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2 **DEET Potentiates the Development and Persistence of Anticholinesterase**
3 **Dependent Chronic Pain Signs in a Rat Model of Gulf War Illness Pain**
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Abstract

Exposure to DEET (N,N-diethyl-meta-toluamide) may have influenced the pattern of symptoms observed in soldiers with GWI (Gulf War Illness; Haley and Kurt, 1997). We examined how the addition of DEET (400 mg/kg; 50% topical) to an exposure protocol of permethrin (2.6 mg/kg; topical), chlorpyrifos (CP; 120 mg/kg), and pyridostigmine bromide (PB;13 mg/kg) altered the emergence and pattern of pain signs in an animal model of GWI pain (Nutter et al., 2015). Rats underwent behavioral testing before, during and after a 4 week exposure: 1) hindlimb pressure withdrawal threshold; 2) ambulation (movement distance and rate); and 3) resting duration. Additional studies were conducted to assess the influence of acute DEET (10-100 μ M) on muscle and vascular nociceptor K_v7 , K_{DR} , $Na_v1.8$ and $Na_v1.9$. We report that a 50% concentration of DEET enhanced the development and persistence of pain-signs. Rats exposed to all 4 compounds exhibited ambulation deficits that appeared 5-12 weeks post-exposure and persisted through weeks 21-24. Rats exposed to only three agents (CP or PB excluded), did not fully develop ambulation deficits. When PB was excluded, rats also developed rest duration pain signs, in addition to ambulation deficits. There was no evidence that physiological doses of DEET acutely modified nociceptor K_v7 , K_{DR} , $Na_v1.8$ or $Na_v1.9$ activities. Nevertheless, DEET augmented protocols decreased the conductance of K_v7 expressed in vascular nociceptors harvested from chronically exposed rats. We concluded that DEET enhanced the development and persistence of pain behaviors, but the anticholinesterases CP and PB played a determinant role.

Introduction

Chronic pain is a common symptom of Gulf War Illness (GWI). More than 60% of US veterans of the 1991 Persian Gulf War developed a highly varied constellation of deep tissue pains that included headache, muscle, joint, and abdominal pain (Haley and Kurt, 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). While GWI typically developed soon after they returned from service in the Persian Gulf, a substantial portion of warfighters reported symptoms while still in theater (~25%; Kroenke et al., 1998). In the years that followed, the symptoms of GWI tended to remain the same or worsen over time (Hotopf et al., 2003). After more than 20 years of research, there is an emerging consensus that excessive exposure to insecticides and related agents contributed to the development of GWI symptoms (White et al., 2016). However, the relationship between particular exposure patterns and symptoms has remained elusive.

During their relatively brief deployment, the soldiers of ODS (Operation Desert Storm) were potentially exposed to 64 insecticides and repellants containing 37 distinct active ingredients (DoD Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008; RAC, 2014). Our laboratory developed a rat model of Gulf War Illness pain as part of an effort to explore cellular and molecular maladaptations associated with prolonged exposure to insecticides that were employed in ODS. Our early investigations focused on the role of three particular agents that possessed a unique potential to interact with membrane proteins expressed by deep

tissue nociceptors. These agents included: 1) permethrin--- a type I pyrethroid that was supposed to be applied by soldiers to their uniforms every 4-5 days. Permethrin is a powerful Na_v (voltage activated sodium) channel deactivation inhibitor that lengthens action potential duration and thereby permits relatively massive amounts of Ca^{++} into intracellular space of nociceptors (Jiang et al., 2013); 2) chlorpyrifos---a powerful acetylcholinesterase (AChE) inhibitor with the potential to alter multiple cholinergic signaling mechanisms and pathways that are present in deep tissue nociceptors (Rau et al., 2005; Nutter et al., 2013; Cooper et al., 2016). Chlorpyrifos was used as an area spray/fogger and was also present in flea collars that soldiers obtained outside of their officially approved panel of agents (Binns et al., 2008); and 3) pyridostigmine bromide (PB)----an acetylcholinesterase inhibitor that soldiers were instructed to use as a prophylactic against potential nerve agent attack (Weinbroum, 2004; Newmark, 2005; Weissman and Raveh, 2011). PB was supposed to be self-administered by soldiers 3 times per day. Compliance with the prescribed doses and application frequencies of these chemicals was highly variable, and some agents were used excessively (Binns et al., 2008).

Exposing rats to various concentrations and durations of these three GW agents failed to produce a pattern of behavior changes consistent with chronic pain (Jiang et al., 2013; Nutter et al., 2013; Nutter and Cooper, 2014). We recently found that an intensified exposure to the AChE inhibitors (chlorpyrifos, PB) could produce pain-like behaviors that appeared and/or persisted up to 12 weeks following termination of exposure (Nutter et al., 2015). Although an 8 week protocol, utilizing an intermittent exposure pattern, and consisting of daily permethrin, chlorpyrifos (twice per month; 7% duty cycle), and PB (14 days per month; 50% duty cycle) could not produce any lasting changes in rat activity levels or superficial pain measurements (Nutter et al., 2013; Nutter and Cooper, 2014), a doubling of the duty cycle of the

anticholinesterases (chlorpyrifos to 14%; PB to 100%), did induce a delayed pain-like syndrome that emerged 9-12 weeks after exposure (Nutter et al., 2015). Using this anticholinesterase intensified protocol, pain-like behaviors were manifested as an increase in resting times and a decrease in free ranging ambulation. Patch clamp studies conducted on dorsal root ganglion neurons harvested from these same rats revealed the development of a variety of cellular and molecular maladaptations to muscarinic receptor (mAChR) signaling pathways and effectors in muscle nociceptors that were consistent with a chronic myalgia (Nutter et al., 2015; Cooper et al., 2016).

DEET (N,N-Diethyl-meta-toluamide) is an insect repellent that was commonly used by troops during their deployment (Binns et al., 2008). There is evidence that the application of DEET covaried with the development of pain symptoms in returning veterans (Haley and Kurt, 1997). DEET has no known direct interaction with the pain system, but has been shown to be a very weak anticholinesterase (Corbel et al., 2009; Wille et al., 2011; Swale et al., 2014). A recent publication demonstrated that high concentrations of DEET could inhibit both Na_v and K_v ion channel current amplitudes in rat cortical neurons (Swale et al., 2014; see also Corbel et al., 2009). Our laboratory has associated enhanced Na_v1.9 and decreased K_v7 activity with the appearance of pain-like signs in rats exposed to GW chemicals (Nutter and Cooper, 2014; Nutter et al., 2015; Cooper et al., 2016). In the experiments described below, we examined the impact of a DEET augmented exposure protocol on the development of pain signs in our rat model. Additional studies clarified the interaction of DEET with nociceptor ion channels implicated in the development of GWI pain.

Methods

Behavioral Studies

Subjects. Fifty (50) young adult male rats were used in the pesticide exposure studies (Sprague-Dawley; Envigo/Harlan). An additional 85 rats were used in physiology experiments. Rats entering the study weighed 90-110 grams. Terminal weights did not differ significantly in any pesticide exposure group (see Table 1). All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO (Animal Care and Use Review Office of the Army Medical Research and Materiel Command). Two rats developed health issues and were euthanized. After chemical exposures had ended, one rat manifested a rigidity of one hindlimb and the second rat developed a ventral midline tumor. There were no signs of acute pesticide toxicity typically associated with permethrin or chlorpyrifos during the execution of these studies.

Chronic Exposure Protocol. Over a period of 4 weeks, rats (n=50) were exposed to permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), DEET (200 or 400 mg/kg; Sigma Aldrich) and pyridostigmine bromide (PB; 13 mg/kg; Sigma Aldrich). Permethrin, in ETOH, was applied every day to a shaved area of the back (~1square inch) between the forelimbs. Chlorpyrifos was administered by a subcutaneous

141 injection (corn oil) once every 7 days. The dose of chlorpyrifos was intended to represent a net
142 exposure to the potentially large and varied anticholinesterases that soldiers were exposed to in
143 the Gulf theater (Binns et al., 2008). Chlorpyrifos was administered in a corn oil formulation
144 that released the agent over a couple of days (Smith et al., 2009). DEET was administered
145 topically in ethanol at one of two concentrations (25% or 50%). PB was administered daily by
146 oral gavage (tap water) based upon a standard military dose that was adjusted to account for
147 faster pharmacokinetics in rodents (Birtley et al 1966; Husain et al., 1968; Aquilonius et al.,
148 1980; Breyer-Pfaff et al., 1985). Rats were weighed once per week throughout the studies and
149 doses were adjusted accordingly. Control rats received only vehicle exposures over the identical
150 time course.

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152 Five distinct groups of rats (n=10) were formed (see Table 1). One group received all 4
153 agents (Group A). Three groups were exposed to DEET at 50% concentration (400 mg/kg;
154 ETOH) while a fourth group received all 4 agents with DEET reduced to half concentration
155 (Group HD; 200 mg/kg; 25% in ETOH). The latter group served as a positive control for DEET
156 potentiation (Haley and Kurt, 1997). Two groups received only 3 agents: Group PB (PB
157 excluded) and Group CP (chlorpyrifos excluded). Group C served as a vehicle control group.
158 Agents were always administered in the same order. On the one day per week that rats were
159 dosed with chlorpyrifos, the order was PB, chlorpyrifos, permethrin and then DEET. On the
160 days in which chlorpyrifos was not administered, the order of administration was PB, permethrin
161 and DEET. There was little indication that any combination of chemical exposures affected final
162 body weight (Table 1).

Assessment of Pain Behaviors. Prior to entering the study, rats were acclimated to the behavioral procedures for 2 weeks. Pain assessments were conducted weekly throughout the entire dosing and post-dosing periods. A pressure-pain withdrawal threshold was measured using a computer monitored, hand held force transducer (PAM; Ugo Basile). Pressure was applied via a 5 mm diameter ball to the semitendinosus and biceps femoris muscles (left hind limb). During force application, the applied pressure was monitored and instantaneously displayed on a video screen. Video feedback enabled the rate of force application to be regulated by comparison to a standard curve. When the rat withdrew its limb, the force at withdrawal was automatically registered and stored. To complement pressure-pain testing, activity levels (movement distance, average movement rate, and rest time duration) were recorded automatically by infrared sensors in a modified activity box (15 min test period; Fusion Systems, AccuScan Instruments Inc.). The 35 by 40 cm test chamber was modified to prevent rearing behaviors. The chamber was cleaned after each 15 minute test period. Behavioral tests were conducted on both chemically exposed (permethrin, chlorpyrifos, DEET, PB) and vehicle treated (ETOH, corn oil, water) animals over an identical time course. Rats were tested once per week on the behavioral tasks. PAM tests were conducted in ‘blinded’ conditions.

Electrophysiological Studies

Preparation of Cells. Dorsal root ganglion neurons (DRG) were harvested from young adult male rats (90-150 grams). Rats were anesthetized (Isoflurane) and rapidly euthanized by decapitation (Harvard Instruments). The spinal column was removed, bisected and the DRG were dissected free from T11 to S1. Ganglia were trimmed, cut into strips and digested in

Tyrode's solution containing collagenase A (2 mg/ml; Roche Chemical) and Dispase II (5 mg/ml; Roche Chemical). A 15 ml centrifuge tube containing the dissected ganglia was placed in a heated, shaking water bath for 90 minutes at 35° C (EDVOTEK Digital Shaking Water Bath). Gentle trituration was then used to break up visible strips of ganglia. The dispersed neurons were then digested for an additional 45 minutes, and then spun at 500 RPM (30 sec). The supernatant was discarded. The remaining pellet was dispersed into 2 ml of Tyrode's, triturated and plated on 9, 35 mm, polylysine coated Petri dishes (Fluorodish). Plated neurons were bathed continuously in a Tyrode's solution, containing (in mM) 140 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. All electrophysiological studies were conducted at room temperature (20 °C) within 10 hours of plating. Only one cell was used per Petri dish. Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). In experiments on K_v channels, the pipette solution contained (in mM): 120 KCl, 5 Na₂-ATP, 0.4 Na₂-GTP, 5 EGTA, 2.25 CaCl₂, 5 MgCl₂, 20 HEPES, adjusted to pH 7.4 with KOH. In experiments on Na_v channels, the pipette solution contained (in mM): 140 CsF, 10 NaCl, 5 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH. The osmolarity was approximately 290 mOsm.

Recording and Characterization of Muscle and Vascular Nociceptors. Whole cell patch clamp recordings were made with an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and a Digidata 1322A. Series resistance (R_s) was compensated 60-75% with Axopatch compensation circuitry. Whole cell

resistance and capacitance were determined by the Clampex software utility. Recorded currents were sampled at 10-20 kHz and filtered at 2 kHz (Bessel filter).

Once the whole cell mode was achieved, neurons were classified as type 5 (muscle) or type 8 (vascular) nociceptors using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2000; 2002; see also Xu et al., 2010; Ono et al., 2010). Categorization of cells by ‘current signatures’ permits relatively simple identification of distinct cell groups with uniform physiological properties and anatomical targets. Categorization procedures have evolved since they were first established by the Scroggs laboratory and subsequently expanded by our laboratory. Using 3 voltage characterization protocols (CP1, CP2 and CP3), we classified small and medium sized neurons as type 5 muscle or type 8 vascular nociceptors. The physiological signature of type 5 nociceptors used in this study included small I_H (1.18 ± 0.22 pA/pF; CP1), a high threshold I_A (0 mV; CP2) that exhibited a prolonged settling time (55.4 ± 0.87 msec) and a high threshold (> -20 mV; CP3), broad (5.47 ± 0.20 msec at baseline; 0 mV test) Na^+ current. Type 5 nociceptors were found in both the small and medium sized cell pool (30-45 μ M diameter; 80.5 ± 2.92 pF). The physiological signature of type 8 nociceptors included small I_H (1.10 ± 0.19 pA/pF; CP1), an I_A threshold of -20 mV with prolonged I_A settling time (57.6 ± 1.60 msec; CP2), and a high threshold (> -20 mV; CP3), broad (4.61 ± 0.23 msec at baseline; 0 mV test) Na^+ current. Type 8 nociceptors were found among the medium sized cell population (35-45 μ M diameter; 78.5 ± 2.68 pF). The main distinguishing feature between type 5 and type 8 cells was the 20 mV difference in the threshold of I_A . Their signatures are very different from other medium sized neurons encountered in DRG recordings. Those neurons typically feature combinations of large I_H , low threshold, fast settling I_A and low threshold Na^+ currents with fast kinetics (Petruska et al., 2000; 2002). Cells not fitting the classification criteria of type 5 or 8

were discarded. Anatomical targets of type 5 and type 8 neurons were determined by a series of anatomic tracing experiments (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). Type 5 and type 8 nociceptors are capsaicin/heat sensitive and co-express vasoactive neuropeptides (substance P and CGRP; Petruska et al., 2000, 2002; Rau et al., 2007).

Isolation of $\text{Na}_v1.8$ and $\text{Na}_v1.9$ Channel Currents. Following cell classification in Tyrode's solution, Na^+ currents were isolated in an external solution (Na_{iso}) containing (in mM): 20 or 70 NaCl, 120 or 70 TEA-Cl, 0.1 CaCl_2 , 0.1 CdCl_2 and 10 HEPES, adjusted to pH 7.4 with TEA-OH. TTX (500 nM) was added prior to the days experiment. $\text{Na}_v1.9$ currents were recorded using the 70 mM Na_{iso} solution while $\text{Na}_v1.8$ currents were recorded using the 20 mM Na_{iso} solution. The pipette solution contained 140 CsF, 10 NaCl, 5 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH.

Evocation and Characterization of $\text{Na}_v1.9$. From a V_h of -120 mV, cells were stepped from -80 to -20 mV in 5 mV steps (300 ms duration). Currents were leak corrected, on line, using the P/4 procedure module of Clampex 9.0. DEET or ETOH was applied, by close superfusion (~ 1 mm), for 2 minutes prior to testing. All Na_v characterizations were performed at room temperature (20°C). Series resistance was corrected 70-80%. Junction offsets were not corrected.

Peak currents of non-desensitizing $\text{Na}_v1.9$ were measured 250 msec from the start of the voltage step to avoid contamination by $\text{Na}_v1.8$. The slow desensitizing $\text{Na}_v1.8$ could appear at -20 mV but it would be fully desensitized within 50 msec of the voltage step. For voltage dependent activation, individual evoked peak currents were transformed into a conductance:

$G = I_{\text{peak}} / (V_m - V_{\text{rev}})$, where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be 49.6 mV. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzmann function of the form: $G = G_{\text{max}} / (1 + \exp((V_{.50} - V_m)/K))$, where $V_{.50}$ is the voltage at which G is half maximal, and K is a slope factor. Average currents were formed from the normalized peak currents observed over the active range (-65 to -40 mV).

Evocation and Characterization of $\text{Na}_v1.8$. Currents were isolated in the Na_{iso} solution as described above. Following a conditioning pulse to -70 mV (1,000 msec; $V_H = -60$ mV) a strongly depolarizing step to 0 mV (60 msec), evoked a large amplitude slowly desensitizing inward current. Currents were leak corrected, on line, using the P/4 procedure module of Clampex 9.0. After a stable baseline current was achieved, DEET or ETOH was applied for 9 minutes by close superfusion. Time dependent changes to the peak $\text{Na}_v1.8$ current were examined over a period of 7 minutes (2 minutes following application of DEET/ETOH; 15 sec intertrial interval). Peak currents were normalized to cell size (pF). The series resistance was corrected 60-70%. Junction offsets were not corrected. The peak $\text{Na}_v1.8$ current was measured from the peak current to a point (2500 msec) following the voltage step to 0 mV.

Isolation of K_{DR} and K_v7 Channel Currents. Following cell classification in a Tyrode's solution, K^+ currents were characterized in an external, K_{iso} , solution containing (in mM): 130 N-methyl-d-glucamine, 4 KCL, 4 MgCl_2 , 0.2 CaCl_2 , 1 CsCl_2 , 2, 4-aminopyridine, 10 glucose, 10 HEPES,

adjusted to pH 7.4 with HCl. The pipette solution contained (in mM): 120 KCl, 5 Na₂-ATP, 0.4 Na₂-GTP, 5 EGTA, 2.25 CaCl₂, 5 MgCl₂, 20 HEPES, adjusted to pH 7.4 with KOH.

Evocation and Characterization of K_v7 Current. A current subtraction method was used to isolate K_v7 mediated currents from other K⁺ currents that were present as deactivation tail currents. The cell size normalized peak and average K_v7 current was assessed as a conductance to eliminate deactivation voltage confounding of the peak current. For the K_v7 deactivation protocol: a 1,000 msec step command to -20 mV was followed by a series of repolarizing 10 mV steps from -20 to -90 mV (1,000 ms; V_H = -60 mV) followed by a return step to -60 mV. A tail current could be measured during the repolarization steps. The K_v7 voltage deactivation protocol tests were conducted 3 minutes following application of the K⁺ isolation solution containing ETOH or DEET. This was followed by application of the K_{iso} solution containing the K_v7 specific antagonist linopirdine (10 μM in ETOH; 3 min application). The K_v7 voltage deactivation protocol was reapplied. The linopirdine sensitive K_v7 current was isolated by subtraction.

The amplitude of the linopirdine sensitive tail current was measured from a point beginning 10 ms after the repolarizing voltage step (-30 to -90 mV) to the point 10 ms prior to the return step to -60 mV. The currents of individual cells were normalized by cell capacitance (pA/pF) and converted into a conductance (G) as described above, where V_{rev}=-86.5 mV. A mean G was computed over the range of functional deactivation steps (-30 to -70 mV) to obtain a mean normalized conductance. The peak conductance was determined by inspection.

Evocation and Characterization of K_{DR} Currents. For the purpose of this study, the K_{DR} current was defined as the total 4-AP insensitive K^+ current following removal of the K_v7 component with linopirdine. The voltage dependent activation of the total K_{DR} current, was assessed, as a tail current, after application of the K_v7 inhibitor linopirdine (10 μ M; 8 min). From a holding potential of -60 mV, a 2,000 msec conditioning pulse (-100 mV) was followed by 12 consecutive command steps from -80 to 20 mV (10 mV increments; 500 msec duration). The amplitude of the tail current at -60 mV was measured from the peak relative to the baseline current recorded 2,500 msec after repolarization. For each recorded neuron, the amplitude of tail current was normalized to the peak evoked current and then plotted against the activation voltage to obtain a current-voltage relationship. A Boltzmann function was fit and a $V_{.50}$ determined for each individual cell. The voltage dependence of activation was determined from a fit of the voltage-current measures to a Boltzmann function of the form: $I=I_{max}/(1+\exp((V_{.50}-V_m)/K))$, where $V_{.50}$ is the voltage at which the current (I) is half maximal, and K is a slope factor.

To assess average amplitude, the K_{DR} tail currents, at each voltage, were normalized for cell size (current amplitude (pA) divided by the cell size parameter (pF)). These normalized amplitudes were averaged across functional activation voltages (-60 to 0 mV) to obtain a mean current amplitude.

Statistics

A repeated measures ANOVA was used to assess influence of GW chemical treatments on the development of pain signs (post-exposure weeks 5-12). In order to assess the persistence of pain behaviors, an additional analysis was conducted on the 4 week span proceeding

euthanasia (post-exposure weeks 17-20 and/or 21-24, Group A and C only). Dependent measures included: 1) muscle pain threshold (PAM; grams); 2) ambulation: movement distance (cm/15 min), average movement rate (cm/sec); and 3) rest duration (sec/15 min). The alpha level was set at .05. As noted above, 2 rats were euthanized for health related issues (one rat from Group A and one rat from Group HD). Both were terminated on the advice of the study veterinarian. To equalize the number of animals in each group, the corresponding rat (by date of entry), was excluded from each group. In order to adjust for the high variance, present in rat behavioral data, the means of movement and resting scores were trimmed (highest and lowest scores) prior to construction of plots and performance of analyses (Lix and Keselman, 1998; Wilcox et al., 2000; Mudholkar et al., 2013).

To determine the influence of DEET on physiology measures, Student's t-tests were used to contrast normalized amplitude, conductance and/or $V_{.50}$ of $Na_v1.8$, $Na_v1.9$, K_{DR} and K_v7 in DEET and vehicle (ETOH) treated cells. The alpha level was set at .05.

Results

Behavior

After a period of acclimation and baseline behavioral testing, rats were divided into 5 groups (n=50). One group (Group A) was treated with all four GWI chemicals for 4 weeks (permethrin 2.6 mg/kg, chlorpyrifos 120 mg/kg, PB 13 mg/kg, DEET, 400 mg/kg; 50% in ETOH). A positive control for the influence of DEET (Group HD) received all 4 agents, but

DEET was administered at half the concentration (200 mg/kg; 25% in ETOH). Studies of GW veterans had indicated that skin application of high (75%), but not low concentrations (25%) of DEET, posed a risk factor for chronic pain (Haley and Kurt, 1997). Our preliminary studies indicated that a 50% level was sufficient to produce enduring pain-like signs. The remaining 2 groups received combinations of 3 agents where either chlorpyrifos (Group CP) or PB (Group PB) was not included in the dosing routine. A final group served as a vehicle treated control (Group C; corn oil, ethanol, water). Details of the dosing schedule are provided in ‘Methods’ and Table 1. Behavior assessment tests were conducted on all rats once per week for 26 weeks (Group PB, Group CP, Group HD) or 30 weeks (Group C, Group A). Tests included muscle pressure withdrawal threshold (PAM; left semitendinosus) and 3 open field activity measures: movement distance (cm), average movement rate (cm/sec), and rest time duration (sec). All PAM measures were carried out under blinded test conditions. Activity measures were assessed over a period of 15 minutes, in a 35 x 40 cm Perspex test chamber where movements were monitored and quantified by an automated infrared detection system (AccuScan).

After the 4 week exposure period had ended, we conducted a repeated measures ANOVA on 8 and 4 week blocks that extended from post-exposure week 5 through 24. We had previously reported that rats exposed to the same concentrations of permethrin, chlorpyrifos and PB, but in the absence of DEET, exhibited only paradoxical shifts in movement rate and transient elevations of rest time scores during a 5 to 12 post-exposure assessment (Nutter et al., 2015). In studies of DEET augmented protocols below, we focused on this 5-12 week post-exposure time period as well as upon test periods extending up to 24 weeks after exposure.

With the addition of DEET (50% in ETOH) to the same 4 week exposure protocol, a consistent pattern of pain-like signs emerged and persisted for up to 6 months (figure 1A, B, C).

Both ambulation measures, movement distance and movement rate, were significantly reduced in the immediate weeks following exposure (weeks 5-12; $F=19.47$, $p<.001$ and $F=27.71$; $p<.001$, respectively). Although the depression of movement distance returned to normal levels by the final assessment period, (weeks 21-24), the slowing of movement rate, due to GWI agents, was still significant 21-24 weeks post-exposure (figure 1B).

While there was a substantial augmentation of ambulation related pain signs when DEET was part of the exposure protocol, there was no evidence that rest durations were similarly affected (figure 1C). We previously reported persistent rest time increases with 8 week exposures to the 3 GW chemicals (chlorpyrifos, permethrin and PB; Nutter et al., 2015; Cooper et al., 2016), but not with a 4 week exposure to the same agents at identical dosages. As in all our investigations, we also failed to find that these GW agents produced any change in the semitendinosus muscle pressure-pain withdrawal test (PAM; data not shown; Nutter et al., 2014; Nutter et al., 2015; Cooper et al., 2016). GWI veterans do not report mechanical allodynia to superficial stimuli.

As shown in figure 1, exposure to the 4 agents produced a pattern of activity suppression *during* the 4 week exposure period that was observed in nearly all experiments below (figures 2-4). Movement distance and average rate were substantially suppressed while resting duration was increased in the presence of DEET, chlorpyrifos, permethrin and PB ($F=62.67$, $F=64.16$ and $F=10.55$ respectively; figure 1A, B and C, 'Exposure'). However, the capacity of a given agent or agents to suppress activity scores during exposure was not necessarily related to whether post-exposure pain-like behaviors would occur or persist 5 to 6 months after exposures had ended (see below).

Consistent with reports on symptomatic veterans, high concentrations of DEET were associated with the development of pain-like behaviors in rats (Haley and Kurt, 1997). When the concentration of DEET was reduced to 25% (Group HD; positive control), neither ambulation nor resting measures were shifted in a manner consistent with pain (figure 2A, B and C). Instead, movement distance and resting scores were paradoxically increased (movement distance) or decreased (resting duration), relative to vehicle treated groups, during the final 4 weeks of testing (post-exposure weeks 17-20). When Group HD was contrasted with Group A (50% DEET), significant pain signs were evident in Group A at both the early phase (5-12 weeks post-exposure) and the late phase of assessment (weeks 17-20; figure 2D, E and F). Interestingly, although no pain-like signs developed with the HD group, we still observed highly significant decreases in ambulation (movement distance and rate), as well as increased rest durations during the exposure period (figure 2A, B and C; $F=48.42$, $F=30.45$ and $F=5.35$, respectively; 'Exposure').

Conditions for the Development of GWI Pain Signs in Rats

Given that DEET substantially enhanced the development and persistence of pain-like behaviors, we conducted a series of experiments in order to determine if the development of these behaviors would appear and persist if the anticholinesterases were excluded from the dosing protocol. Our previous report had shown that doubling the anticholinesterase duty cycle had been critical to the development of pain signs in the 8 week protocol. In order to assess the role of AChE inhibitors, we contrasted Groups A and C with groups in which either chlorpyrifos (Group CP) or PB (Group PB) was excluded from the exposure regimen.

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410 The Contribution of Chlorpyrifos. CP was originally included in the exposure set in order to
411 represent the large variety of anticholinesterase insecticides that warfighters were exposed to
412 during their deployment (Binns et al., 2008). Statistical analyses comparing vehicle treated rats
413 (Group C) and rats exposed to all agents, except chlorpyrifos (Group CP), confirmed that CP was
414 an essential component of those GW chemicals that induced persistent pain-like behaviors.
415 When CP was omitted from the exposure protocol (Group CP), ambulation deficits were not
416 sustained at post-exposure weeks 17-20 (figure 3A and B). Although transient shifts in
417 movement rates were present through post exposure weeks 5-12 (figure 3B), movement distance
418 scores also failed to emerge during weeks 5-12 in the absence of CP. When comparisons were
419 made between rats exposed to all 4 agents (Group A) and Group CP, rats that were not exposed
420 to CP exhibited significantly improved ambulation during all phases of the study (figure 3D and
421 E).

422 It also appeared that CP contributed significantly to the suppression of movement
423 distance and, to a lesser extent, movement rate that occurred during the 4 week exposure period
424 (figure 3; 'Exposure'). As noted above, rats receiving all 4 GWI chemicals typically manifested
425 a substantial reduction of ambulation scores (and elevation of rest times) during the 4 week
426 exposure session (figure 1A, B and C; 'Exposure'). In the absence of CP, the suppression of
427 both movement distance and rate were significantly relieved relative to Group A (figure 3D and
428 E; 'Exposure'; $F=21.99$ and $F= 42.11$). Nonetheless, some weaker suppression of movement
429 rate and induction of resting could still be detected relative to Group C (figure 3B; 'Exposure';
430 $F=12.12$).

The Contribution of Pyridostigmine Bromide. Exclusion of PB from the chemical exposure protocol substantially altered the development of pain-like behaviors. Like the other anticholinesterase in the exposure set (CP), the inclusion of PB in the 4 week exposure period was necessary for the development of pain signs of movement distance and rate decreases that persisted into weeks 17-20; it was also required for development of these pain behaviors during post-exposure weeks 5-12 (Group PB vs Group C; figure 4A and B). Moreover, in the absence of PB, ambulation scores were significantly shifted towards normal levels relative to animals exposed to all 4 compounds (Group PB vs Group A; figure 4D and E).

Unlike CP, the absence of PB from the exposure protocol did not affect movement scores *during* the exposure protocol (figure 4A, B and C, ‘Exposure’), and more importantly proved to be permissive for the development and persistence of resting pain signs over the course of post-exposure testing. Rats that did not receive PB treatment developed significant and substantial increases in resting behaviors over weeks 5-12 (Group C vs Group PB). These behaviors persisted into weeks 17-20 (figure 4C). Moreover, when Group PB resting times were compared to Group A, scores were not shifted toward normal levels. Instead, resting pain signs were further increased relative to the group that received all 4 agents (figure 4E). Therefore, the contribution of PB was to exert a protective influence against the development of pain associated with increased resting. Given that PB was prescribed to soldiers as a prophylactic against nerve agent anticholinesterases (i.e., Soman/Sarin), it is not surprising that such a finding could emerge. However, it is not a simple matter to reconcile this finding with the influence on

movement distance and rate, in which PB contributed to impairments over the entire post-treatment period.

The Influence of DEET on K_v and Na_v Ion Channel Physiology

Our previous studies indicated that an 8 week exposure to 3 GWI chemicals (chlorpyrifos, permethrin and PB) increased rest time durations and lowered movement distance scores 9-12 weeks post-exposure. Patch clamp physiology performed on cells harvested from those rats revealed that muscle nociceptors exhibited decreased net activity of K_v7 and other K_{DR} ion channels (Nutter et al., 2015). We further demonstrated that muscle nociceptors manifested a unique action potential burst discharge in response to activation of muscarinic receptors (mAChR); and that these action potential bursts were modulated by K_v7 and potentiated in rats exposed to the GWI chemicals (Nutter et al., 2014; Nutter et al., 2015). The low voltage activated K^+ channel, K_v7 , is known to be important for governing neuronal excitability (Brown and Passmore, 2009).

As the inclusion of DEET in the exposure protocol intensified and shifted the pattern of behavioral outcomes, we hypothesized that DEET might also influence the activity of K_v (and other ion channels) channels that have been associated with the development of pain-signs in rats (K_{DR}). It was recently shown that DEET (~100 μ M) significantly diminished the amplitude of the K_{DR} in cultured rat cortical neurons (Swale et al., 2014). Therefore, we initiated a series of studies which examined the influences of DEET on nociceptor K_v7 ion channels and the residual K_{DR} (that K_{DR} remaining after K_v7 and 4-AP sensitive channels were removed). Studies were focused on muscle nociceptors as these were the class of neurons that had been shown to be

modified by GW agent protocols that produced persistent pain-like behaviors in our rat model (Nutter et al., 2015; Cooper et al., 2016).

The Influence of DEET on Nociceptor K_v7 and K_{DR} . Young adult male rats served as subjects. Cells were harvested and plated on the morning of the experiment, and discarded afterwards. Recordings began approximately 2 hours after plating. Neurons identified as muscle or vascular nociceptors were exposed to DEET (10 or 50 μ M). K_v channels were isolated from other voltage activated currents using a K_{iso} solution described in ‘Methods’. In control cases, equal volumes of vehicle (ethanol/ETOH) were substituted for DEET. In the case of K_{DR} , Boltzmann functions were used to describe the voltage dependent data. Individual cases in which Boltzmann functions could not be fit to K_{DR} currents were excluded from the analysis.

As shown in figure 5, we were unable to detect any influence of DEET (10-50 μ M) on voltage dependence or amplitude of either K_v7 or K_{DR} ion channels expressed in muscle nociceptors. There were no influences of DEET on average (figure 5A) or peak conductance (not shown) of K_v7 . Nor were changes observed in the calculated $V_{.50}$ or average currents evoked from K_{DR} channels (figure 5B and C). Accordingly, the amplification of behavioral pain signs exhibited 5-24 weeks after DEET exposure, could not be attributed to a direct influence of DEET on K_v channels during the exposure.

The Influence of DEET on Nociceptor Na_v . Acute exposure to pyrethroid insecticides, such as permethrin, have powerful influences on the properties of TTX_{sensitive} ($Na_v1.6$, $Na_v1.7$) and TTX_{insensitive} ($Na_v1.8$) ion channels expressed in DRG (Ginsburg and Narahashi, 1993;

Tatebayashi and Narahashi, 1994; Tabarean and Narahashi, 1998; Tabarean and Narahashi, 2001; Jiang et al., 2013). $\text{Na}_v1.8$ is expressed in a high proportion of nociceptors and is the principle Na_v that forms action potentials in these neurons (Djoughri et al., 2003; Jiang and Cooper, 2011). Prolonged exposure to permethrin, chlorpyrifos and PB changes inactivation characteristics of $\text{Na}_v1.8$ and prolongs action potential duration (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al., 2015). Given that high concentrations of DEET ($>500 \mu\text{M}$) were recently shown to reduce the amplitude of mixed cortical Na_v , we examined whether such effects could be identified in DRG nociceptors at levels which might be physiologically significant (Swale et al., 2014).

The Influence of DEET on $\text{Na}_v1.8$. Cells were plated as described above. Following identification of a neuron as a muscle or vascular nociceptor, $\text{TTX}_{\text{insensitive}}$ Na_v were isolated from other voltage activated currents using the Na_{iso} solution ($[\text{Na}^+] = 20 \text{ mM}$) and 500 nM TTX (described in ‘Methods’). Time dependent changes to $\text{Na}_v1.8$, in the presence of DEET, were examined. Following a conditioning pulse to -70 mV a strongly depolarizing step to 0 mV (60 msec), evoked powerful inward currents (figure 6A insert). In the presence of vehicle (ETOH), a stable peak current was established over 6-10 evocations (pre-test series). Subsequently, DEET containing solutions were presented for 2 minutes by close superfusion ($100 \mu\text{M}$) and the protocol was then restarted and continued for 7 additional minutes in the continuous presence of DEET (post-test series; 15 second intervals; 28 total tests). In separate experiments, pre and post-test solutions contained only ETOH vehicle.

We compared the percentage change in leak corrected peak amplitudes of $\text{Na}_v1.8$ between ETOH baseline and DEET treated cases (post/pre). We found no evidence that a 9 minute presentation of 100 μM DEET could reduce the amplitude of $\text{Na}_v1.8$ in either muscle or vascular nociceptors (figure 6).

The Influence of DEET on $\text{Na}_v1.9$. Following an 8 week exposure to three GWI chemicals, the amplitude of $\text{TTX}_{\text{insensitive}}$, $\text{Na}_v1.9$ was significantly increased (Nutter et al., 2014). Although $\text{Na}_v1.9$ does not contribute to the formation of action potentials (Cummins et al., 1999; Dib-Hajj et al., 2002), it is an important contributor to nociceptor discharge properties (Fang et al., 2002; Jiang and Cooper, 2011). Due to its ultraslow kinetics and hyperpolarized voltage dependence, $\text{Na}_v1.9$ could mediate burst discharges via the formation of long duration ‘plateau’ potentials (Copel et al., 2009, Herzog et al., 2001; Maingret et al., 2008). If DEET influenced the amplitude of $\text{Na}_v1.9$ during exposure, it might amplify the post-exposure influence of GWI chemicals (permethrin, chlorpyrifos and PB) on the physiology of $\text{Na}_v1.9$. Accordingly, we examined whether an acute presentation of DEET would modify this unique $\text{TTX}_{\text{insensitive}}$ Na_v channel.

Muscle and vascular nociceptors were isolated as described above. A Na_{iso} solution ($[\text{Na}^+] = 70 \text{ mM}$; 500 nM TTX; see ‘Methods’) containing either DEET or vehicle (ETOH) was applied for 2 minutes. After this conditioning period, a family of voltage dependent currents were generated using stepped pulses from -80 to -20 mV ($V_H = -120 \text{ mV}$, 5 mV steps, 300 ms duration). Leak corrections were performed on line and activation curves were constructed by fitting a Boltzmann function. Individual cases in which Boltzmann functions could not be fit were excluded from the analysis.

DEET exhibited some weak and inconsistent influences on Na_v1.9. Following application of DEET to muscle and vascular nociceptor Na_v1.9, there was some indication that high doses (>100 µM) might modulate the current. When the averaged peak amplitudes of the DEET treated currents were compared to vehicle treated cases, it appeared that trends favored a decrease in amplitude at doses exceeding 100 µM. All comparisons to vehicle treated cases were non-significant; however, a significant difference was observed between tests at 50 and 100 µM (figure 7C). In addition to the averaged current, we observed a significant ~-2 mV shift in the voltage dependence of muscle nociceptor Na_v1.9 (V₅₀; 50 µM; figure 7A). However, we could not reproduce the voltage shift at 100 µM DEET. No effects of DEET were observed for voltage dependence of vascular nociceptor Na_v1.9 (figure 7A and B). As these shifts all occurred at what were clearly non-physiological doses, we did not pursue these trends any further.

Nociceptor Physiology in Rats Chronically Exposed to Combinations of DEET, Permethrin, PB and Chlorpyrifos.

We examined whether the, DEET augmented, 4 week exposure protocol altered nociceptor physiology. Experiments were conducted on Group A, Group PB and Group C rats. Twelve weeks after exposures ended, DRGs were excised from rats for whole cell patch clamp studies. Recordings were obtained from vascular nociceptor (type 8) K_v7 ion channels using solutions and protocols described in 'Methods'.

As shown in figure 8, the conductance of nociceptor K_v7 ion channels harvested from Group A and Group PB rats exhibited decreased activity relative to vehicle exposed controls. Although distinct exposure protocols led to distinct behavioral outcomes (figures 1 and 4), there were no differences in K_v7 activity in neurons harvested from Group A versus Group PB (figure 8A and 8B).

Discussion

We previously identified a protocol in which an 8 week, but not a 4 week, exposure to 3 GWI agents (chlorpyrifos, permethrin, PB) produced a pattern of behavior that could be interpreted as a delayed chronic pain syndrome that resembled a subset of symptoms that afflicted veterans following their return from the 1991 Persian Gulf War (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al., 2015). A key factor leading to the development of those pain-like behaviors was a doubling of the anticholinesterase duty cycle from 7% (twice per month) and 50% (15 times per month) to 14% and 100% (chlorpyrifos, PB; respectively). Given that DEET usage has been statistically associated with the development of the GWI pain syndrome (syndrome 3; Haley and Kurt, 1997), we examined whether the addition of this repellent to our exposure protocol would promote the appearance of pain behaviors in our rat model. Our studies have now shown that DEET hastened the development and extended the persistence of pain-like behaviors that appeared following a 4 week exposure to chlorpyrifos, permethrin and PB. Relative to either vehicle or positive control groups, rat ambulatory

behaviors were suppressed as early as 5-12 weeks post-exposure and remained suppressed out to the 17-24 week test periods. These behavior shifts were accompanied by a decreased conductance in vascular nociceptor K_v7 . Despite the powerful influence of DEET, the development and persistence of pain signs were shown to be mainly dependent, albeit in a complex fashion, on the anticholinesterase components of the exposure protocol.

Experiments in which chlorpyrifos or PB were eliminated from the exposure set gave clear indications of the importance of these anticholinesterases for the development and maintenance of pain behaviors. In the absence of CP, ambulation deficits were substantially reduced or prevented. Although the removal of PB had much less impact on behavior patterns occurring during the exposure, the extent of reversals of post-exposure ambulation measures were even more complete in the absence of PB.

DEET Potentiates the Development of a Chronic Pain Condition

Despite the critical contribution of the anticholinesterases to the development of pain signs in our model, the fact that any ambulatory deficits appeared, following a 4 week exposure, seemed to be attributable to the inclusion of DEET in the exposure set. When the concentration level of DEET was halved from 50 to 25%, there was no indication that DEET potentiated or altered the pattern of pain signs. This finding is consistent with the observation that veterans that applied a 75%, but not a 25% concentration of a commercial DEET preparation were more likely to develop GW pain symptoms (Haley and Kurt, 1997). The means by which DEET amplified the impact of GW chemicals remains uncertain, but it is unlikely to be due to its weak anticholinesterase activity (Corbel et al., 2009; Wille et al., 2011; Swale et al., 2014).

We had reported previously that exposure to chlorpyrifos, permethrin and PB, sufficient to produce ambulatory and resting pain signs, was accompanied by diminished conductance through muscle, but not vascular, nociceptor, K_v7 , ion channels (Nutter et al., 2015). As a voltage dependent brake on membrane potential, a reduction in K_v7 activity increases neuronal excitability (Brown and Passmore, 2009). Moreover, we have recently demonstrated muscarinic agonist induced, K_v7 modulated, action potential bursts in muscle nociceptors of GW agent exposed rats (Nutter et al., 2015; Cooper et al., 2016). In the DEET augmented protocol, we again report decreased conductance of K_v7 channels, but they are now manifested in vascular nociceptors. Potentially, an expanded impact on vascular nociceptor populations, in the presence of DEET, altered the development and pattern of behavioral outcomes. The removal of PB from the exposure protocol also shifted the pattern of behavioral signs, but there was no indication that this was reflected, in any way, by K_v7 activity. The decline in K_v7 activity was not influenced by the presence or absence of PB.

While DEET clearly potentiated behavioral maladaptations, and may have potentiated shifts in vascular nociceptor physiology, we could not demonstrate any acute influences of DEET on K_v7 activity. Nor did we observe acute influences, by DEET, on other ion channels that have been implicated in GWI agent-induced maladaptations (i.e., $Na_v1.8$, $Na_v1.9$ and K_{DR} ; Nutter and Cooper, 2014; Nutter et al., 2015; Cooper et al., 2016). The absence of effects of DEET on known and suspected GW agent maladapted ion channel proteins does not preclude a direct molecular level interaction of DEET with deep tissue nociceptors through other pathways; nor does it rule out an indirect interaction through secondary pathways yet to be identified.

Chlorpyrifos is an AChE inhibitor, but one of its hepatic metabolites, chlorpyrifos-oxon is 1,000 fold more potent in that role (Huff and Corcoran, 1994). In a direct assessment of the

hepatic interaction of these to GW agents, Usmani and colleagues reported that DEET increased the hepatic conversion of chlorpyrifos to its oxon form by a factor of 2.4 (Usmani et al., 2002). Potentially the amplification of pain signs, by DEET, occurs through its capacity to increase the peak levels of the more potent oxon form of CP (Abou-Donia et al., 1996; Abu-Qare and Abu-Donia, 2008). Moreover, it has been shown that co-administration of PB with DEET, slows hepatic DEET metabolism, and as a result could further enhance the DEET potentiated conversion of chlorpyrifos to chlorpyrifos-oxon (Abu-Qare and Abu-Donia, 2008; see also Chaney et al., 2000). Therefore, the amplification of pain signs by addition of DEET, in an otherwise ineffective protocol, could simply be due to an increase in the effective peak concentration of chlorpyrifos-oxon. Just as increasing the duty cycle of the anticholinesterases potentiated pain signs with an 8 week exposure (Nutter et al., 2015), increasing the functional concentration of chlorpyrifos-oxon, during a 4 week exposure, could have potentiated and prolonged ambulatory deficits.

It is well known that single high dose exposures to certain organophosphate anticholinesterases have been linked to the development of a progressive motor and sensory neurodegenerative disorder known as OPIDN/OPIDP (organophosphate induced delayed neuropathy/polyneuropathy; Johnson, 1975; Vale and Lotti, 2015). One to three weeks after a single exposure, subjects develop a variety of symptoms that include progressive ataxia, paresthesias, and muscle pain. These are accompanied by a peripheral axonopathy characterized by slowed action potential conduction and evidence of Wallerian degeneration (Lotti and Moretto, 2005). Most animal studies have been conducted on 'susceptible' species (chickens and mice). Severe inhibition of NTE (neuropathy target esterase) and induction of intracellular phosphorylation pathways are key events leading to the development of the axonopathy

associated with OPIDN (Abou-Donia and Lapadula, 1990; Pope et al., 2005; Choudhary et al., 2006; Emerick et al., 2012). However, rats are considered to be highly resistant to OPIDN (but see Padilla and Veronesi, 1988; Moretto et al., 1992; Choudhary et al., 2001), and there is little evidence that chickens, mice or rats, develop classic signs of OPIDN following single or multiple exposures to either chlorpyrifos or PB (Richardson, 1995; Lotti, 2002a; Wilson et al., 2002; Kropp and Richardson, 2003; Wang et al., 2014). Although sequential exposure to some organophosphates (including chlorpyrifos) with certain ‘non-neuropathic’ NTE inhibitors potentiates organophosphate inhibition of NTE and ultimately leads to the development of OPIDN (Pope and Padilla, 1990; Pope et al., 1993; Lotti et al., 1991; Lotti, 2002b), we cannot point to any specific evidence linking PB, permethrin or DEET to promotion of a chlorpyrifos dependent OPIDN.

Complex Influences of Chlorpyrifos and PB on Pain Signs

The evidence that linked the anticholinesterases to the development of ambulation deficits was relatively straightforward. The contributions of CP and PB were internally consistent on these measures: exclusion of either CP or PB blocked the development of ambulation pain-signs and significantly shifted ambulation scores that accompanied 4 agent exposures, toward normal levels. The exclusion of PB was more definitive in this regard, as its presence was required for the development of both movement distance and rate impairments (weeks 5-12). However, both the anticholinesterases were required for the persistence of ambulation deficits out to 20 weeks post-exposure. Unexpectedly, we observed that the development of rest duration pain signs diverged from those of ambulation. Resting increased

during the post-exposure phase *only* in the absence of PB. Moreover, resting pain signs were further increased relative to groups receiving all four chemicals. The divergent influence of the two anticholinesterases on rat activity measures must be considered, not only with respect to their interactive anticholinesterase activity, but also in the broader context of their non-cholinesterase interactions with the nervous system.

PB, a reversible inhibitor of AChE, was approved for use in ODS due to its capacity to act as a prophylactic, and when combined with antidotes (2-PAM; atropine) to reduce the lethality of highly potent and irreversible anticholinesterase nerve agents such as Soman (Maxwell et al., 1988; von Bredow et al., 1991; Adle et al., 1992; Koplovitz and Stewart, 1994; Kassa and Fusek, 1998; Weinbroum, 2004; Newmark, 2005; Weissman and Raveh, 2011). Chlorpyrifos-oxon is an irreversible anticholinesterase whose anticholinesterase activity is significantly reduced by pre-treatment with PB (Henderson et al., 2012). When PB was left out of our dosing protocol, the loss of this prophylactic action might be manifested in the development of resting pain signs. In partial support of this, we have shown that increasing the duration of exposure to CP to 8 weeks, also increases resting scores in weeks 9-12 (Nutter et al., 2015; Cooper et al., 2016).

While PB has a demonstrated capacity to oppose the anticholinesterase effects of chlorpyrifos-oxon (Henderson et al., 2012), it has no capacity to oppose or prevent the extra-anticholinesterase effects of chlorpyrifos-oxon. The latter are considerable and well documented. Independent of any inhibition of AChE, chlorpyrifos-oxon, as well as some other organophosphates used in the Gulf War (i.e., malathion/maloxon) can directly bind to, activate, and cause internalization of muscarinic receptors (Ward et al., 1993; Huff et al., 1994; Ward and Mundy, 1996; Bomser and Casida, 2001; Olivier et al., 2001; Howard and Pope, 2002; Liu et al.,

2002; Zou et al., 2006; Mirajkar and Pope, 2008; Udarbe et al., 2008; see also Smulders et al., 2004). Chlorpyrifos-oxon also modulates the activity of a variety of G-protein coupled protein kinases and receptor protein kinases (Huff et al., 1995; Huff et al., 2001; Bomser and Casida, 2000; Bomser et al., 2002; Zhang et al., 2002; Torres-Altoro et al., 2011; Suriyo et al., 2015). Repeated exposure to chlorpyrifos ultimately alters the expression of muscarinic receptors in the CNS (Nostrandt et al., 1997; Liu et al., 1999; Huff et al., 2001; Abou-Donia et al., 2003; Zhang et al., 2002; Padilla et al., 2005; Pung et al., 2006; Proskocil et al., 2010) and modifies the functional consequences of mAChR activation in PNS nociceptors (Nutter and Cooper, 2015; Cooper et al., 2016).

How these multiple cholinesterase and non-cholinesterase actions contribute to the development of specific pain signs cannot be readily resolved in this report. Our examination of vascular nociceptor K_v7 did not reveal any differential influences attributable to the presence or absence of PB in the exposure protocol (see above). Soldiers suffering from GWI pain describe highly diverse symptom patterns (i.e., muscle pain, back pain, joint pain, abdominal pain, headache) that suggest pathology in multiple, functionally distinct, nociceptor pools that innervate these diverse tissues (Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). In the absence of PB, additional nociceptive pools, under the full influence of both the anticholinesterase and extra-cholinesterase actions of CP, could suffer significant maladaptations that are manifested as distinct forms of pain.

Summary and Conclusions

DEET substantially potentiated and prolonged the pain signs that developed after a 4 week exposure to GW agents. The pattern of pain signs associated with a DEET augmented exposure set diverged from those observed after an 8 week exposure to the same GW agents in the absence of DEET (Nutter et al., 2015; Cooper et al., 2016). While we were unable to identify a specific linkage between nociceptor ion channel physiology and acute exposure to DEET, such interactions may yet be found. At present, it is more likely that DEET indirectly amplifies the physiological impact of chlorpyrifos on a variety of molecular targets.

Soldiers deployed to the Persian Gulf were potentially exposed to a large variety of insecticides, repellants, nerve agents, adjuvants, depleted uranium, and other toxins (Binns et al., 2008; RAC, 2014). A high percentage developed symptoms while still in theater (~25%; Kroenke et al., 1998), but most developed a wide variety of pain symptoms that were delayed in onset and worsened over time (Hotopf et al., 2003). The variations of the symptoms, as well as the timing of their onset, could represent different exposure patterns (and degrees of exposures) and how they ultimately interacted with the genetic makeup of each individual. Acknowledging that, it is likely that there were common risk factors that set into motion a definable set of maladaptations that resulted in the symptoms of GWI. Most of our research points to the fundamental role of the anticholinesterases, CP and PB, as primary risk factors for pain. Although it is not clear that it was specifically their anticholinesterase activity that posed the greatest risk, the manifestations of pain-like behaviors appeared after doubling the exposure duty cycle of PB and CP (Nutter et al., 2015; Cooper et al., 2016). Adding DEET to the exposure set accelerated the development, altered the pattern and prolonged the persistence of pain-like behaviors that were ultimately dependent upon the presence of chlorpyrifos and/or pyridostigmine bromide. The impact of DEET augmentation on vascular nociceptor K_v7

physiology might account for some of the resultant behavioral signs, but could not account for the differential influences of PB on these signs.

PB was prescribed to soldiers to protect them from nerve agents such as Soman or Tabun (Gordon et al., 1978; Gall, 1981; Ray et al., 1991; Adler, et al., 1992; Kassa and Vachek, 2002; Kassa and Krejeova, 2003; Maselli et al., 2011; but see Shiloff and Clement, 1986). The full benefit of PB pre-treatment required timely administration of antidotes, such as 2-PAM and atropine (Maxwell, et al., 1988; von Bredow et al., 1991; Adler, et al., 1992; Koplovitz and Stewart, 1994; Kassa and Fusek, 1998; Kassa and Vachek, 2002; Layish et al., 2005). Ironically, Soman was never encountered in the Persian Gulf; and while Sarin nerve agent was encountered, PB had not been shown to be a useful prophylactic against Sarin (Koplovitz et al., 1992; Worek and Szinicz, 1995; Wilson et al., 2002; but see Tuovinen et al., 1999). As this could not be known beforehand, measures were taken that were believed to offer the best margins of safety for the warfighters. Probably half of the soldiers deployed to the Persian Gulf self-administered PB, without antidote, for several weeks. The antidotes were not to be taken unless there was an indication that a nerve gas attack was imminent or in progress (Binns et al., 2008). Accordingly, soldiers took PB routinely in anticipation of attacks that rarely, if ever, materialized and for which its prophylactic action was documented to be of little use. As a result, they may have been self-administering an agent that accentuated the toxic effects of insecticides and repellants through an hepatic overload (Abou-Donia et al., 1996). Nevertheless, as the present data indicates, routine administration of PB did afford a degree of protection against the physiological impact of some of the anticholinesterase insecticides to which the soldiers were overexposed, and whose toxicity was amplified by what was thought to be a harmless repellant (DEET). Yet, PB could not protect them from, and may have actually amplified the actions of, the oxon

765 metabolites of the organophosphates that asserted their deleterious actions through pathways that
766 were independent of anticholinesterase activity but had the capacity to derange important
767 components of the nervous system.

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Figure Captions

Figure 1. A DEET Augmented Exposure Protocol Produced Long Lasting Pain-Like Behaviors. **A)** Movement distance was significantly decreased at 5-12 weeks post exposure ($F=19.47$; $p<.001$). Movement distance pain signs approached significance 17-20 weeks post exposure ($F=3.72$; $p<.06$), but faded in the final month to testing (weeks 21-24). **B)** Average movement rate was significantly decreased 5-12 weeks post exposure ($F=22.71$; $p<.001$). Significant rate decreases were maintained out to weeks 21-24 ($F=4.00$; $p<.05$). **C)** Resting duration was unchanged during all post exposure test periods. Tests were not conducted on any measure 1-4 weeks post exposure. B: baseline testing; A: DEET, chlorpyrifos, PB, permethrin. C/Vehicle: (ethanol, corn oil, ethanol, water). *significantly different by ANOVA.

Figure 2. Pain-Like Behaviors Did Not Develop Nor Persist When DEET Concentration was Halved. **A)** Relative to control animals, movement distance was paradoxically increased in during post-exposure weeks 17-20 ($F=5.50$). **B)** Average movement rate was unchanged compared to vehicle exposed controls. **C)** Resting duration was paradoxically decreased relative to control rats in Group HD (weeks 17-20; $F=19.51$). There were no indications of pain signs during the early post-exposure period in any measure (weeks 5-12). **D and E)** When compared

to Group A (50% DEET), the reduction of DEET concentration to 25% produced movement distance and rate scores that were significantly higher (more normal) during the last observation period (post-exposure weeks 17-20; $F=12.4$, $p<.001$ and $F=17.2$, $p<.001$, distance and rate respectively). Pain behaviors appeared during the early testing phase in Group A ($F=16.7$ and $F=16.1$; $p<.001$, movement distance and rate, respectively). **F)** At week 17-20, resting was significantly reduced in the 25% DEET concentration group ($F=11.24$; $p<.002$), but there were no accompanying shifts during the early post-exposure phase (weeks 5-12). B: baseline testing; A: DEET (400 mg/kg; 50%), PB, chlorpyrifos, permethrin; C/Vehicle: (ethanol, corn oil, ethanol, water); HD: DEET (200 mg/kg; 25%), chlorpyrifos, PB, permethrin; *significantly different by ANOVA.

Figure 3. Excluding the AChE Inhibitor, Chlorpyrifos, from the Exposure Protocol Prevented Development of Persistent Pain Behaviors. **A)** The omission of CP prevented the suppression of movement (distance) by GW chemicals. **B)** Average movement rate was still significantly reduced in the early post-exposure phase in the absence of CP (weeks 5-12; $F=6.11$). Persistent changes in movement rate did not develop in the absence of CP (post-weeks 17-20). **C)** Rest durations remained unaffected when CP was absent. **D and E)** In the absence of chlorpyrifos, movement rate scores were shifted significantly towards vehicle exposure levels relative to groups that were exposed to all 4 agents. Significant rescue was observed over post-exposure weeks 5-12 (rate: $F=4.37$) and 17-20 ($F=4.67$ and $F=7.84$, movement distance and rate respectively). **F)** The exclusion of CP shifted resting scores toward vehicle levels only during the period of exposure ($F=42.11$). No other shifts were observed relative to Group A rats. B:

baseline testing; A: DEET, chlorpyrifos, PB, permethrin; C: (ethanol, corn oil, ethanol, water);
CP: DEET, PB, permethrin. *significantly different by ANOVA.

Figure 4. Excluding Pyridostigmine Bromide from the Exposure Protocol Differentially Contributed to the Development and Persistence of Pain Behaviors. A) Movement distance was unaffected in the absence of PB. B) Except for a paradoxical increase in weeks 17-20 ($F=5.05$), the average movement rate was also unaffected by GWI chemicals when PB was excluded from the exposure set. C) In the absence of PB, significant increases in rest duration scores emerged during the early post-exposure phase (weeks 5-12; $F=11.60$) and persisted into the final month of measurement (post-weeks 17-20; $F=7.27$). D and E) In the absence of PB, final movement distance and rate scores were shifted significantly towards vehicle exposure levels relative to groups that were exposed to all 4 agents (weeks 17-20: $F=6.04$ and $F=17.34$, movement distance and rate respectively). Movement distance and rate scores were also rescued during the early post-exposure phase (weeks 5-12: $F=4.00$ and $F=23.69$, respectively). F) The exclusion of PB accentuated the influence of the remaining 3 GW chemical on rest durations during both post-exposure assessment periods (weeks 5-12: $F=18.49$; weeks 17-20; $F=6.19$). B: baseline testing; A: DEET, chlorpyrifos, PB, permethrin; C: (ethanol, corn oil, ethanol, water); PB: DEET, chlorpyrifos, permethrin. *significantly different by ANOVA.

Figure 5. Voltage Activated K^+ Channels were Unaffected by Acute Exposure to DEET. A) The average conductance of muscle nociceptor K_v7 channels was not altered by DEET (10-50 μ M); B) and C) Following a 12 minute exposure, there was no indication that either the voltage

dependence or the average K_{DR} currents were modified by DEET (10-50 μ M). Insert B: a representative family of K_{DR} current traces (-80 to 30 mV). The voltage activation curves shown were formed from the mean tail currents of all cells averaged at a given voltage. Statistical tests were performed on $V_{.50}$'s computed from individual curve fits. For K_v7 , the average currents were determined as the mean linopirdine sensitive current from -40 to -70 mV. For K_{DR} , the average currents were determined as the mean tail current from -60 to 0 mV. Data was collected from 33 rats.

Figure 6. Time Dependent Modification of $Na_v1.8$ by DEET. A) The amplitude of muscle nociceptor $Na_v1.8$ was not changed by DEET (100 μ M). A representative $Na_v1.8$ current is inserted. B) Vascular nociceptor $Na_v1.8$ amplitude was not altered by DEET (100 μ M). Baseline records were taken prior to DEET or ETOH exposure. The average of the last three pre-tests was used as a baseline score. DEET was pre-applied for 2 minutes prior to 7 minutes of continuous post-test recording (15 sec test intervals). This data was collected from 19 rats.

Figure 7. Voltage Activated, $Na_v1.9$, Channels were Weakly Modulated by Acute Exposure to DEET. A) The voltage dependent activation of muscle nociceptors was hyperpolarized at 50 but not at 100 μ M DEET. A representative trace of a $Na_v1.9$ current (step to -50 mV; V_H =-120 mV) is included as an insert. B) The voltage dependent activation of vascular nociceptors was unaffected by DEET. C) Average currents of muscle nociceptors were unaffected by DEET ($p<.13$, Vehicle vs 100 μ M; $p<.05$, 50 vs 100 μ M); D) Vascular nociceptor average currents were unchanged ($p<.17$, Vehicle vs 100 μ M; $p<.08$, 50 vs 100 μ M). The voltage-activation

curves were formed from the mean conductances of all cells averaged at a given voltage. Statistical tests were performed on V_{50} 's computed from individual curve fits. Average currents were determined as the mean, cell size normalized, current over the activation range (-65 to -40 mV). **significantly different from vehicle treated cases; + significantly different from 50 μ M tests. Thirty-two rats contributed to these graphs.

Figure 8. The Conductance of K_v7 Ion Channels Was Reduced in Vascular Nociceptors of Exposed Rats. **A)** The average conductance (-30 to -70 mV) was significantly reduced in both Group A and Group PB nociceptors. **B)** The peak conductance was significantly reduced only in cells harvested from rats that were not exposed to PB during the 4 week period. GRP A: PB, permethrin, chlorpyrifos and DEET; GRP PB: permethrin, chlorpyrifos and DEET VEH: water, ETOH, corn oil, ETOH. The insert in panel 'A' is a schematic representation of the voltage protocol test sequence. Cells were depolarized to -20 mV for 1000 msec and repolarized (-30 to -90 mv) for 1000 msec. The linopirdine sensitive current was determined by subtraction. Eighteen rats contributed to this experiment. ** significantly different from vehicle exposed cases.

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Figure 2

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Figure 4

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Figure 8

Table 1

Group	Permethrin	Chlorpyrifos	PB	DEET	Body Weight
A	2.6*	120	13	400	489 ± 7.0 [#]
HD	2.6	120	13	200	486 ± 5.2
CP	2.6	0	13	400	476 ± 6.07
PB	2.6	120	0	400	514 ± 11.0
C	0	0	0	0	489 ± 8.0

*all doses in mg/kg # final weight in grams