



## A review of pre-clinical models for Gulf War Illness

Ana C.R. Ribeiro<sup>a</sup>, Laxmikant S. Deshpande<sup>a,b,\*</sup>

<sup>a</sup> Departments of Neurology, School of Medicine, Virginia Commonwealth University, Richmond, VA, USA

<sup>b</sup> Departments of Pharmacology and Toxicology, School of Medicine, Virginia Commonwealth University, Richmond, VA, USA



### ARTICLE INFO

Available online 22 June 2021

Editor: S. J. Enna

#### Keywords:

Chemical exposures  
Pesticides  
Insecticides  
Nerve agents  
Depression  
Cognitive dysfunction  
Pain  
Fatigue  
Neuroinflammation  
Oxidative stress  
Neuronal injury  
Calcium homeostasis  
Rats  
Mice

### ABSTRACT

Gulf War Illness (GWI) is a chronic multisymptomatic disorder that afflicts over 1/3rd of the 1991 GW veterans. It spans multiple bodily systems and presents itself as a syndrome exhibiting diverse symptoms including fatigue, depression, mood, and memory and concentration deficits, musculoskeletal pain and gastrointestinal distress in GW veterans. The etiology of GWI is complex and many factors, including chemical, physiological, and environmental stressors present in the GW arena, have been implicated for its development. It has been over 30 years since the end of the GW but, GWI has been persistent in suffering veterans who are also dealing with paucity of effective treatments. The multifactorial aspect of GWI along with genetic heterogeneity and lack of available data surrounding war-time exposures have proved to be challenging in developing pre-clinical models of GWI. Despite this, over a dozen GWI animal models exist in the literature. In this article, following a brief discussion of GW history, GWI definitions, and probable causes for its pathogenesis, we will expand upon various experimental models used in GWI laboratory research. These animal models will be discussed in the context of their attempts at mimicking GW-related exposures with regards to the variations in chemical combinations, doses, and frequency of exposures. We will discuss their advantages and limitations in modeling GWI followed by a discussion of behavioral and molecular findings in these models. The mechanistic data obtained from these preclinical studies have offered multiple molecular pathways including chronic inflammation, mitochondrial dysfunction, oxidative stress, lipid disturbances, calcium homeostatic alterations, changes in gut microbiota, and epigenetic modifications, amongst others for explaining GWI development and its persistence. Finally, these findings have also informed us on novel druggable targets in GWI. While, it has been difficult to conceive a single pre-clinical model that could express all the GWI signs and exhibit biological complexity reflective of the clinical presentation in GWI, animal models have been critical for identifying molecular underpinnings of GWI and evaluating treatment strategies for GWI.

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**Abbreviations:** ACh, Acetylcholine; AChE, Acetylcholinesterase; AChR, Acetylcholine receptor; BBB, Blood Brain Barrier; BChE, Butyrylcholinesterase; BDNF, Brain Derived Neurotrophic Factor; Ca<sup>2+</sup>, Calcium ions; CDC, Center for Disease Control; CMI, Chronic Multisymptom Illness; CORT, Corticosterone; CPF, Chlorpyrifos; DEET, N, N-Diethyl-meta-toluamide; DFP, diisopropyl fluorophosphate; DMSO, dimethyl sulfoxide; DOD, Department of Defense; DU, Depleted Uranium; FDA, Food and Drug Administration; GW, Gulf War; GWI, Gulf War Illness; GWS, Gulf War Syndrome; GWV, Gulf War Veterans; i.p., intraperitoneal; IOM, Institute of Medicine; LD<sub>50</sub>, Lethal Dose 50%; mg/kg, milligram per kilogram; NAS, National Academy of Sciences; PB, Pyridostigmine bromide; PER, Permethrin; PON, paraoxonase; RAC, Research Advisory Committee; s.c., sub-cutaneous; S-D, Sprague-Dawley; US, United States; VA, Veterans Affairs.

\* Corresponding author at: Virginia Commonwealth University, School of Medicine, PO Box 980599, Richmond, VA 23298, USA.

E-mail address: [laxmikant.deshpande@vcuhealth.org](mailto:laxmikant.deshpande@vcuhealth.org) (L.S. Deshpande).

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## 1. Introduction

In the early days of August 1990, Iraqi armed forces attacked the neighboring country of Kuwait. With the support of United Nations resolution 660, the United States (US) along with a coalition of nations led a military response to this Iraqi incursion. Code named Operation Desert Shield, US and coalition soldiers from 34 other nations began a massive troop buildup in the Persian Gulf region. By January 1991, an intense six-weeks of air attacks on Iraqi forces had begun. Code named Operation Desert Storm, this air assault was followed by land attack that began on February 23, 1991 and brought a swift end to the conflict. Following a ceasefire on February 28, 1991, most of the troops finally returned home by June 1991. Over the course of the entire operation, around 700,000 US soldiers and about 250,000 coalition soldiers were present in this region of Asia. With relatively few injuries and deaths, many consider this war as an example of excellent tactical planning and military acumen. Despite its military success, the 1991 Gulf War (GW) is known for the chronic health effects that veterans of this war suffer from to date. It has been 30 years since the end of the GW and anywhere between 25 and 32% of the deployed soldiers continue to exhibit a constellation of chronic health symptoms that are popularly known as the Gulf War Illness (GWI) (Committee on Gulf War and Health, 2013, 2016). Other names for this condition include Gulf War Syndrome (GWS) and the chronic multi-symptom illness (CMI). In addition to the US forces, soldiers from the coalition nations deployed during this war also exhibit GWI (Gwini, Forbes, Sim, & Kelsall, 2016). [Table 1] Elegant review articles have comprehensively covered the epidemiology, causes, pathology, and therapeutic approaches for GWI (Belgrad et al., 2019; Committee on Gulf War and Health, 2013, 2016; Dickey, Madhu, & Shetty, 2021; Freeman et al., 2021; Gwini, Forbes, et al., 2016; Jeffrey et al., 2019; Michalovicz, Kelly, Sullivan, & O'Callaghan, 2020; O'Callaghan & Miller, 2019; Trageser, Sebastian-Valverde, Naughton, & Pasinetti, 2020; White et al., 2016). The purpose of this review article is to discuss various animal models used in GWI laboratory research in the context of their mimicking of the GW-related exposures, and their advantages and limitations. This will be followed by molecular and behavioral findings in such models that could help explain GWI development, its persistence, and what they inform us about novel druggable targets and therapeutic indications.

## 2. GWI definitions and clinical presentation

GWI is a multi-system and a multi-symptom chronic health condition seen in over 1/3rd of the GW veterans (Committee on Gulf War and Health, 2010, 2016; U.S. Department of Veterans Affairs Research Advisory Committee, 2008). These symptoms appeared shortly after soldiers returned state-side. Commonly reported symptoms include chronic fatigue, musculoskeletal pain, gastrointestinal distress, respiratory difficulties, headaches, mood disorders, memory and concentration impairments, and insomnia (Committee on Gulf War and Health, 2016). The Institute of Medicine (IOM, now, National Academy of Sciences, NAS) recommends two case definitions for diagnosing GWI (Institute of Medicine, 2014). To meet the criteria for the Center for Disease Control (CDC) definition (Fukuda et al., 1998), a GW veteran must experience at least one symptom in two or more of the following three categories for more than 6 months: 1) Fatigue, 2) Mood and cognitive dysfunction, and 3) Musculoskeletal problems including pain. The Kansas definition

(Steele, 2000) requires that a GW veteran experience symptom in three of the following six areas: 1) Pain, 2) Cognitive and mood symptoms, 3) Fatigue and sleep problems, 4) Gastrointestinal distress, 5) Respiratory symptoms, and 6) Skin symptoms. The CDC definition is recommended for clinical use but is considered overly inclusive resulting in greater prevalence rates of GWI including CMI detection in control population. The Kansas definition is recommended for clinical research and while it predicts consistent prevalence rates, it can inadvertently exclude GW veterans from receiving a GWI diagnosis on account of multiple co-morbidities. In addition to these two definitions, a third more restrictive definition covering variants of GWI has also been described. This third diagnostic criteria is also known as Haley Syndromes (Haley, Kurt, & Hom, 1997). Using a two-stage factor analysis on answers to a survey conducted in GW veterans, ambiguous symptoms were disentangled and some syndromes were identified (Haley et al., 1997; Haley & Kurt, 1997). In the absence of specific biomarkers and the overlap of symptoms with other diseases and conditions, recognizing GWI has proven to be difficult (Kaimal & Dieterich-Hartwell, 2020). Greater emphasis is to be placed on improving provider education and the Veterans Affairs (VA) healthcare system's response to identify, treat, and provide appropriate coverage to GW suffering veterans (Baldwin et al., 2019).

## 3. Probable causes for GWI pathogenesis

GW soldiers experienced a myriad of exposures including chemical, biological, physiological, psychological, and environmental factors in the GW theatre. These exposures ranged from heat and dust, smoke from oil-well fires, repeated exposures to pesticides and insecticides, low-level exposure to nerve gas, pharmaceuticals such as pyridostigmine bromide, vaccines, depleted uranium, and industrial solvents and paints [Table 2]. Complex interactions between environmental and biological factors is thought to underlie GWI development. However, what makes evaluating contribution of these factors for GWI pathogenesis complicated is the lack of rigorous data on the dose, frequency, and durations of such exposures. In addition to the variety of toxicants, it is not known how various toxicants would have interacted with each other in the war theatre or how multiple exposures of the same agent would behave under war and deployment related stressors. The NAS Committee on Gulf War and Health and the VA Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses have extensively studied potential impacts of deployment-related exposures on veteran's health (Brimfield, 2012; Committee on Gulf War and Health, 2016;

**Table 1**  
Overview of GW events leading to GWI.

Date	Event
Aug 2, 1990	Iraqi forces invade Kuwait
Aug 8, 1990	US military and 34 other nations send troops to the Persian Gulf (Operation Desert Shield)
Jan 16, 1991	Operation Desert Storm commences with air attacks
Feb 23, 1991	Operation Desert Storm land attacks
Feb 28, 1991	Iraqi forces surrender
Apr 6, 1991	Ceasefire signed
Jun 1991	Majority of troops return home
< 1 yr later	Returning troops complain of multiple symptoms afflicting multiple bodily systems [Ultimately diagnosed as Gulf War Illness]

**Table 2**  
Potential Exposures in the 1991 Gulf War Theatre of Operations.

Category	Factors
Environmental	<ol style="list-style-type: none"> <li>1. Dust storms</li> <li>2. Intense heat</li> <li>3. Smoke from oil-well fires</li> <li>4. Sand flies, desert insects, mosquitoes</li> </ol>
Physiological	<ol style="list-style-type: none"> <li>1. Deployment and combat stress</li> <li>2. Psychological stressors</li> <li>3. Gene-environment interactions</li> </ol>
Chemicals	<ol style="list-style-type: none"> <li>1. Insecticides and Pesticides               <ol style="list-style-type: none"> <li>A. Organophosphates azamethiphos, chlorpyrifos, diazinon, dichlorvos, malathion</li> <li>B. Carbamates bendiocarb, methomyl, propoxur</li> <li>C. Pyrethroids Permethrin, d-phenothrin</li> <li>D. Insect repellents DEET</li> <li>E. Organochlorine Lindane</li> </ol> </li> <li>2. Industrial solvents and gases CARC paint, heated metal fumes, chlorinated hydrocarbons, and other occupational exposures</li> <li>3. Depleted Uranium</li> <li>4. Combustion products Fumes from kerosene stoves, tent heaters, burn pits</li> </ol>
Chemical and Biological warfare related	<ol style="list-style-type: none"> <li>1. Pharmaceuticals Pyridostigmine bromide tablets</li> <li>2. Nerve agents Sarin and Cyclosarin</li> <li>3. Vesicating agent Mustard gas</li> <li>4. Vaccines Anthrax vaccine and Botulinum toxoid vaccine</li> </ol>

U.S. Department of Veterans Affairs Research Advisory Committee, 2008). A brief discussion of some of the well-researched causative factors in the context of GWI animal modeling is given below.

### 3.1. Pesticides and insecticides

It is estimated that soldiers were exposed to over 35 different types of pesticides and insecticides during the GW (Cecchine, Golomb, Hilborne, Spektor, & Anthony, 2000; Fricker et al., 2000; Institute of Medicine, 2003). While there are no exact records of levels of pesticide exposure amongst the soldiers, epidemiological studies have shown significant association between pesticide exposure and symptoms of GWI in multiple GW veteran populations (Cecchine et al., 2000; Institute of Medicine, 2003; Steele, Sastre, Gerkovich, & Cook, 2012). As shown in Table 2, these included a variety of chemicals in organophosphate, pyrethroid, and repellent categories. Organophosphate pesticides such as chlorpyrifos and malathion were liberally used in the camp grounds and as a fumigating agent (Cecchine et al., 2000). Pyrethroid such as permethrin was applied by soldiers to their skin and to their uniforms every 4–5 days. It was provided to the soldiers as 0.5% spray and its usage is reported to be significantly higher than the recommended labeling instructions (Binns, Bloom, & Clauw, 2008; Fricker et al., 2000; Plapp Jr., 1999). It was supposed to be applied this way but it varied widely and was used much more often by some troops and much less by others. There were also military personnel whose job was to treat all the uniforms for their units thus making their exposure much higher (Steele et al., 2012; Sullivan et al., 2018; Winkenwerder, 2003). The repellent DEET was also commonly used by the soldiers. Some of these compounds were also present in flea collars worn by the troops. However, only some veterans wore these flea collars and they were not

supplied by the Department of Defense. Pharmacologically, these compounds have diverse mechanisms of action and affect multiple signaling systems. Organophosphate compounds are inhibitor of the enzyme acetylcholinesterase (AChE). Inhibition of this enzyme leads to build up the neurotransmitter acetylcholine (ACh) at the synapses. Dependent on the organophosphate type, dose, duration, frequency, and degree of exposure; symptoms range from acute expression of headache, confusion, blurring of the eyes to foaming of the mouth, seizures, bradycardia and even death (Bajgar, 2004; Brimfield, 2012). Chronic effects of acute or repeated organophosphate exposure include mood and memory disturbances, vision problems, and even increased risk for suicide (Jaga & Dharmani, 2007; Parron, Hernandez, & Villanueva, 1996; Steenland et al., 1994; Wesseling et al., 2002). Permethrin is a sodium channel blocker (Vais, Williamson, Devonshire, & Usherwood, 2001) while DEET is reported to possess weak AChE activity along with a capability to block sodium and potassium channels that contributes to its neurotoxicity (Swale, Sun, Tong, & Bloomquist, 2014). Sustained use of permethrin in combination with other GW causative agents has been demonstrated to affect pain behavior and neurological outcomes in rodents (Cooper, Flunker, Johnson, & Nutter, 2018; Flunker, Nutter, Johnson, & Cooper, 2017; Nutter & Cooper, 2014; Nutter, Jiang, & Cooper, 2013; Nutter, Johnson, & Cooper, 2015).

### 3.2. Pyridostigmine bromide

It was well known that Iraq had possessed chemical weapons as demonstrated by their use against Kurdish civilians prior to the GW. There was a legitimate concern that US forces could come under nerve gas attack. Thus, pyridostigmine bromide (PB) tablets were distributed amongst the soldiers as a prophylactic against possible attack with nerve agents. Each tablet had a strength of 30 mg PB and they were in blister packs of 21 with a recommendation of taking one tablet every 8 h. PB is a reversible AChE inhibitor and thus was used with the intent of shielding the enzyme from subsequent exposure to nerve agents that are irreversible inhibitors of AChE. It is estimated that over half the troops consumed PB tablets at some point during deployment but, there are no complete records of actual levels of PB exposure amongst veterans. However, similar to pesticide exposure, there is a strong correlation between PB use and GWI symptoms (Committee on Gulf War and Health, 2000; Golomb, 1999; Haley et al., 1997; Haley & Kurt, 1997; Unwin et al., 1999).

### 3.3. Nerve agents

While the deployed soldiers did not come under direct attacks with nerve gas, there is now sufficient evidence using meteorological and satellite imaging that some soldiers were accidentally exposed to sarin and cyclosarin during the destruction of Iraqi munitions storage sites after the cessation of GW (U.S. Department of Defense, 2002a; Directorate of Deployments., 1997; Haley & Tuite, 2013; Special Assistant to the Secretary of Defense for Gulf War Illnesses, 2001; Tuite & Haley, 2013). Dispersion modeling of sarin plume suggests that over 100,000 soldiers could have been exposed to nerve gas and it is estimated that this low-level exposure lasted for a 4-day period from March 10 to 13, 1991 (U.S. Department of Defense, 2002b). While there are no correct estimates available for levels of nerve gas exposure, it is believed that there were no troops in the vicinity of “first-noticeable effect” zone, where the effects of nerve gas exposure would have been most pronounced. The majority of plume exposed soldiers were estimated to be in the “low-level hazard” zone (Proctor, Heaton, Heeren, & White, 2006). Similar to the OP pesticides and PB, sarin is also an AChE inhibitor (Golomb, 2008). Low-level exposure to sarin produces symptoms that are consistent with some symptoms experienced by GWI veterans (Brown & Brix, 1998; Chao, Rothlind, Cardenas, Meyerhoff, & Weiner, 2010).

### 3.4. Vaccines and depleted uranium

Role of vaccines and depleted uranium (DU) have also been a subject of speculation for GWI symptoms (Committee on Gulf War and Health, 2000). Some soldiers received anthrax and botulinum toxoid vaccines before their deployment. It was hypothesized that immune response to these vaccinations or the components in these vaccines were inducing GWI symptoms. Several recent studies have concluded that there were no associations between GWI development and vaccines (Hornby, Pearce, Bowditch, Scott, & Griffiths, 2006; Peakman, Skowera, & Hotopf, 2006; Phillips et al., 2009; Steele et al., 2012). DU from the munitions and dust has also been a contentious factor for GWI symptoms. Epidemiologic studies have not been able to confirm an association between DU and GWI health effects. A more recent study used isotope mass spectroscopy analysis on urine samples from GWI veterans and concluded that even the highest likely levels of DU inhalation played no role in the development of GWI (Parrish & Haley, 2021).

### 3.5. Other factors

In addition to the factors briefly discussed above, several other putative factors were present during the GW including various organic solvents, combustion products, fuels, chemical agent-resistant coating (CRAC) paints. These have also been investigated and no associations have been found between their exposure and GWI symptoms (Institute of Medicine, 2005). One another factor that is gaining recognition as a possible contributor for GWI susceptibility is the genetic variability amongst the (soldier) population. Genetic differences in response to wartime exposures are increasingly being recognized as important factors in GWI etiology (Georgopoulos et al., 2016; O'Callaghan, Michalovicz, Miller, & Kelly, 2017). For example, a significant interaction between prior PB use and butyrylcholinesterase (BChE) genotype for GWI severity has been reported (Steele, Lockridge, Gerkovich, Cook, & Sastre, 2015). Similarly, low activity of the enzyme paraoxonase1 (PON1) that metabolizes OPs has also been attributed to GWI susceptibility in veterans (Haley, Billecke, & La Du, 1999; Hotopf et al., 2003; Mackness, Durrington, & Mackness, 2000).

## 4. Attributes of an animal model for GWI

In general, animal models are geared towards replicating human disease condition and should ideally exhibit similarities between the pathophysiology, histopathology, biomarkers, disease progression, and response to drug therapies (Mergenthaler & Meisel, 2015). In this context, an ideal animal model should exhibit good validation with the clinical condition it is trying to replicate (Denayer, Stöhr, & Van Roy, 2014; Nestler & Hyman, 2010). Four such validators include 1) *face validity* that refers to the similarities in the biology, pathophysiology, and symptoms between the animal model and the human disease, 2) *predictive validity* is the demonstration that the model responds to treatments and interventions in a way that predicts the effects of those treatments in humans, 3) *target validity* refers to similarities in the role of the target under investigation to its role in the disease condition and, finally 4) *construct validity* refers to the relevance of the methods by which the disease is constructed in the model to reflect the clinical situation.

The majority of preclinical research that has significantly contributed to our understanding of the multifactorial etiology of GWI has heavily relied on rodent models. The general scheme for this research approach is to employ various neurotoxicants present in the GW theatre in an attempt to mimic GW-like exposures. At various time points following these exposures, rodents are subjected to molecular studies and/or a battery of behavioral tests to assess for signs that are indicative of some of the symptoms reported by GW veterans. These models have also been utilized to study brain, gut, muscles, blood to identify mechanisms underlying the GWI symptoms afflicting different bodily systems.

Consequently, these animal models are used to evaluate potential therapeutic strategies and interventions for GWI treatment.

There are, however, several challenges when modeling GWI in rodents and, a deeper appreciation of the deployment circumstances and current status of GW veterans is essential when designing studies and interpreting the experimental data. An ideal animal model for GWI should consider the myriad factors that were present in the GW theatre. Not only should the model then recapitulate the different chemicals and conditions surrounding GW deployment, considerations should also be made regarding the dose, duration, frequency, and possibility of interaction between different factors including physiological, psychological and, pharmacological factors. This is particularly challenging since there are major gaps regarding the availability of data around these exposures. Thus, the inability to accurately determine the exposures limits the construct validity of GWI models. The interpretation of behavioral data is also challenging since some subjective GWI symptoms are hard to measure in animal models. In addition, as will be discussed below, not all the GWI signs have been evaluated in all the animal models thereby limiting the face validity of such modeling. The predictive validity of GWI pre-clinical models could also be affected if the time points chosen for experimental studies do not align with the chronic nature of GWI. It has been over three decades since the GW and therefore, studying the latent effects of toxic GW chemical exposures would more closely approximate the current age status of GWI suffering veterans. Careful considerations of the rodent aging and its correlation to human age is critical for determining experimental time points for GWI studies. Given these challenges, an all-encompassing pre-clinical model of GWI is difficult to be established. Despite this, researchers have developed laboratory models that produces aspects of GWI signs in rodents. Studies in these models have revealed physiological alterations in response to exposure to GW neurotoxicants and are providing mechanistic information that could explain the development, progression, and persistence of GWI. In the sections below, we will highlight some of the widely used pre-clinical models for GWI research. Studies that have utilized GW neurotoxicants at concentrations that are relevant to GW-related exposures are being discussed below.

## 5. Mouse models of GWI

### 5.1. (PB + PER ± CPF) models

This model mimicked war-time neurotoxicant exposures by exposing CD1 mice to PB (2 mg/kg, i.p.) and PER (200 mg/kg, i.p.) in dimethyl sulfoxide (DMSO) over a period of 10 consecutive days. The actual GW veterans' exposures to PB are not known. While some veterans took the PB pills leading up the war, some of the available records indicate that soldiers were instructed to take PB pills during the ground war. Given the short duration of ground conflict, a 10-day exposure regimen in mice could potentially mimic GW-like exposures to PB and PER whose exposure correlated with GWI symptoms (Fricker et al., 2000; Golomb, 1999; GulfWarandHealth, 2000; Hilborne, 2005). This is an important consideration for animal modeling since intake of 21 or more pills by GW veterans (minimum 7-day exposure period) have been associated with increased rates of GWI (Steele et al., 2012; Wolfe, Proctor, Erickson, & Hu, 2002). At various time points post exposure, mice were analyzed for behavioral deficits. It was noted that at early time-points (8-days post exposure), these mice did not exhibit any significant behavioral phenotype nor any neuronal loss or astrogliosis. However, as time elapsed, behavioral and neuropathological changes emerged. An increased anxiety-like behavior was seen at 30 days with no evidence of motor incoordination. While, delayed cognitive impairments were noted at 5-months post exposure. Proteomic analyses revealed alterations in lipid metabolism particularly reductions in the fatty acid-binding protein 3 (FABP3) along with alterations in endocrine function, immune and inflammatory pathways (Abdullah et al., 2011). Lipidomic analysis at this chronic time-point post PB + PER exposure also revealed



increased phosphatidyl choline and sphingomyelin in the brain along with increased catalase and decreased lysoplatelet activating factors indicative of lysosomal and peroxisomal alterations (Abdullah et al., 2013).

Another study in mice investigated acute neuropathological effects of a cocktail of GW neurotoxicants. Here, C57/BL6 mice were exposed to a mixture of CPF (5 mg/kg) + PB (0.7 mg/kg) + PER (200 mg/kg) administered i.p. as a single injection in a volume of 50  $\mu$ L DMSO for the same 10-day exposure regimen. These doses were approximately 1/5th to 1/2 of the LD<sub>50</sub> doses for PB and PER respectively, and thus were sub-toxic. The PB dose was revised lower to prevent greater PB toxicity and mortality due to increased cholinergic sensitivity of this strain of mice. At 3 days post exposure, persistent increase in ACh levels, astrogliosis, and synaptic impairment due to reductions in hippocampal synaptophysin along with decreased neuronal differentiation in the limbic system and signs of vascular injury were noted (Ojo et al., 2014).

This model was further optimized in follow up studies where C57/BL6 mice were exposed to a lower PB dose (0.7 mg/kg) along with PER (200 mg/kg) administered in DMSO i.p. for the same 10-day period. In the first set of studies, testing was conducted at approximately 2.5-weeks and 5-months postexposure. While no cognitive deficits or significant neuropathological changes were detected at the early time point, however, long-term cognitive deficits associated with increased astrogliosis and reduction of cortical and hippocampal synaptophysin were noted at 5 months post exposure (Zakirova et al., 2015). A longitudinal characterization of neurobehavioral and neuropathological changes in these PB + PER exposed mice was subsequently carried out and at almost two-years post exposure, chronic cognitive deficits and persistent neuroinflammatory changes were noted (Zakirova et al., 2015). These are important findings since it indicates that GW-related exposures create life-long changes in behavior and memory function. The neuropathological changes observed at these chronic time points are highly relevant given the advancing age of GW veterans since such a model system could be utilized to screen therapeutics that have the potential to halt the progressive neuroinflammatory changes with the intent to reverse the life-long GWI neurobehavioral signs. Such an approach has great translational potential for finding mechanisms underlying GWI and screening therapies that could effectively treat GWI symptoms (Abdullah et al., 2016; Joshi et al., 2018; Joshi et al., 2020). Indeed, proteomics, lipidomics, and metabolomics studies in this PB + PER mouse model (Abdullah et al., 2011; Abdullah et al., 2012; Abdullah et al., 2013; Zakirova et al., 2017) have exhibited impaired lipid metabolism and neuroinflammation that is in line with lipid changes detected in GWI veterans blood specimen (Emmerich et al., 2017). Additional studies have found similar accumulation of very long chain fatty acids in plasma from veterans with GWI and GWI mouse models (Joshi et al., 2018).

### 5.2. (PB + PER + DEET + stress) model

The PB + PER model has been expanded to include additional GW-related exposures in the form of DEET and stress. This type of modeling approach is in line with a rat model which will be discussed in later sections (Abdel-Rahman, Abou-Donia, El-Masry, Shetty, & Abou-Donia, 2004; Abdel-Rahman, Shetty, & Abou-Donia, 2002). In the expanded mouse model, C57BL6 mice chronically received PB (1.3 mg/kg orally) along with dermal applications of PER (0.13 mg/kg) and DEET (40 mg/kg). Stress was induced *via* daily 5-min restraint. These exposures + stress continued for 28 days (Abdullah et al., 2012). At 4-weeks post GW exposures, increased anxiety and sensorimotor deficits were noted. These behavioral findings are similar to those reported in combined GW-agents + stress rat model (Abdel-Rahman et al., 2004). Subsequent studies conducted at 6-weeks post exposures revealed increased astrogliosis in the cerebral cortex along with increased phosphocholine containing brain lipids which agrees with similar phospholipid alterations seen in PB and PER model (Abdullah et al., 2013).

### 5.3. (PB + DEET + CORT + DFP) model

In order to study effect of GW-related nerve gas exposure, a surrogate of sarin, diisopropyl fluorophosphate (DFP) was used in combination with other known GW chemicals. In this model, male C57BL6 mice were treated with PB (3 mg/kg, s.c.) with DEET (30 mg/kg, s.c.) for 14 days. During this exposure, between days 8–15, CORT (300 mg/l in drinking water) was supplemented to mimic physiological effects of war-time stress. Finally, on the 15th day, mice were acutely injected with DFP (4 mg/kg, i.p.) and then euthanized on the same day for molecular studies (O'Callaghan, Kelly, Locker, Miller, & Lasley, 2015). Results of these studies showed that CORT+DFP exposure resulted in a minimal neuroinflammatory response at 2-h post DFP dosing which increased by 6-h and then returned to control levels at 12-h. These inflammatory effects were observed in the absence of any neurodegeneration, astrogliosis, or microglial activation (O'Callaghan et al., 2015). Interestingly, combined pre-treatment with PB and DEET did not exacerbate neuroinflammatory responses following exposure to CORT and DFP (O'Callaghan et al., 2015). This suggested that CORT + DFP exposures could be sufficient to produce GWI-like neuroinflammatory changes in rodents. A mouse model describing this possibility was subsequently proposed and is discussed below.

### 5.4. (CORT + DFP) model

In this model, male C57BL/6J mice received 4 days of CORT (400 mg/l) in the drinking water followed by a single dose of DFP (4 mg/kg, i.p.). Mice were euthanized by 6-h post DFP exposure for molecular analysis. CORT + DFP treatment produced increases in cytokines and chemokines expressions similar to earlier models using these agents (Locker et al., 2017; O'Callaghan et al., 2015). Furthermore, AChE inhibition did not appear to drive neuroinflammation in this GWI model suggesting a role for non-cholinergic mechanisms for GWI symptoms (Locker et al., 2017). This stress priming also produced epigenetic changes particularly DNA methylation and histone modifications in multiple genes known to modulate immune and neuronal function (Ashbrook et al., 2018). One of the important advances in GWI modeling occurred when this "two-hit" treatment model was revised and expanded to include several other mice strains in an attempt to establish the genetic variability for the development of GWI symptoms amongst GWI veterans (Gao et al., 2020; Jones et al., 2020). Here, mice were treated with CORT (20 mg% in drinking water) for 8 days. On the final day, mice received DFP (4 mg/kg, i.p.) and were euthanized 6-h later for subsequent molecular analysis. Analysis in 30 BXD strains identified variability in susceptibility to developing GWI neuroinflammatory response following CORT + DFP exposures. In comparing the phenotypic responses, IL1 $\beta$  and TNF $\alpha$  were reported to be better indices of the treatment effect of CORT+DFP. Given the high probability of overlap between the candidate genes identified in this study to human genome, this study has the potential to identify a possible factor underlying individual differences to the conditions that produce GWI by locating a plausible candidate gene that underlies the strain differences in a genetic reference population of mice (Gao et al., 2020; Jones et al., 2020; Xu et al., 2020).

One of the confounds of this model is the possible seizure activity associated with a high-dose DFP exposure (O'Callaghan et al., 2015). It is well known that seizure activity due to cholinergic activation is associated with wide-spread neuroinflammation and also produces significant mortality (Guignet et al., 2020). Whether the observed changes are secondary to DFP-induced seizure activity cannot be ruled out. Moreover, whether the acute neuroinflammatory changes seen following DFP exposures persist over a longer period to more accurately reflect the chronic nature of GWI symptoms remains to be seen. Overall, these data indicated that nerve agent exposure combined with high physiological levels of stress hormone produced an early neuroinflammatory

response that could help explain later development of some of the symptoms exhibited by GWI veterans.

## 6. Rat models of GWI

### 6.1. (PER + DEET) model

In one of the pioneering models for studying GWI development, contribution of GW neurotoxicant PER and DEET was first assessed. Adult male rats were dermally exposed to sub-chronic doses of DEET (40 mg/kg) or PER (0.13 mg/kg) or a combination of the two agents for 60 days (Abdel-Rahman, Shetty, & Abou-Donia, 2001). Histopathological assessments carried out 24-h after the last exposure to GW chemicals revealed diffuse neuronal death and significant cytoskeletal abnormalities in diverse regions of brains including the motor cortex, hippocampus, and cerebellum that play a role in maintaining normal motor, mood, and memory function. Indeed, alterations in these functions constitute some of the GWI symptoms (Abdel-Rahman et al., 2001). This model was subsequently built upon with additional GW chemicals and physiological factors to mimic GW-like exposures in rats as discussed below.

### 6.2. (PB + PER + DEET ± stress) model

This model utilizes some of the common and liberally used GW toxicants to mimic chemical exposure from the GW arena. Here, chronic exposure to GW chemicals are also paired with physical stress to simulate pharmacological and physiological interactions between the GW factors. Addition of stress has been a debatable factor for GW agents' toxicity. It was hypothesized that stress could affect the permeability of the blood-brain-barrier thereby allowing for greater uptake of GW-chemicals particularly PB in the brain (Friedman et al., 1996). However, multiple studies using different stressors and animal species have found no evidence for enhanced PB uptake into the brain following stress (Amourette et al., 2009; Kant et al., 2001; Shaikh & Pope, 2003; Song et al., 2004; Song, Tian, Bressler, Pruett, & Pope, 2002). This is in contrast with some other studies that found stress to be an essential factor for exacerbating the effects of GW neurotoxicants. Unlike the mouse models, here war-time stress was mimicked by restraining the rats daily from anywhere between 5 and 15 min. Additional studies revealed that a 5-min restraint was optimal in producing stressful response in rats in combination with GW chemicals (Abdel-Rahman et al., 2002). It was postulated that addition of stress potentiated the neurochemical and neuronal alterations induced by GW chemicals either by inducing liver damage and thereby decreasing detoxification and increasing "effective concentration" of GW chemicals in the brain, or by producing reactive oxygen species, resulting in oxidative stress. Indeed, more recent studies have confirmed signs of chronic inflammation in the liver (Petrescu et al., 2018), brain (Madhu et al., 2019), and presence of oxidative stress (Shetty et al., 2017) in GWI rat model that combined stress with GW chemicals.

In the current and more widely used iteration of this model, adult male Sprague-Dawley rats were administered PB (1.3 mg/kg, orally) along with dermal application of DEET (40 mg/kg) and PER (0.13 mg/kg) in conjunction with a daily 5 min of restraint stress over a 4-week period (Abdel-Rahman et al., 2002; Abdel-Rahman et al., 2004; Parihar, Hattiangady, Shuai, & Shetty, 2013). In contrast to the mouse models discussed above, exposures in the rat model are over a 4-week timeframe to mimic a potentially longer-term exposure of veterans to the GW agents. In the earlier studies, rats were sacrificed one day after the combined chemical + stress exposures. Neurochemical and histological analyses revealed a significant inhibition of AChE, downregulation of muscarinic AChR, diffuse neuronal cell death, and cytoskeletal abnormalities in various regions of the brain including the cerebral cortex, hippocampus, and cerebellum. It was proposed that these profound neurochemical and neuropathological changes could

underlie behavioral abnormalities seen in GWI (Abdel-Rahman et al., 2002; Abdel-Rahman et al., 2004).

In subsequent studies, rats were assessed for mood and cognitive function at 3 months following the GW chemicals + stress procedures and were found to exhibit depressive- and anxiety-like behavior along with spatial learning and memory dysfunction (Hattiangady et al., 2014; Parihar et al., 2013). Additional molecular studies found that these behavioral changes were associated with reduced hippocampal volume, reduced neurogenesis, chronic oxidative stress, mitochondrial dysfunction, and chronic neuroinflammation (Madhu et al., 2019; Megahed, Hattiangady, Shuai, & Shetty, 2014; Parihar et al., 2013; Shetty et al., 2017). Phospholipid profiling of plasma in this rat model bears resemblance to alterations in lipids seen in a mouse model of GWI and in the plasma from GW veterans (Emmerich et al., 2017). These phospholipids have been reported to play an important role in inflammatory processes. Given the congruity of observations across pre-clinical models and the clinical data with regards to enhanced inflammation in GWI, phospholipid analysis could prove to be reliable biomarkers for GWI.

### 6.3. (PB + PER + CPF ± DEET) models

In these models, initially (Bajgar, 2004), rats were exposed to varying concentrations and durations of three common GW agents namely PB, PER, and CPF. The treatment protocol consisted of 60 days dermal exposure to PER (2.6 mg/kg), CPF (120 mg/kg, s.c.) once every 14 days and PB (13 mg/kg; orally) for 14 consecutive days, beginning at day 1 (Nutter et al., 2013). In a variation of this model, PB was administered again for 14 more days on day 30 (Nutter & Cooper, 2014). These doses were selected based on estimations of insecticides and repellants and pharmaceuticals to which veterans could have been exposed in the GW arena. Behavioral and electrophysiological experiments carried out at 8- and 12- weeks after the end of the dosing period did not exhibit a consistent pattern of pain related changes. However, when the duration of exposure to this GW chemical combination was increased to 60 days, a delayed pain-like syndrome was noted 12 weeks post exposure (Nutter et al., 2015). This is an important point that adds to the translational ability of this model since it is reported that the majority of veterans began experiencing pain symptoms following their return stateside from GW deployment (Hotopf et al., 2003; Kroenke, Koslowe, & Roy, 1998). Thus, the delayed emergence of pain-like behavior in this model is consistent with the GW experience.

Given the involvement of DEET for GW veterans pain symptoms (Haley & Kurt, 1997), contribution of DEET for the development and persistence of GWI pain system in rats has been carried out in these models. DEET was incorporated to the above PB + PER + CPF combination at 200 or 400 mg/kg over a 4-week period. DEET augmentation produced a more consistent pattern of pain-like signs and potentiated the development of a chronic pain-like conditions since such behavioral and electrophysiological alterations were noted for up to 6 months post exposure (Flunker et al., 2017). Similar to DEET, PB was also found to be essential for developing a pain-like conditions in these rats since omission of PB from the exposure regimen prevented the emergence of pain deficits (Cooper et al., 2018). Thus, using varying doses, combinations, and duration of exposure, these models have been useful in deciphering contributions of GW chemicals to the chronic GWI pain-like condition.

### 6.4. (PB + PER + DEET) model

The role of GW chemicals PB, PER, DEET in affecting motor function was investigated in a series of studies where combinations of the above GW neurotoxicants were administered. In the first study, male Sprague-Dawley rats were dermally treated with DEET (40 mg/kg) or PER (0.13 mg/kg), for 45 consecutive days either alone or in combination with PB (1.3 mg/kg) which was administered orally for last 15 days of the

45 days exposure regimen (Abou-Donia et al., 2001). On days 30 and 45 following the treatment, sensorimotor performance on a beam-walk, incline plane, and forepaw grip strength was assessed. Treatment with either DEET or PER, alone or in combination with each other, did not have a significant effect on beam-walk score but addition of PB to this combination produced a significant deficit in beam-walk score and beam-walk time. Interestingly, all GW chemicals, alone or in combination, resulted in a significant impairment in incline plane testing. These neurobehavioral changes were also associated with significant inhibition in AChE in brainstem and midbrain (Abou-Donia et al., 2001).

In a follow-up study, rats were now treated with similar doses of DEET and PER as the earlier study but the exposure continued for 60 days with PB administered during the last 15 days (Abou-Donia et al., 2004). Neurobehavioral performance was assessed on day 60 following the beginning of the treatment with DEET and permethrin. It was observed that PB alone, or PB + DEET, or PB + DEET + PER all resulted in deficits in beam-walk scores and also caused impairment in incline plane performance and forepaw grip strength.

In yet another study, varying combinations and doses of PB (3.75, 7.5, or 10 mg/kg, orally) + PER (3.75, 15, 20, 30 or 60 mg/kg, i.p.) + DEET (50, 100, 200, or 500 mg/kg, orally) were administered to rats once or daily for one-week. Analyses of locomotor activity 30 min or 24 h post last exposure showed reductions in locomotor speed with PB + PER and PB + DEET combinations (Hoy et al., 2000; Hoy, Cornell, Karlix, Tebbett, & van Haaren, 2000). These studies point towards importance of PB for the development of pain and musculoskeletal issues reported by GW veterans.

#### 6.5. Repeated, low-dose DFP or sarin or CPF models

Nerve agents like sarin and soman are chemically OPs and pharmacological inhibitor of the AChE enzyme (Bajgar, 2004). Accidental exposure to sarin and cyclosarin in GW arena and its role in GWI symptoms is now well documented (Haley & Tuite, 2013; Tuite & Haley, 2013). The compound diisopropyl fluorophosphate (DFP) is also an OP and an AChE inhibitor and has been used as a nerve agent surrogate in preclinical research (Guignet et al., 2020). In one such DFP-based model, adult, male Sprague-Dawley rats were exposed to DFP (0.5 mg/kg, s.c.) over a 5-day period (Phillips & Deshpande, 2016). The DFP doses employed were low doses at about 1/5th the LD50 dose for DFP. This is an important consideration since it is known that nerve gas exposures in the GW arena were at a very low-level and there are no reports of acute cholinergic symptoms amongst soldiers. Repeated DFP exposures in this model did not produce overt cholinergic symptoms, did not require any pharmacological interventions, and were not associated with mortality. These rats were assessed for GWI neurological signs at 3- and 6- months post DFP exposures and revealed a depressive phenotype associated with impairment on memory tasks (Phillips & Deshpande, 2016, 2018). Additionally, hippocampal injury (Phillips & Deshpande, 2016), chronic alterations in intracellular calcium homeostatic mechanisms (Phillips & Deshpande, 2020), and reductions in BDNF expressions (Ribeiro et al., 2020) have been reported in this model. Variations exist with respect to DFP dosing and frequency of administration in an attempt to mimic OP-based GW exposures (Naughton & Terry Jr., 2018; Terry Jr., 2012). One such variation in male albino Wistar rats, used a lower dose of DFP (0.25 mg/kg, s.c.), administered daily but the duration of exposure was increased to 2-weeks (Prendergast, Terry Jr., & Buccafusco, 1997). Here, rats were observed to exhibit protracted deficits in cognition on spatial learning tasks when tested at two-weeks post DFP exposures. Another variation to this DFP-based model employed even lower doses (0.1–0.5 mg/kg, s.c.) for the same two-week period (Naughton et al., 2018). These sub-threshold DFP exposures were demonstrated to be associated with persistent decreases in axonal transport as well as alterations in myelin structure in the rat brain when assessed at 30-days post DFP exposures.

In a yet another variation, a DFP dose (0.5 mg/kg, s.c.) was administered every other day for 30 days (Terry Jr. et al., 2014). These repeated DFP exposures were found to produce persistent impairments of inhibitory response control. Together, these observations could help explain mechanisms underlying some of the long-term neurological deficits in GW veterans following repeated exposure to OP agents.

Studies also exist on low-dose sarin exposures in mice, rats, and guinea pigs. In one such study, male C57BL/6 mice were exposed to a single subclinical dose of sarin (0.4× LD50). At various timepoints for up to 8-weeks post sarin exposures studies were conducted for neurochemical analysis and revealed region-specific effects on the monoaminergic neurotransmitter systems. While a decrease in dopamine turnover was noted at 1-week, no such changes were evident at 4- and 8-weeks post sarin exposures. Interestingly, increases in serotonin levels were seen at both early and late timepoints in cortex and putamen but decreases in amygdala were also noted (Oswal, Garrett, Morris, & Lucot, 2013). In the guinea pig study, animals were injected 1× daily for 5 days/week (Monday-Friday), for 2 weeks with various sub-toxic sarin doses (0.3×–0.6× of the LD50 dose of sarin). Doses above 0.3× LD50 sarin were associated with significant alterations in brain cholinergic system when tested for 12-days post repeated, low-dose sarin exposures (Shih, Hulet, & McDonough, 2006). It has also been observed that repeated low-level sarin inhalation in rats at clinically asymptomatic doses caused long-term memory impairments when assessed up to 6 weeks post sarin exposures (Kassa, Koupilova, & Vachek, 2001a, 2001b). Similarly, performance and cognitive deficits were noted in a 11-week period following multiple low-level inhalation exposures to sarin (4.0 mg/m<sup>3</sup> for 60 min/day for 3 days) in rats (Genovese, Benton, Shippee, Jakubowski, & Bonnell, 2006; Genovese, Mioduszewski, Benton, Pare, & Cooksey, 2009).

GW veterans were exposed to higher ambient temperatures in the desert environment. Effect of heat stress and chronic sarin exposure has also been studied. In this study, rats were exposed to two different temperatures, a normal room temperature (25 °C) and heat stress (32 °C) for 1 h every day for 1, 5, or 10 days of inhalational exposure to sarin (0.2–0.4 mg/m<sup>3</sup>). When assessed one-month later, no changes in locomotor function or evidence of neuropathology were noted but a significant decrease in AChE staining was observed pointing towards the role of sustained cholinergic dysfunction for GWI symptoms (Conn et al., 2002; Henderson et al., 2002).

Repeated low-dose CPF has also been assessed for chronic effects on behavioral and molecular systems. In one such study, male Wistar rats were injected with CPF (10 or 18 mg/kg, s.c.) every other day for 30 days (Middlemore-Risher, Buccafusco, & Terry Jr., 2010; Terry Jr., Beck, Warner, Vandenhuerk, & Callahan, 2012). At about 7-weeks post CPF exposures, rats showed spatial learning impairments when tested on radial arm maze. These cognitive impairments were also present when rats were assessed on a water maze task at about 20-weeks post CPF exposures (Terry Jr. et al., 2012). Another study employed even lower doses of CPF (5 mg/kg, i.p. for 5 days) and assessed synaptic plasticity at 3 months following CPF administration (Speed et al., 2012). A 50% reduction in synaptic transmission with a concomitant decrease in CA1 pyramidal neuron synaptic spine density was noted (Speed et al., 2012), suggesting that chronic alterations in synaptic plasticity could underlie the persistent behavioral and cognitive deficits following repeated exposures to OP agents.

While these models used sub-toxic DFP or sarin or CPF exposures and studied latent effects in some cases, it could be argued that it does not take into consideration multiple exposures from the GW arena and therefore underappreciates possible complex interactions between GW-related chemicals for the development GWI symptomatology. Recent studies are excluding role of some putative GW chemicals for GWI symptoms (Parrish & Haley, 2021) and are pointing towards a greater role for cholinesterase-inhibiting toxicants in GWI (Michalovicz et al., 2020; Parrish & Haley, 2021). Thus, the OP-alone models provide a system to further tease out the role of sarin exposures



in GWI pathogenesis. However, it remains to be seen whether additional physical and molecular signs of GWI such as increased sensitivity to pain or changes in the gut microbiota or immune dysfunction that are reported in the other GWI model that utilizes multiple toxicants are recapitulated in this model.

### 6.6. (CORT + DFP) model

This rat model is an extension of a similar mouse model (O'Callaghan et al., 2015) that aims at mimicking the physiological stress and sarin exposure from the GW arena. Here, male Sprague-Dawley rats are administered corticosterone (CORT, 200 mg/l in 0.6% EtOH in drinking water) for 4-days followed by a single injection of DFP (1.5 mg/kg, i.p.) (Koo et al., 2018). Rats were then sacrificed 6-h later and subsequent molecular analyses revealed a neuroinflammatory phenotype characterized by increase in several proinflammatory mediators. In this model, DFP alone rats also exhibited increases in the expression of several inflammatory genes, but CORT exposure significantly exacerbated this neuroinflammatory response. Interestingly, these neuroinflammatory alterations were not associated with brain damage. Further, brain MRI studies in this model suggested morphological or connectivity changes in glia and neurons which, could possibly indicate dendritic remodeling due to DFP + CORT exposures. The early timepoint for the assessment is suggestive of the role of the GW chemicals and the subsequent molecular changes for initiating GWI events. It remains to be seen whether this neuroinflammatory profile is consistent with the chronic nature of GWI. Thus, assessments at a later, chronic time point for both signs of GWI and neuroinflammation in this model are awaited.

In a recent modification to this model, to assess neuroinflammatory basis for pain behavior in GWI, rats were subjected to 7-days of CORT (200 mg/l in 0.6% EtOH in drinking water) exposure followed by DFP (1.5 mg/kg, s.c.) injection on the 7th day (Lacagnina et al., 2021). It was observed that increasing the duration of CORT exposure did not precipitate nociceptive hypersensitivity. There is evidence that fatiguing exercise or muscle injury can cause tissue acidosis which, could increase risk of musculoskeletal pain. In order to simulate this scenario, 7 days after DFP, a single injection of acidic saline (100 µl, pH = 4.0 into the left gastrocnemius muscle) was employed. This challenge resulted in robust and persistent hind paw allodynia associated with neuroinflammation in the dorsal spinal cord and dorsal root ganglia, as well as induction of cytokines in the gastrocnemius muscle (Lacagnina et al., 2021). This modeling paradigm raises an interesting possibility. It is well-known that not all veterans with GWI suffer from chronic pain (GulfWarandHealth, 2016). The GW theatre exposures and experiences could have led to increased vulnerability to pain conditions such that a latter sub-threshold stimulus exacerbates the pain response in veterans. These subsequent stressors in addition to the GW exposures could therefore be required for nociceptive hypersensitivity (Kelly et al., 2018; Lacagnina et al., 2021). [Tables 2–5].

## 7. Additional rodent studies with GW neurotoxicants

While the majority of preclinical data around GWI exist with the models discussed above, there are additional animal studies where the role of GW neurotoxicants have been studied. In one such study, the combined effect of PB (or another AChE inhibitor physostigmine) and stress on working memory was assessed in rats. PB (25 mg/ml) or physostigmine (20 mg/ml) was delivered to rats using osmotic minipumps while stress was applied using avoidance-escape paradigm. Yoked stress was also included to enhance CORT levels. No effect

**Table 3**  
CNS effects of GW-related toxicants in mice.

Reference study	Experimental setup	Key Findings
(Friedman et al., 1996)	Mice received 0.1 mg/kg PB or physostigmine 10 min after stress.	Stress decreased the dose of PB required to inhibit central AChE activity.
(Grauer, Alkalai, Kapon, Cohen, & Raveh, 2000)	Mice received PB (0.4 mg/kg) or physostigmine (0.2 mg/kg) ± stress	Stress did not alter PB effect on brain ChE activity irrespective of stress type, gender or mouse strain.
(Husain & Somani, 2004)	Mice received either sarin (0.01 mg/kg, s.c.), pyridostigmine (1.2 mg/kg, p. o.) or both	Exercise augmented the persistent/ delayed toxic effects of low-dose sarin and pyridostigmine in specific tissues of mice.
(Dubovicky, Paton, Morris, Mach, & Lucot, 2007)	Mice received PB (10 mg/kg) by osmotic minipumps for 7 days.	Administration of PB to mice resulted in sensorimotor alterations and decreased locomotor activity. There was no delayed effect of PB and/or stress on sensorimotor reactions.
(Mach et al., 2008)	Mice received sarin (64 µg/kg) for 3 days, s.c. ± stress	Sarin combined with chronic shaker stress caused delayed behavioral (excessive grooming behavior) and endocrine (decreased catecholamine concentration in adrenal) changes.
(Abdullah et al., 2011)	Mice were given 2 mg/kg PB and 200 mg/kg of PER in a single i.p. injection or to the same volume of DMSO alone (control) for 10 consecutive days.	These findings provide a model of GW agent exposure that recapitulates a cognitive and psychomotor-based CNS symptom profile associated with GWI, and proteomic and neuropathologic studies suggest possible biological mechanisms for these symptoms.
(Abdullah et al., 2012)	Mice were given 1.3 mg/kg PB orally in water, 0.13 mg/kg PER and 40 mg/kg DEET (both dermal), over a period of 28 days.	Lipid changes suggest that alterations in peroxisomal pathways and stearyl-CoA desaturase activity accompany neurobehavioral and neuropathological changes after GW agent exposure.
(Abdullah et al., 2013)	Same as (Abdullah et al., 2011)	These studies suggest peroxisomal and lysosomal dysfunction in the brain at a chronic post-exposure timepoint following GW agent exposure.
(Ojo et al., 2014)	Mice received either 5 mg/kg CPF or 5 mg/kg CPF + 0.7 mg/kg PB + 200 mg/kg PER, delivered in a single i.p. injection	Exposure to CPF, either alone or in combination with PB + PER results in a persistent increase in ACh levels, astrogliosis, impairments to synapses, neuronal differentiation and subtle vascular injury at an acute time point post-exposure
(O'Callaghan et al., 2015)	Mice were dosed 1× daily for 14 days with PB (2 mg/kg, s.c.) and DEET (30 mg/kg, s.c). They also received CORT in the drinking water (200 mg/L) and a single injection of DFP (4 mg/kg, i.p.).	The findings indicate a possible critical interaction between GW physiological stressors and GW agents; exposures in which the CNS is primed to amplify future exposures to pathogens, injury or toxicity and result in symptoms of sickness behavior characteristic of GWI.
(Zakirova, Tweed, et al., 2015)	Mice received 0.7 mg/kg of PB and 200 mg/kg PER, or saline, i.p. daily for 10 days.	Impaired cholinergic function observed in GWI that may in part contribute to deficits observed in long-term memory formation.



Table 3 (continued)

Reference study	Experimental setup	Key Findings
(Abdullah et al., 2016)	Same as (Abdullah et al., 2011)	This paper presents mitochondrial lipid disturbances which corresponded with neurobehavioral deficits long after subacute exposure to GW agents.
(Locker et al., 2017)	Mice were treated with CPF (8 mg/kg), DFP (4 mg/kg), PB (3 mg/kg), PHY (0.5 mg/kg), all i.p. CORT (400 mg/L) was given orally.	Exposure to CORT at levels associated with high physiological stress prior to DFP and CPF, results in a robust neuroinflammatory response that can serve as the basis of sickness behavior-like symptoms associated with GWI. These effects are not related to the AChE inhibition induced by these agents.
(Carreras et al., 2018)	The mouse model used followed the rat model from (Abdel-Rahman et al., 2002).	GWI mice exhibited increased anxiety, decreased hippocampal levels of <i>N</i> -acetyl aspartate, GABA, GAD-67 and microglial activation.
(Ashbrook et al., 2018)	CORT was given in drinking water (200 mg/L) for 4 days. On day 5, a single i.p. injection of DFP (4 mg/kg) or saline was administered.	The authors show a range of transcriptome changes in the GWI mouse, many of which reflect gene expression seen in GWI veterans. Alterations in H3K27ac and DNA methylation were also reported. Early epigenetic and transcriptional changes are thought to contribute for chronic GWI pathology.
(Miller et al., 2018)	Mice were treated with CPF (8 mg/kg), DFP (4 mg/kg), PHY (0.5 mg/kg), all i.p. CORT (400 mg/L) was given orally.	Amelioration of ACh accumulation via CORT priming is unique to DFP mice in brain, whereas CORT priming had little to no effect on CPF- or PHY-induced ACh levels in the same brain areas.
(Carpenter et al., 2020)	Two different mice models were used: one following (Zakirova et al., 2015), and the other following (O'Callaghan et al., 2015).	Overall, centrally, in both models, serotonergic and dopaminergic dyshomeostasis was present in multiple brain regions. However, the effects observed varied between models and were more pronounced in Model 1. Peripherally, splenic 5-HT and NE were altered by GWI chemicals, primarily in Model 2. Further, GWI chemicals in both models produced some shared (IL-1 $\beta$ , CCL-2, TNF $\alpha$ and YM-1) and model specific elevations in hippocampal inflammatory markers.

of PB and stress combination was noted on memory performance (Kant et al., 2001).

Role of PB and sarin has also been evaluated. PB (1.3 mg/kg) was administered for 15 consecutive days followed by sarin exposure (50–100  $\mu$ g/kg) on the last day. The combination of PB + sarin was associated with impairment of motor function (Abou-Donia, Dechkovskaia, Goldstein, Bullman, & Khan, 2002). Another study in rats with different PB and sarin doses (PB at 80 mg/l orally and sarin at 62.5  $\mu$ g/kg, s.c. administered 3 $\times$ /week) could not find evidence for long term alterations on avoidance behavior but noted an increase in nociceptive threshold when assessed at 4 months post exposure (Scremin et al., 2003).

Table 4

CNS effects of GW-related toxicants in rats.

Reference study	Experimental setup	Key Findings
(Buchholz, Pawley, Vogel, & Mauthe, 1997)	One group of adult male SD rats was treated with PB chow for 10 days (7.75 mg/kg), another group was injected with 3 mg/kg PER i.p.	No chemical species containing PER were detected in the brain.
(Hoy, Cornell, Karlix, Schmidt, et al., 2000; Hoy, Cornell, Karlix, Tebbett, & van Haaren, 2000; Van Haaren et al., 2000)	SD rats received 15 or 60 mg/kg PER (i.p.). Other groups received 1.5 mg/kg PB (gavage), or 1.5 mg/kg PB and 15 mg/kg PER or 1.5 mg/kg PB and 60 mg/kg PER.	Repeated PB administration disrupts response acquisition in male and female rats. Such disruption is not observed after PER administration, and that there is no significant behavioral interaction when PB and PER are simultaneously administered.
(Kant et al., 2001)	SD rats were treated with 25 mg/kg of PB delivered by osmotic minipumps, and 20 mg/ml of physostigmine.	Chronic PB administration at doses that inhibit blood AChE by 50% does not penetrate the blood brain barrier in chronically stressed rats or affect a test of working memory in rats.
(Abdel-Rahman et al., 2001)	Male S-D rats received daily dermal dose of 40 mg/kg DEET, or 0.13 mg/kg PER, or DEET and PER for 60 days.	Sub chronic dermal application of these chemicals led to diffuse neuronal cell death and significant neuronal cytoskeletal abnormalities in the motor cortex, the hippocampal formation, and the cerebellum.
(Abou-Donia et al., 2001)	Male S-D rats received PB (1.3 mg/kg/day, oral). DEET (40 mg/kg/day, dermal). PER (0.13 mg/kg/day, dermal) alone or combined for 30 or 45 days	Exposure to these chemicals, alone or in combination, produced sensorimotor deficit. DEET and PER alone did not inhibit the CNS AChE activity, but PB combination inhibited brainstem and midbrain activity.
(Abdel-Rahman et al., 2002)	Male S-D rats received PB (1.3 mg/kg, oral). DEET (40 mg/kg, dermal), PER (0.13 mg/kg, dermal) alone or combined for 28 days	Disruption of the BBB caused the entry of PB into the brain, and the consequent inhibition of brain AChE activity
(Song et al., 2002; Tian, Song, Bressler, Pruett, & Pope, 2002)	In all acute and repeated restraint studies, male adult S-D rats were given either saline only (saline, 1 ml/kg, p.o.), PB only (30 mg/kg, p.o.), restraint only (saline), or restraint + PB.	Stress does not generally increase the AChE actions of PB in the CNS.
(Abou-Donia et al., 2002)	Male S-D rats were treated with either saline (i.m.), PB (1.3 mg/kg, gavage), sarin (50, 75, 90 and 100 $\mu$ g/kg, i.m.) or a combination of PB and sarin, for 15 days.	There are significant effects of sarin and PB, alone or in combination, on sensorimotor performance as well as changes in cholinergic system in rats.
(Shaikh & Pope, 2003)	Young (6 weeks of age) male S-D rats received PB in dosages of 10 or 30 mg/kg (p.o.) Paraoxon in dosage of 0.1 mg/kg (i.m.)	Pre-exposure to the OP paraoxon, either by itself or along with forced running stress, had no detectable effect on acute PB neurotoxicity.
(Shaikh, Karanth, Chakraborty, Pruett, & Pope, 2003)	Young (5–7 weeks of age) male S-D rats were PB challenged (0, 3 or	Repeated physical stress and daily sub- clinical paraoxon exposures have

Table 4 (continued)

Reference study	Experimental setup	Key Findings
	10 mg/kg per day, p.o.) for 14 consecutive days. Other set of rats was given paraoxon (0.05 or 0.1 mg/kg).	little effect on subacute PB toxicity. Repeated low-dose PO exposures also had little effect on functional effects of subacute PB dosing.
(Abdel-Rahman et al., 2004)	Male S-D rats were given PB (1.3 mg/kg, oral), DEET (40 mg/kg, dermal) and PER (0.3 mg/kg, dermal) for 28 days.	Significant damage to the cerebral cortex, hippocampus, and cerebellum, even in the absence of BBB damage.
(Abou-Donia et al., 2004)	Male S-D rats were given these drugs, either individually or in combination: PB (0.13, 1.3 or 13 mg/kg, gavage), DEET (4, 40 or 400 mg/kg, dermal) and PER (0.013, 0.13 or 1.3 mg/kg, gavage).	Exposure to various doses of PB alone or in combination with DEET or a combination of PB, DEET, and permethrin resulted in sensorimotor deficits and alteration in the cholinergic system in rats.
(Scremin et al., 2003; Scremin et al., 2005)	Male S-D rats were given saline (s.c.), or PB in drinking water (10 mg/kg), or sarin (62.5 µg/kg, s.c.).	Changes in nociceptive threshold or cerebral blood flow and glucose utilization were present only up to 4 weeks post exposures. By 16 weeks no detectable changes in these parameters were noted.
(Megahed et al., 2014; Parihar et al., 2013; Shetty et al., 2017)	Three-month-old male S-D rats were treated with chemicals (CHEM): PB (1.3 mg/kg, gavage), DEET (40 mg/kg, dermal), PM (0.13 mg/kg, dermal) or vehicle, for 4 weeks.	Significant mood and cognitive dysfunction along with decreased neurogenesis, partial loss of principal neurons and interneurons. Mild inflammation, oxidative stress, Nrf2 activation and mitochondrial impairment.
(Phillips & Deshpande, 2016, 2018)	Nine-weeks old male S-D rats were injected with DFP (0.5 mg/kg, s.c.) once-daily for 5-days	Chronic behavioral and cognitive deficits along with protracted neuronal calcium elevations in rats.
(Pierce et al., 2016)	S-D rats received PB (1.3 mg/kg, oral in water), DEET (40 mg/kg, dermal), and permethrin (0.13 mg/kg, dermal) for 28 days.	Long-term epigenetic alterations characterized by increased expression of miR-124-3p and miR-29b-3p in the hippocampus and regional alterations in global 5mC and 5hmC content along with differential expression of circulating piR-007899 and piR-019162 one-year after GW exposures.
(Koo et al., 2018)	Male S-D rats received CORT in drinking water (200 mg/L) for 4 days, followed by DFP (1.5 mg/kg, i.p.).	CORT-enhanced DFP-induced neuroinflammatory changes similar to the mouse model (O'Callaghan et al., 2015).
(Macht et al., 2018; Macht et al., 2019; Macht et al., 2020)	Adult male S-D rats were gavaged in the morning with either 1.3 mg/kg PB or vehicle from days 1–14.	CORT levels, like cytokines and ChE activity, exhibit a homeostatic shift over time following chronic stress and PB. In addition, PB and stress interact to impair fear conditioning one week following the cessation of treatment. PB and stress also interact to impact excitatory neurotransmission.

## 8. *In vitro* approaches to GWI modeling

In addition to the inherent drawbacks with *in vivo* models (Bracken, 2009; Henderson, Kimmelman, Fergusson, Grimshaw, & Hackam, 2013; Pound et al., 2004; Robinson et al., 2019), as discussed in section 4 above, GWI animal modeling has some specific limitations. Briefly, lack of data on neurotoxicant exposures and the physiological and pharmacological interactions between the multiple GW specific chemicals has been hard to predict. The need for incorporating the latent effects of chemical exposure to more closely reflect the current status of GW veterans adds significant delays before animals could be studied. This adds greater costs towards the maintenance of GW-chemicals exposed animals. Age-related progressions in physiology and its effects on the underlying GWI state also complicates the interpretation of animal data. Further, the number of animals required for rapid drug screening and hypothesis driven research makes this *in vivo* approach both costly and time consuming. Finally, a lack of appreciation of genetic variability amongst GW veterans into the animal models is also a major limitation.

Novel *in vitro* approaches to GWI modeling can effectively overcome some of these disadvantages. A major effort on these lines has resulted in generation of human induced pluripotent stem cells (hiPSC) from the blood of veterans with GWI (Qiang et al., 2017). These cells can then be complemented with specific growth factors that induce them into various cell types including neuronal and non-neuronal cells of the nervous system, as well as myocytes. Since the hiPSCs are derived from human cells they express human proteins and retain epigenetic priming that could not be accurately mimicked in an animal model. This *in vitro* approach could allow for rapid-throughput studies of existing FDA approved drugs for their applicability in GWI treatment. While the use of cell-lines brings advantages for GWI testing, there are also many unknowns to the use of GW-veterans derived hiPSC model system for GWI research. It is not clear whether such hiPSCs would “retain the disease” or these newly differentiated cells would require GW toxicant exposure to make them express GWI molecular signatures. Further, every carefully designed experiment needs an appropriate control. Here, there is a wide variety of control blood samples to choose from. An important question is whether the control group be hiPSCs derived from age-matched human samples, or non-deployed GW era veterans, or obtained from deployed but not sick GW veterans. The cell bank has started by inducing hiPSC obtained from deployed GW veterans blood samples that did not develop GWI. As this approach gains more popularity and produces greater collaborations with scientists, it will allow for testing novel hypotheses for GWI development and screen potential therapeutics (Qiang et al., 2017).

In addition to the novel approach of reprogramming blood cells from GW veterans into neurons, a more traditional approach where neuronal cultures from rodents are exposed to GW toxicants have also been employed in GWI research. In one such study, primary cortical neurons obtained from Sprague-Dawley rats were cultured for one-week, exposed to CORT on the 7th day and, 24-h later exposed to varying concentrations of DFP (20 and 200 nM) (Rao et al., 2017). A reduction in the ratio of acetylated to total tubulin after DFP-alone exposure was noted which was exacerbated by pre-treatment with CORT. This stress priming is in line with the *in vivo* modeling where pre-exposure with CORT has been found to exacerbate neuroinflammatory profile following DFP exposures (Ashbrook et al., 2018; Locker et al., 2017; O'Callaghan et al., 2015). Further molecular characterization revealed mitochondrial dysfunction and decreased dopamine release as a downstream target for DFP toxicity (Rao et al., 2017). Recent studies in hippocampal slices treated with sub-toxic doses of DFP have revealed non-cholinergic mechanisms for reduced synaptic transmission which provides a candidate mechanism for cognitive deficits seen in GW veterans (Brown, Filipov, & Wagner, 2020). A combination of *in vivo* and *in vitro* studies is also being employed to assess GWI related physiological changes (Garg, Ethridge, Miklasevich, & Rohan, 2021a). Thus,

**Table 5**  
Additional systemic effects of GW-related toxicants in mice and rats.

Related to GI			
Reference Study	Species	Experimental setup	Key Findings
(Alhasson et al., 2017)	TLR4 KO C57BL/6 J mice	Mice were dosed 3 times with PB (2 mg/kg) and permethrin (200 mg/kg), both orally. They were then given CORT (100 µg/mice/day), i.p. for 5 days.	This study proves the existence of an alternative immune network that arises from the ability of GW chemicals to alter the microbiome and enrich several gram-negative bacterial populations. Further the altered microbiome-induced gut leakiness and endotoxemia might contribute to neuroinflammation and intestinal injury that are TLR4 dependent. The present study also is likely to support the notion that antibacterial like minocycline and probiotics might be a good therapeutic regimen in GWI.
(Seth et al., 2018)	TLR4-KO mice	Same as (Alhasson et al., 2017)	GW-chemical exposure in mice and subsequent systemic inflammation following a dysbiosis in the gut could cause significant changes in the way the liver metabolizes lipid and carbohydrate with no detectable pathology while butyrate resists those changes.
(Michalovicz et al., 2019)	Adult male C57BL/6 J	Mice received a single s.c. injection of PB (2 mg/kg) and DEET (20 mg/kg) each day for 14 days, and then CORT (200 mg/L) in the drinking water during 7–14 days. On day 15, mice received a DFP (4 mg/kg, i.p.)	The increased inflammation seen in the liver is the result of DFP-induced tissue damage that is suppressible by CORT exposure. GWI is largely a neuroimmune illness instigated by exposure to several conditions experienced during the 1991 Persian Gulf War, particularly high physiological stress, and irreversible AChE inhibitors.
(Petrescu et al., 2018)	Male S-D rats	Rats received PB (2 mg/kg, gavage), DEET (60 mg/kg, dermal) and permethrin (0.2 mg/kg, dermal), for 28 days.	The exposure to GWI-related chemicals and restraint stress resulted in a low-level inflammation of the liver which, after cholestasis challenge, caused an increased inflammatory reaction, with very high recruitment of CD11b/c + F/80 – CD68 – macrophages, enhanced proliferation of cholangiocytes, activation of HSCs, and ultimately increased liver fibrosis.
Related to pain			
Reference	Species	Experimental setup	Key Findings
(Conn et al., 2002)	Male Fischer 344 rats, 10–13 weeks old	Rats were exposed to nose-only sarin (0.2 or 0.4 mg/m <sup>3</sup> ) for 1 h each day, for 1, 5 or 10 days.	Exposure of rats to low levels of sarin, regardless of whether they were undergoing heat stress, did not significantly alter their temperature regulation or locomotor activity. The rats housed at 32 °C showed signs consistent with mild heat stress, but this stressor did not exacerbate their response to sarin gas relative to those rats housed in their thermoneutral zone.
(Scremin et al., 2003)	Male S-D rats	Rats were injected with either saline (0.5 ml/kg, s.c.) or sarin (0.3, 0.4, 0.5 or 0.6 LD50, s.c.). They also had PB in the drinking water (80 mg/L).	PB did not produce adverse delayed neurobehavioral effects. Moreover, at 2 weeks post-treatment, simultaneous administration of PB and sarin prevented the development of decreased exploratory activity and enhanced response to an acoustic startle test that were associated with sarin exposure without PB protection. Thus, this study gives further support to the use of PB as one of the therapeutic resources against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association, with low-level nerve agent exposure.
(Nutter et al., 2013)	Male S-D rats	Rats were exposed to PER (2.6 mg/kg, dermal), CPF (120 mg/kg, s.c.) and PB (13 mg/kg, gavage) for a total of 60 days.	A mixture of PER, CPF and PB can produce molecular adaptations in vascular and muscle nociceptors that last up to 8 weeks after exposure has ended. These changes include an increase in Kv7 conductance and an increased tendency to emit spontaneous discharge, both at physiological temperatures and after 'release' of the reactive inhibition by Kv7 channel proteins. Molecular defects subside by 12 weeks.
(Nutter et al., 2015)	Male S-D rats (-6 weeks)	Rats were exposed to PER (2.6 mg/kg, dermal) daily, CPF (120 mg/kg, sc) 1× every 7 days and PB (13 mg/kg, gavage) daily, for 30 or 60 days.	Increasing chronic exposure to certain anticholinesterases (PB, CPF), in the presence of PER, produces delayed pain-like behaviors and long-lasting maladaptation in muscle and vascular nociceptor ion channel function.
(Flunker et al., 2017)	Male S-D rats	Rats were presented with PER (2.6 mg/kg, dermal), CPF (120 mg/kg, s.c.), DEET (200 or 400 mg/kg, dermal) and PB (13 mg/kg, gavage).	Routine administration of PB did afford a degree of protection against the physiological impact of some of the AChE insecticides to which the soldiers were overexposed, and whose toxicity was amplified by what was thought to be a harmless repellent (DEET). Yet, PB could not protect them from, and may have actually amplified the actions of, the oxon metabolites of the OPs that asserted their deleterious actions through pathways that were independent of AChE activity but had the capacity to derange important components of the nervous system.
(Cooper et al., 2018)	Male S-D rats	Rats were treated with DEET (400 mg/kg), PER (2.6 mg/kg) [both dermal] and CPF (120 mg/kg, s.c.). A different group was treated with all 3 plus PB (13 mg/kg, gavage).	DEET potentiated behavioral changes that appeared and persisted following exposure to GW agents. PB played a critical role in the emergence of pain-deficits, as the presence or absence of PB during the exposure period determined whether ambulatory signs of chronic pain would occur and persist. Certain molecular events (Nav1.9 and TRPA1), and associated changes to nociceptor excitability, varied with behavioral outcomes related to PB.
(Lacagnina et al., 2021)	Male S-D rats	Rats were treated with CORT (200 mg/L in 0.6% ethanol in drinking water for 7 days) followed by a single injection of DFP (1.5 mg/kg, s.c.) on the 7th day of CORT treatment.	No significant allodynia was observed with DFP + CORT exposures. An additional sub-threshold challenge with acidic saline produced long-lasting, bilateral allodynia associated with increased inflammation in spinal cord regions. Allodynia was reversed by treatment with either minocycline, the TLR4 inhibitor (+)-naltrexone, or IL-10 plasmid DNA.

(continued on next page)



Table 5 (continued)

Related to Fatigue			
Reference	Species	Experimental setup	Key Findings
(Somani, Husain, Asha, & Helfert, 2000; Verma-Ahuja, Husain, Verhulst, Espinosa, & Somani, 2000)	Male NIH Swiss mice	Mice were administered with either saline or PB (1.2 mg/kg, p.o.) on week 5 and 6 of the experiment.	Delayed effects of a sub-chronic PB plus exercise resulted in a significant decrease in plasma BChE and triceps muscle AChE activity. An increase in plasma CPK activity, indicating possible perturbation in the integrity of the neuromuscular system; and an enhanced lipid peroxidation in triceps muscle of mice, indicating an oxidative stress response was also noted.
(Jagannathan, Husain, & Somani, 2001)	Male NIH Swiss mice	Mice were administered with either saline or PB (1.2 mg/kg, p.o.) on week 5 and 6 of the experiment.	The combination of PB ingestion for 2 weeks and exercise training for 10 weeks caused significant alterations in antioxidant enzyme activities, enzyme protein levels and lipid peroxidation, leading to oxidative injury. Thus, physical stress amplified the delayed effects of PB in skeletal muscle of mice.

*in vitro* studies have the potential to inform on the molecular changes following exposure to GW neurotoxicants and could provide a screening tool for discovering targeted drugs for GWI treatment.

### 9. Mechanistic and therapeutic insights from GWI models

A major contribution of GWI animal models has been in providing mechanistic data regarding GWI pathogenesis and identifying candidate therapies for GWI treatment. Recent review articles have also discussed some of these findings (Belgrad et al., 2019; Dickey et al., 2021; Trageser et al., 2020; White et al., 2016). Animal models have been able to recapitulate various GWI signs including mood disorders, memory dysfunction, depressive symptoms, chronic pain, and musculo-skeletal changes following exposure to GW neurotoxicants (Abdullah et al., 2011; Abou-Donia et al., 2001; Middlemore-Risher et al., 2010; Nutter et al., 2015; Parihar et al., 2013; Phillips & Deshpande, 2016; Ramirez-Sanchez et al., 2020; Scremin et al., 2003; Terry Jr. et al., 2012). Several putative molecular mechanisms have been elucidated for the expression of these GWI-related signs. A recurring theme across animal models and multiple GWI ailments has been the demonstration of an inflammatory state. Thus, chronic neuroinflammation typified by presence of activated microglia and reactive astrocytes have been reported in rat (Parihar et al., 2013) and mouse models of GWI (O'Callaghan et al., 2015; Zakirova, Crynen, et al., 2015; Zakirova, Tweed, et al., 2015). Further, increased levels of several proinflammatory cytokines and inflammatory factors such as interleukin 1beta (IL-1 $\beta$ ), IL-6, tissue necrosis factor-alpha (TNF- $\alpha$ ), and High Mobility Group Box 1 (HMGB1) have also been reported in DFP rat brains primed with CORT (Koo et al., 2018) and those expressing mood and cognitive impairment following exposure to GW neurotoxicants (Madhu et al., 2019; Parihar et al., 2013). Increases in inflammatory factors such as Toll-like receptor 4 (TLR4) along with IL-1 $\beta$  and IL-6 have been found in (CORT + DFP) exposed GW rats displaying pain-like symptoms (Lacagnina et al., 2021). Systemic inflammation in GWI rat model has also been reported as evidenced by presence of markers of oxidative stress and inflammatory cytokines in circulating blood (Madhu et al., 2019; Shetty et al., 2017). Thus, it has been hypothesized that strategies aimed at taming this inflammation could provide relief from GWI symptoms. Indeed, proof-of-concept studies with anti-inflammatory compounds such as curcumin (Kodali et al., 2018), and monosodium luminol (Shetty et al., 2020) have recently been shown to alleviate GWI behavioral signs by lowering inflammation, reinstating redox homeostasis, and promoting neurogenesis in a rat model of GWI. Therapeutic strategies that target lipid imbalances in GWI could also potentially alleviate inflammation, oxidative stress, mitochondrial dysfunction and improve GWI outcomes. This strategy has shown some pre-clinical efficacy where treatment with oleoylethanolamide, a peroxisome proliferator-activated receptor (PPAR $\alpha$ ) agonist, was found to reduce neurobehavioral deficits and neuropathology (Joshi et al., 2018). Similar approach has also identified nicotinamide riboside (Joshi et al., 2020) to improve mood, fatigue, and memory function in GWI rodent models. Lacto-N-ducopentaose III (LNFPIII), a proteoglycan found in

human breast milk, has also been reported to produce an anti-inflammatory response in rodent models of GWI (Carpenter et al., 2020). Similarly, treatment with another anti-inflammatory compound minocycline and a TLR4 antagonist naltrexone reversed allodynia in a GWI rat model (Lacagnina et al., 2021).

Clinical trials in GW veterans based on this antioxidant and anti-inflammatory hypotheses have tested many nutraceuticals for GWI treatment. Nutritional supplementation with L-carnosine, an endogenous antioxidant and free radical scavenger, was found to have a beneficial effect on cognitive function with the added benefit of reducing diarrhea associated with irritable bowel syndrome seen in GWI veterans (Baraniuk, El-Amin, Corey, Rayhan, & Timbol, 2013). Coenzyme Q10 (CoQ10) was found to significantly improve physical function (Golomb et al., 2014) while concord grape juice (CGJ) significantly improved executive function (Helmer et al., 2020) in GWI veterans. Additional clinical trials with larger samples are needed to further confirm these findings (Chester, Rowneki, Van Doren, & Helmer, 2019). However, not all the interventions are amenable to prior rodent testing. In fact, certain alternative therapies and non-pharmaceutical approaches such as acupuncture, transcranial magnetic stimulation, diet modification, probiotics, behavioral therapies, and others are also being trialed in GWI veterans without any data from prior studies in GWI animal models [clinicaltrials.gov, keyword: GWS/ GWI and reviewed in: (Dickey et al., 2021)].

In addition to the anti-inflammatory approach, a highly complex disorder like GWI would also require some unconventional approaches for identifying effective treatment modalities. Psychedelics and psychotomimetics such as psilocybin and ketamine are being tested as rapid acting antidepressants (Kadriu et al., 2021). While ketamine has recently been approved by the FDA for the treatment of therapy-resistant depression (Kaufman, 2019), it is not known whether GWI veterans with possibly refractory depression (Blore, Sim, Forbes, Creamer, & Kelsall, 2015) could benefit from ketamine therapy. Indeed, ketamine and its enantiomers at low non-anesthetic doses have been shown to produce a rapid and sustained antidepressant-like effect in a rat model of GWI-related depression (Ribeiro et al., 2020; Zhu, Hawkins, Phillips, & Deshpande, 2020). A clinical trial for ketamine in GWI is in early phase-1 [ClinicalTrials.gov Identifier: NCT04712071].

Alterations in gut microbiota have been reported in GWI veterans (Janulewicz et al., 2019). Approaches to restore the gut microbiota and improve the abundance of beneficial microbial species has been proved successful in GWI models. Thus, treatment with LNFPIII, an immunotherapeutic from human milk, was reported to have long-lasting beneficial effects on gut microbiota in a mouse model of GWI (Mote et al., 2020) while treatment with sodium butyrate or spartolonin B, a nutraceutical derived from a Chinese herb, was reported to increase abundance of butyrogenic bacteria and lower inflammation *via* TLR antagonism (Bose et al., 2020; Seth et al., 2018).

Vagal nerve stimulation (VNS) has been reported to diminish chronic pain in humans (Chakravarthy, Chaudhry, Williams, & Christo, 2015), however, VNS paradoxically lowered nociceptive threshold in a GWI mouse model (Nizamutdinov, Mukherjee, Deng, Stauss, &

Shapiro, 2018). VNS is also FDA approved for the treatment of epilepsy and depression (Johnson & Wilson, 2018). It remains to be seen if VNS could improve depressive signs in GWI rodent models. A VNS therapy for treatment of migraine in GWI veterans is currently underway [ClinicalTrials.gov Identifier: NCT02791893].

Alterations in epigenetic mechanisms including DNA methylation along with hippocampal microRNA-124-3p (miR-124) upregulation (Pierce, Kurata, Matsumoto, Clark, & Farmer, 2016) were first reported in GWI models and subsequently also observed in GWI veterans (Trivedi et al., 2019). Inhibition of miR-124 has been shown to enhance synaptic plasticity and improve memory and depression (Roy, Dunbar, Shelton, & Dwivedi, 2017; Wang et al., 2018), symptoms that are also features of GWI. A proof-of-concept study tested miR-124 inhibition using an antisense oligonucleotide approach and found an increase in synaptic plasticity and neurogenesis related genes within the hippocampus of GWI rats (Laferrriere, Kurata, Grayson 3rd, Stecklow, & Pierce, 2019). *In vitro* GWI modeling has also identified novel target for drug discovery in the form of HDAC6 which is also a tubulin deacetylase. Preliminary study indicated that treatment with tubacin, an HDAC6 and tubulin deacetylase inhibitor, restored the microtubule acetylation status, improved mitochondrial transport and normalized dopamine release in cultured neurons (Rao et al., 2017). Interestingly, HDAC6 is also an epigenetic regulator of chromatin function and alterations in histone modifications have been noted in a GWI mouse model (Ashbrook et al., 2018). Thus, drugs targeting histone pathways in GWI could offer beneficial profile for the treatment of GWI symptoms.

AChE inhibition following exposures to OP-based GW toxicants has been implicated for GWI symptoms (Golomb, 2008). Coincidentally, an effect of this sustained inhibition was the enhancement of glutamatergic transmission. In fact, glutamatergic dysfunction has been reported in GWI rodent models (Gargas, Ethridge, Miklasevich, & Rohan, 2021b; Joyce & Holton, 2020; Torres-Altora et al., 2011) and treatment with an experimental compound LDN/OSU -215,111 significantly ameliorated mood and memory deficits in a GWI mouse model along with normalization of hippocampal pathological changes (Wang et al., 2020). A downstream target of glutamatergic activity is neuronal calcium ion ( $\text{Ca}^{2+}$ ) influx. Studies in GWI models have shown protracted elevations in hippocampal  $\text{Ca}^{2+}$  levels (Phillips & Deshpande, 2018) that had their origins in “leaky” ryanodine receptors releasing  $\text{Ca}^{2+}$  from intracellular stores (Phillips, Santos, Blair, & Deshpande, 2019). Consequently, treatment with  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release blockers such as dantrolene and levetiracetam was shown to reduce elevated  $\text{Ca}^{2+}$  levels and improve GWI neurological signs in a rat model (Phillips et al., 2019).  $\text{Ca}^{2+}$  signaling based drugs could possibly offer effective therapeutics for GWI treatment.  $\text{Ca}^{2+}$  targeting could also have additional beneficial effect of lowering downstream mitochondrial dysfunction, although that remains to be demonstrated (Phillips & Deshpande, 2020).

Another approach to GWI drug discovery has explored drug re-purposing by using a combination of systems biology and bioinformatics techniques with pharmacogenomic information to compare gene expression patterns in GWI to known drug targets and expression patterns found in a set of human diseases. These efforts have offered immunotherapeutics and hormones-based therapy for GWI treatment (Craddock et al., 2015). Thus, newer candidate drugs and novel targets identified from these preclinical studies could offer a starting point for their further assessments in clinical trials with GWI veterans for possible amelioration of GWI symptoms.

## 10. Considerations for modifications to existing GWI models

One of the major challenges in advancing GWI research will be maintaining the relevance of existing rodent models to the current status of GWI veteran population. The GW ended almost 30 years ago and most of the GW veterans have aged significantly. According to a 2020

U.S. Census Bureau report, the median age of a GW veteran is around 50 years (<https://www.census.gov/newsroom/press-releases/2020/veterans-report.html>). Given the advancing age of the GW veterans, it is likely that they could have developed age-related co-morbidities. In fact, a recent epidemiological study reported that GW veterans showed higher risk for heart-attacks, high blood pressure, diabetes, arthritis, and chronic bronchitis compared to general population (Zundel et al., 2019) suggestive of “accelerated” aging patterns than their non-deployed peers. Another longitudinal study reported that GWI-related diverse symptoms including chronic fatigue, persistent pain, headaches, GI disturbances, and neurological symptoms such as loss of concentration increased in GW veterans 20 years after their return on stateside (Gwini, Forbes, Kelsall, Ikin, & Sim, 2015) and that these GWI symptoms increased over time (Yee et al., 2020). Additionally, changes to lifestyle, diet, onset of chronic health conditions, stress related to work-life all could be potential risk factors for modifying progression of GWI (Gwini et al., 2016). Such life-style related risk factors that contribute to poor health could play important roles in exacerbating the severity of GWI symptoms and may help explain its persistence (Gwini et al., 2015; Li, Mahan, Kang, Eisen, & Engel, 2011). Thus, existing GWI rodent models could possibly be further modified to simulate aspects of latter life conditions that GW veterans might be experiencing in order to better mimic the influence of current life stage factors on GWI progression and its persistence.

One such modifying factor that could be incorporated in GWI research with relative ease is the age of the animal. It is important to maintain congruency for when rodents receive GW-related exposures and the latency period before which they are included for behavioral and molecular studies such that their age approaches current ages of the majority of GW veterans. Rodents and humans age differently and multiple studies exist that suggest age-related correlations (Agoston, 2017; Jackson et al., 2017; Sengupta, 2013). Generally speaking, GW-related exposure in adulthood and a latency period of 6 months or longer post GW exposures have been used to approximate ages during deployment and the current age of GW veterans. Many GWI models including the (PB + PER + DEET + Stress) rat model (Kodali et al., 2018; Madhu et al., 2019; Madhu et al., 2021; Parihar et al., 2013), repeated, low-dose DFP rat model (Phillips & Deshpande, 2016, 2018), (PB + PER) mice model (Abdullah et al., 2016; Zakirova, Crynen, et al., 2015) have demonstrated age-dependent longitudinal progression of GWI symptoms and molecular mechanisms.

A second modifying factor that could be considered for GWI modeling is to address the influence of diet and body weight on GWI progression. Life-style factors such as western diet and sedentary behavior affect obesity outcomes that are highly prevalent in GW-era veterans (Coughlin, 2016; Coughlin, Kang, & Mahan, 2011) along with greater preponderance of metabolic dysfunction (Naviaux et al., 2019). A recent study incorporated western diet (high in fats and carbohydrates) in the (PB + PER) mouse model of GWI and observed that ensuing obesity worsened GWI pathology and the GWI persistence was associated with a chronic inflammatory condition affecting multiple bodily systems including the liver, alterations in gut microbiome, persistent neuroinflammation, and decreases in neurotrophic factors in the brain (Bose et al., 2020). Another study incorporated high-fat diet in the PB + PER mouse model and observed that this diet accentuated the effects of GW chemicals on gut microbiome complexity and diversity (Angoa-Perez et al., 2020). Interestingly, this high-fat diet induced enhancement of gut microbiome dysbiosis was reversible which, raises an interesting possibility of incorporating dietary modifications or other non-invasive treatments such as probiotics that may provide relief from GWI symptoms (Angoa-Perez et al., 2020). Indeed, clinical trials are underway in GW veterans to assess effects of dietary modifications for GWI treatment (Dickey et al., 2021).

A third modifying factor that could affect GWI severity and persistence is the contribution of stress in the lives of GW veterans. It is well known that GWI veterans continue to experience challenges during

their daily functioning which significantly affects their physical and mental health (Gade & Wenger, 2011; Jeffrey et al., 2019; Wachen et al., 2013). In addition, there is a small prevalence for GWI and co-morbid post-traumatic stress disorder (PTSD) (Bierer et al., 2015; Jeffrey et al., 2021; Weiner et al., 2011). Furthermore, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in enhanced glucocorticoid responsivity are also reported in GWI veterans (Golier et al., 2016; Golier, Schmeidler, Legge, & Yehuda, 2006). Thus, a heightened stress is probably experienced by GWI veterans in their day to day lives. Indeed, GWI veterans reported of enhanced perceived stress compared to general population (Carter & McKenzie, 2021). Both mice (Kelly et al., 2018; Koo et al., 2018; Locker et al., 2017; Michalovicz et al., 2019; O'Callaghan et al., 2015) and rat (Abdel-Rahman et al., 2004; Shetty et al., 2017) models have shown that addition of stress during the GWI modeling paradigm exacerbates the response to organophosphate and neurotoxicant exposures. Indeed, inclusion of an additional stressor in the form of acidic saline produced nociceptive hypersensitivity in rats that otherwise did not exhibit such behavior following CORT + DFP exposures (Lacagnina et al., 2021). However, it remains to be seen whether inclusion of stressor in existing rodent models after GWI has been established in order to mimic ongoing stress in the lives of GW veterans would modify GWI progression and persistence.

## 11. Summary and future perspectives

In the GW theatre, various chemicals with diverse modes of action interacted with soldiers' physiology that was in a state of heightened stress in an environment that also exhibited daily climatic variations. Added to this are the complicating factors of genetic variability and epigenetic background amongst the deployed veterans' population. Given this diversity of GW exposures and other modifying factors, there is heterogeneity in the expression of the GWI symptoms. Together this complexity is unlike any other acquired disorder and has proven challenging in developing an ideal animal model for GWI. This has contributed to the development of several animal models that have employed GW neurotoxicants alone or in combination at varying doses and durations to mimic GW exposures. Characterization of these preclinical models has been critical for improving our understanding of the contributions of various chemical, environmental and physiological stressors in GWI etiology. Laboratory models have helped in deciphering the molecular mechanisms underlying both the development of GWI and their role in persistence of chronic GWI symptoms afflicting multiple bodily systems. This information has consequently offered newer targets for drug development and have also allowed screening of novel compounds and existing FDA approved drugs for identifying candidate therapies for GWI.

However, the multifactorial aspect of GWI etiology and heterogeneity of symptoms also presents unique challenges. It has been difficult to conceive a single pre-clinical model that could express all the GWI signs and exhibit biological complexity reflective of the clinical presentation in GWI. Animal data is also difficult to interpret for many subjective GWI signs and that limits the applicability of animal models for GWI research. Aging and co-morbidities in GWI veterans is also making the already complex nature of GWI even more complicated (Naviaux et al., 2019; Zundel et al., 2020). Another major limitation of GWI animal studies has been an almost exclusive focus on male-only rodents in their experimental designs. This is particularly important since studies have begun to show that GW women veterans report GWI symptoms at a higher frequency and are also at a greater risk of developing a more severe form of GWI (Gray, Reed, Kaiser, Smith, & Gastanaga, 2002; Heboyan et al., 2019; Kregel et al., 2021; Sullivan et al., 2020). Thus, research is needed to examine sex-specific effect on the development, mechanisms, and progression of GWI-related symptoms of female GW veterans in pre-clinical models.

Thus, the choice of the pre-clinical model for GWI research becomes important if the study is to address to role of various GW neurotoxicants in GWI pathogenesis. The outcome of GWI experimental studies would also vary depending upon the timing of the observations with the early time points offering clues to latter development of GWI and chronic time-points offering data regarding persistence of GWI signs. It is also possible that certain molecular signatures observed in one model of GWI may not be present in another model of GWI. Indeed, heterogeneity of GW exposures drive the heterogeneity of GWI signs. We propose an all-inclusive approach where molecular mechanisms identified in one preclinical model developed with certain GW toxicants are also validated in additional GW models that uses other sets of GW toxicants. This could help confirm some key pathogenic mechanisms that are conserved across multiple GWI models. This has the potential to offer a more reliable target for therapy development. One such mechanism that has been found to be altered across diverse GWI models and which participates in many signaling cascades in GWI progression is the brain derived neurotrophic factor (BDNF). Decreases in BDNF levels and its receptors have been reported in multiple GWI models (Carreras et al., 2018; Kimono et al., 2020; Ribeiro et al., 2020; Brown et al., 2021). Given the role of BDNF in modulating synaptic plasticity and its correlation with depression, memory, pain, and sleep disorders (Autry & Monteggia, 2012; Cappoli, Tabolacci, Aceto, & Dello Russo, 2020; Poon, Heng, & Lim, 2021; Schmitt, Holsboer-Trachsler, & Eckert, 2016; Zhang, Yao, & Hashimoto, 2016), alterations in BDNF signaling could at least, in part, help explain some key GWI neurological symptoms. Similarly, congruity in lipid-based biomarker identification between GW patients and laboratory models has revealed that GW animal models could be employed for biomarker discoveries too (Emmerich et al., 2017). In addition, mitochondrial dysfunction that is noted in GWI veterans (Chen et al., 2017; Koslik, Hamilton, & Golomb, 2014) has also been confirmed with proteomic analysis in preclinical studies (Zakirova et al., 2017) and has further offered a candidate therapy for targeting this dysfunction (Joshi et al., 2020). Multiple signaling cascades converge on mitochondrial dysfunction suggesting that interventions at this molecular level or along this pathway could offer an effective therapeutic approach. In addition to back translating data from clinical findings into preclinical research to further optimize the predictive validity of GWI models, the typical forward translation has also been able to provide pathways to hypothesis testing and clinical trials. A link between the altered gut microbiota and neuroinflammation has been shown in mouse models of GWI (Alhasson et al., 2017; Kimono et al., 2020; Seth et al., 2018; Seth et al., 2019) and significantly different gut microbiome patterns have also been reported in GW veterans (Janulewicz et al., 2019) with additional studies planned on assessing gut microbiome alterations in GWI veterans (Keating et al., 2019). Such back and forth translation between clinical world and pre-clinical models, replication of data, and validation of experimental findings is needed to improve applicability of animal models for GWI research. Future studies also need to start including females in the pre-clinical studies to assess sex as a biological variable in GWI development. Despite these challenges, animal models will retain its importance for GWI research in identifying molecular underpinnings of GWI and evaluating treatment strategies for GWI.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgments

This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs, through the Gulf War Illness Research Program under Award No. W81XWH-14-1-0478, W81XWH-17-1-0573, and W81XWH-20-1-0278. Opinions, interpretations, conclusions, and



recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

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