Center for Complementary and Integrative Health, the National Institutes of Health (K01AT004916).

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