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14. ABSTRACT

Soldiers returning from the 1991 Persian Gulf War suffered from a variety of cognitive, motor, sensory and autonomic symptoms. Warfighters that applied highly concentrated DEET creams were more likely to exhibit more sensory and autonomic symptoms (Haley and Kurt, 1997). We previously developed a rat model of GWI in which multiple pain-like symptoms emerged. Following an 8 week exposure to permethrin, chlorpyrifos and pyridostigmine bromide (PB), rats exhibited decreased ambulation and increased resting that persisted out to 12 weeks after exposures ceased (Nutter et al., 2015). We have now demonstrated that the addition of DEET to a 4 week exposure protocol of permethrin, chlorpyrifos and pyridostigmine bromide altered the pattern and increased the persistence of pain signs that lasted up to 24 weeks post-exposure. Moreover, measureable autonomic dysfunctions were now present up to 8 weeks after the exposure. Studies in which either pyridostigmine bromide or chlorpyrifos were excluded from the full exposure protocol demonstrated that these anticholinesterases were essential to the development of both pain and autonomic dysfunctions. It appeared that DEET accentuated the influence of the anticholinesterases, PB and chlorpyrifos. Electrophysiological experiments revealed that sensory and autonomic maladaptations were accompanied by increased conductance of $Na_v1.9$ and decreased activity of K_v7 ion channels in vascular nociceptors. Such effects will alter excitability in these nociceptors and could affect their influence on sensory and autonomic events.

15. SUBJECT TERMS

Pain, pesticides, pyridostigmine bromide, ion channels, DEET, chlorpyrifos, Gulf War Illness

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1. Introduction

Pain and autonomic dysfunction are common symptoms associated with Gulf War Illness. Thousands of soldiers returning from the Persian Gulf War developed unusual complexes of headache, joint, muscle and abdominal pains (GWI; Haley Syndrome 3; Haley and Kurt, 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). Chronic deep tissue pain was often accompanied by dizziness, night sweats, diarrhea and a variety of other signs of autonomic dysfunction that were also manifested with the cognitive and motor symptom complexes of GWI (Haley Syndromes 1 and 2; Haley and Kurt, 1997; Haley et al., 2013). To investigate the pathophysiology of this disorder, our laboratory developed a rat model of GWI pain. Following a series of studies that utilized a variety of exposure protocols, we recently demonstrated that pain-like behaviors emerged 12 weeks after following an 8 week exposure to a combination of permethrin, chlorpyrifos and pyridostigmine bromide. These behavioral signs were associated with decreased activity of nociceptor K_V7 and maladapted reactivity to muscarinic acetylcholine receptors (mAChR; Nutter et al., 2015; Cooper et al., 2016). In the project covered by this report, we examined the contribution of DEET to the development and persistence of post-exposure pain behaviors. A special emphasis was placed on the evaluation of autonomic dysfunction that can accompany pain symptoms in veterans with GWI.

2. **Keywords:** pain, autonomic, nociceptor, blood flow, pesticides, pyridostigmine bromide, DEET, Gulf War Illness

3. Accomplishments

Exposure to Sarin gas, depleted uranium, vaccination adjuvants and organophosphate insecticides are a few of the many risk factor that may have contributed to GWI. However, no single factor can explain the wide ranging symptoms of this complex disease. The Research Advisory Committee on Gulf War Illness concluded that pesticides may have contributed to the development of the symptoms of GWI (Binns et al., 2008; RAC, 2014). During the brief course of the Gulf War, veterans were potentially exposed to 64 insecticides and repellants containing 37 distinct active ingredients (DoD Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008). Organophosphate, organochlorine, dialkylamide, carbamate and pyrethroid pesticides and repellants were used liberally in the Gulf theater. Soldiers were exposed to these agents while simultaneously self-administering a nerve gas prophylactic, pyridostigmine bromide (PB). PB shares the anticholinesterase properties of many insecticidal chemicals. The insect repellant DEET (N,N-Diethyl-meta-toluamide) was widely used in the Persian Gulf to supplement the numerous insecticides that proved to be inadequate to their purpose. Exposure to DEET and pyridostigmine bromide was subsequently shown to be strongly associated with the development of pain symptoms in GW veterans (Haley and Kurt, 1997). In a preliminary study, we observed a substantial acceleration of pain signs when a DEET augmented protocol was used.

In the report below, we examined how the addition of DEET to the exposure protocol, modified the pattern and persistence of pain behaviors in rats exposed to chlorpyrifos, pyridostigmine and permethrin. We also examined whether the development of pain signs was accompanied by changes to autonomic function, and the specific role played by anticholinesterases in the pattern

of pain-like behaviors. Our SOW is presented in the Appendix (page 52). The objectives are outlined below:

Objectives:

1) Document the contribution of DEET to an animal model of GW chronic pain.
2) Identify the influence of DEET on molecular targets associated with GWI pain.
3) Determine how pyridostigmine bromide, chlorpyrifos and permethrin differentially contribute to the development and persistence of pain signs
4) Examine whether autonomic nervous system function is modified by exposure to GWI chemicals
5) Characterize molecular changes that occur in nociceptors following exposure to a DEET augmented exposure protocol
6) Characterize alterations of vascular nociceptor function, in vivo, in GW chemical exposed rats.
7) Determine whether contemporary and experimental drugs can reverse pain behaviors of rats in our model of GWI pain.

Specific Aim 1. Reversing Signs of GWI Pain Behaviors Maintained by Vascular Nociceptors

This aim was designed to define the role played by various components of the exposure protocol in the persistence and/or development of behavioral and autonomic signs of dysfunction. Once those aspects were defined, outcome linked treatments would be explored.

TASK 1.1: Optimize the Chemical Exposure Protocol

Timeline: Months 1-5

We determined the contributions of DEET, chlorpyrifos, permethrin and pyridostigmine bromide to the development and persistence of pain behaviors. A brief presentation of this data is found below. A complete presentation appears in the attached manuscript (Appendix, p. 79).

Rats were divided into 5 groups (n=50; see Table 1). One group 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 second group (HD) received all 4 agents, but DEET was administered at half the concentration (200 mg/kg; 25% in ETOH). The remaining 3 groups received combinations of 3 agents where either chlorpyrifos (Group CP), permethrin (P) or pyridostigmine bromide (Group PB) was not included in the dosing routine.

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

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 open field activity measures of ambulation (movement distance (cm/15 min); average movement rate (cm/sec)) and resting (sec/15 min). All

PAM measures were carried out under blinded test conditions. Activity measures were assessed over a period of 15 minutes, in a covered 35 x 40 cm Perspex test chamber where movements were monitored and quantified by an automated infrared detection system.

Animals were exposed to GW chemicals for 4 weeks and followed for up to 24 weeks post exposure. Data was contrasted with a 4 week exposure to the same three agents in the absence of DEET. We observed that the DEET augmented protocol accelerated the development and lengthened the persistence of pain behaviors (figure 1).

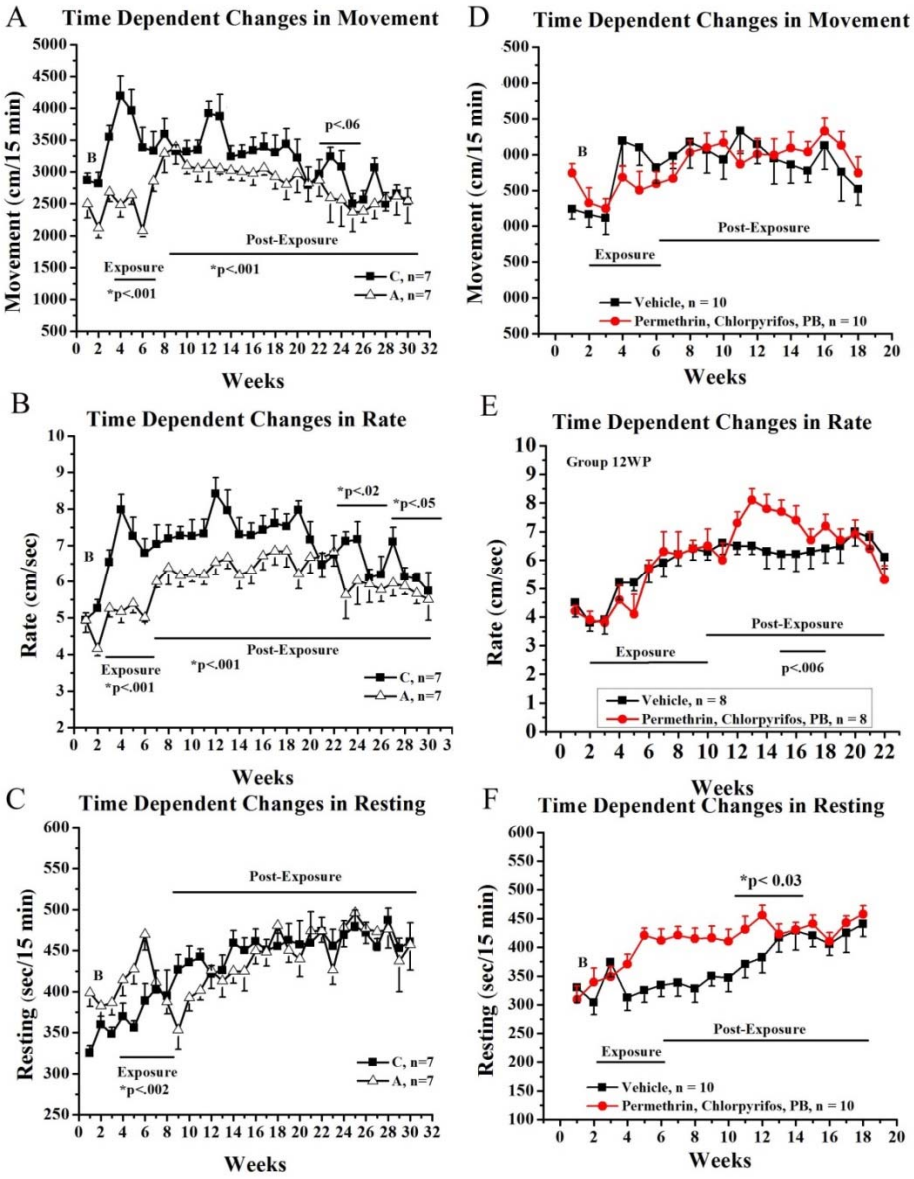


Figure 1. The Inclusion of DEET in the Exposure Protocol Produced Long Lasting Pain-Like Behaviors. A) Movement distance was significantly decreased at 5-12 weeks post exposure ($F=19.47$; $p<.001$). Movement distance pain signs approached significance 17-20 weeks post exposure ($F=3.72$; $p<.06$), but 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. **D and E)** In the absence of DEET, a 4 week exposure to chlorpyrifos, permethrin and PB did not produce any lasting pain-like behaviors. Movement distance was unchanged while average movement rate was paradoxically increased at post 9-12 weeks ($F= 7.23$; $p<.009$). **F)** Resting times were transiently increased during the post-exposure period (5-8 weeks; $F=4.70$; $p<.03$), but these shifts did not persist as far as post exposure weeks 9-12. 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). Panels D, E and F were reprinted from Nutter et al., 2015, and are presented as they were originally analyzed.

Reducing the concentration of DEET by half (200 mg/kg; 25%) prevented the development of pain signs (figure 2). Some paradoxical changes were observed (i.e., increases in movement rather than decreases; or decreases in resting rather than increases). We concluded that the full concentration of DEET (400 mg/kg; 50%) was required to accelerate development and persistence of pain signs.

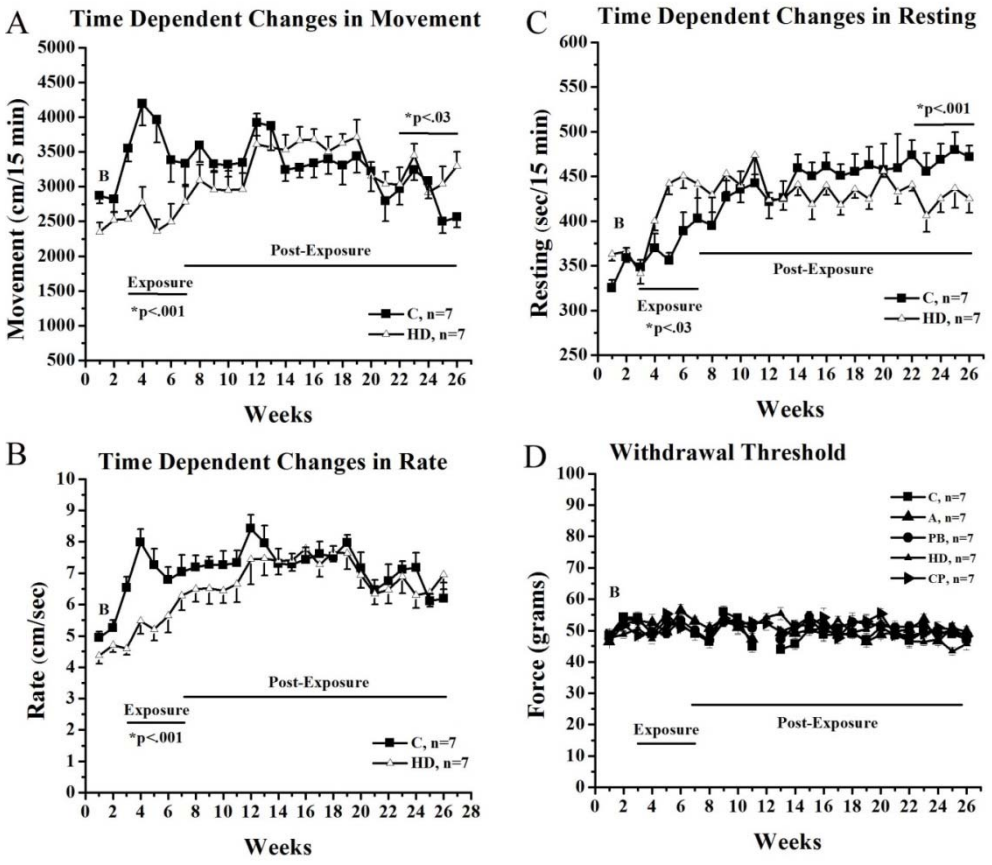


Figure 2. Pain-Like Behaviors Did Not Appear When DEET Concentration was Halved. A) Movement distance was paradoxically increased in during weeks 17-20 (F=5.50). **B)** Average movement rate was unchanged. **C)** Resting duration was paradoxically decreased (weeks 17-20; F=19.51). **D)** PAM withdrawal threshold tests were unchanged in all conditions of the experiment. B: baseline testing; A: DEET (400 mg/kg; 50%), PB, chlorpyrifos, permethrin; C/Vehicle: (ethanol, corn oil, ethanol, water); CP: DEET, PB, permethrin; HD: DEET (200 mg/kg; 25%), chlorpyrifos, PB, permethrin; PB: DEET (400 mg/kg), chlorpyrifos, permethrin.

The Contribution of Chlorpyrifos to the Development and Persistence of Pain Signs

Exclusion of chlorpyrifos (CP) from the exposure protocol blocked persistence of pain signs and weakened their development (figure 3A and B). In the absence of CP, movement and rate deficits were significantly shifted toward normal levels (figure 3D and E). We concluded that despite the capacity of DEET to accelerate the development and lengthen the persistence of pain behaviors, these pain-signs were ultimately dependent on exposure to anticholinesterases.

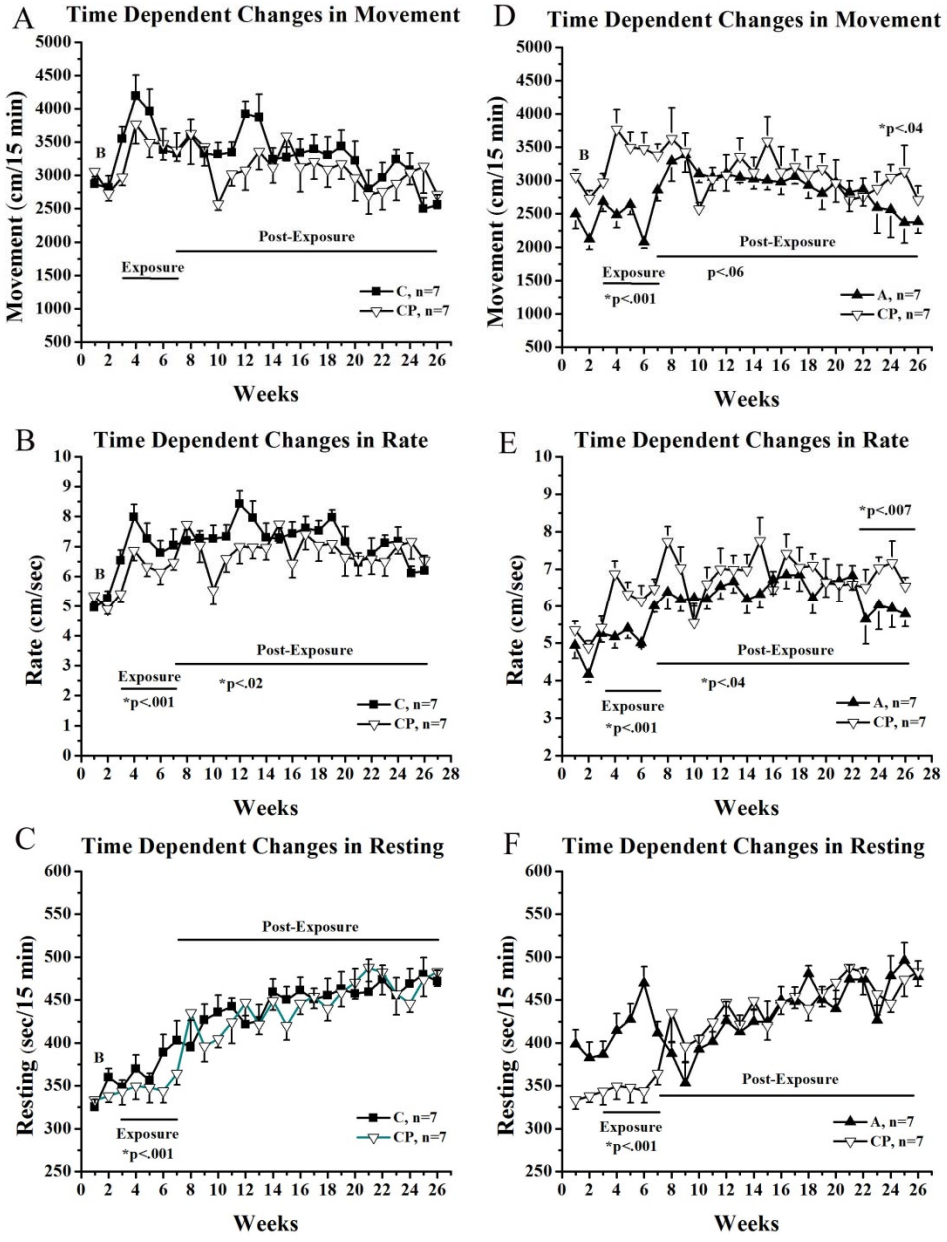


Figure 3. Excluding the AChE Inhibitor, Chlorpyrifos, from the Exposure Protocol Prevented Development of Persistent Pain Behaviors. A) The omission of CP prevented the suppression of movement (distance) by GW chemicals. **B)** Average movement rate was still significantly reduced in the early post-exposure 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 (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.

The Contribution of Pyridostigmine to the Development and Persistence of Pain Signs

When the other anticholinesterase, pyridostigmine bromide (PB), was excluded from the protocol, rats failed to develop any ambulation deficits (figure 4A and B); moreover, ambulation deficits were significantly normalized relative to rats that received all 4 GW agents (figure 4C and D). In addition, the exclusion of PB was shown to be permissive for the emergence of resting deficits. Absent PB, resting deficits appeared in addition to ambulation deficits (figure 4C). We concluded that PB was required for the development and persistence of ambulation deficits, but served a protective role with respect to resting deficits. A thorough consideration of the interpretation and implications of this data are presented below and in the attached manuscript (Discussion; Appendix, p. 112)

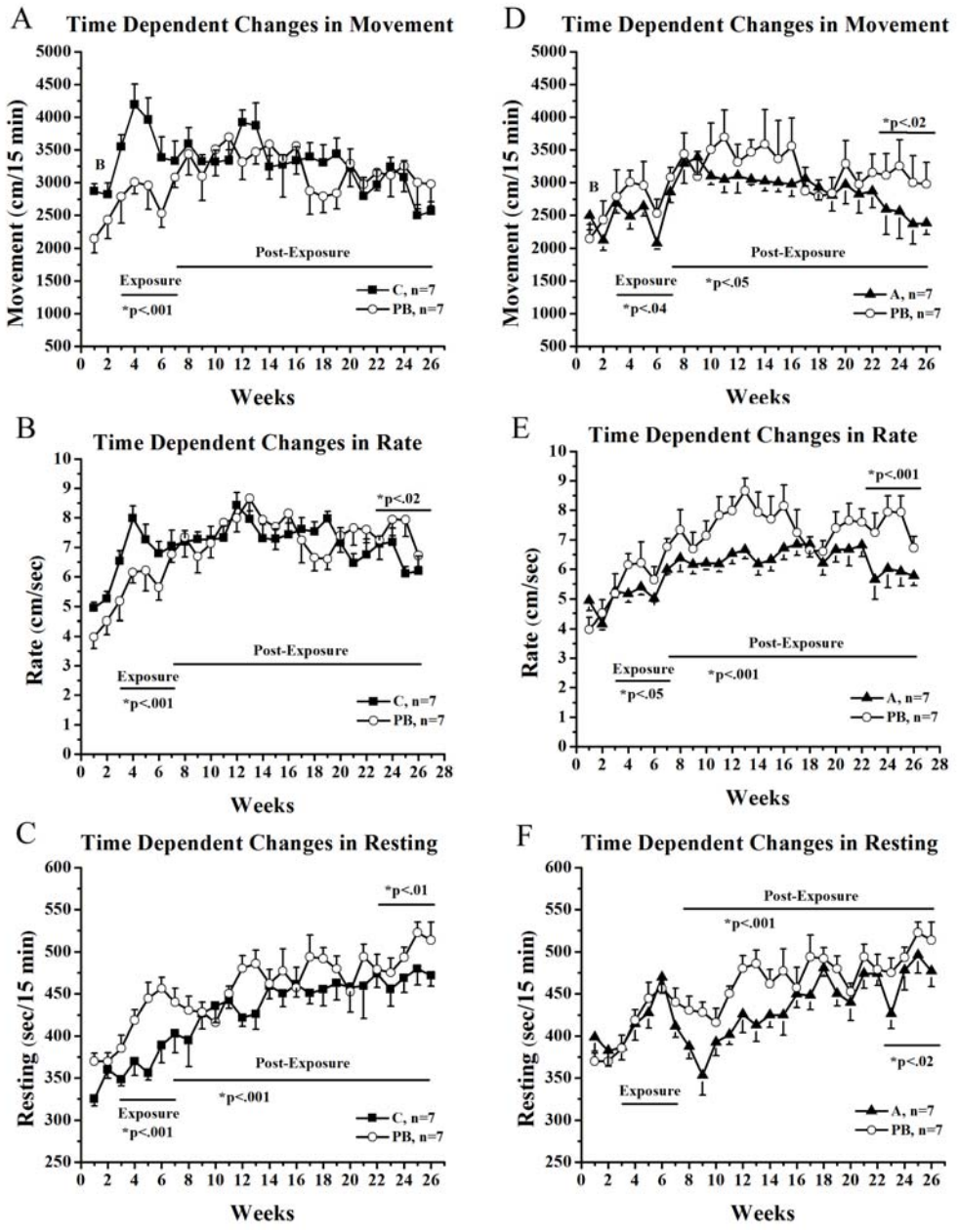


Figure 4. Excluding Pyridostigmine Bromide from the Exposure Protocol Differentially Contributed to the Development and Persistence of Pain Behaviors. A) Movement distance was unaffected in the absence of PB. **B)** Except for a paradoxical increase in weeks 17-20 (F=5.05), the average movement rate 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 (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.

The Contribution of Permethrin to the Development and Persistence of Pain Signs

When permethrin was excluded from the exposure protocol there was little evidence that it contributed to the development of ambulation or rest deficits (weeks 5-12; figure 5A, B and C). Importantly, there was consistent evidence that permethrin contributed to the persistence and/or emergence of ambulation deficits at weeks 17-20 (figure 5A and B). All late phase ambulation deficits failed to appear in the absence of permethrin; while both the development of, and late phase rate scores were shifted toward normal levels relative to groups receiving all 4 agents (figure 5E). Permethrin also significantly reduced the influence of GW agents on resting, but only during the early phase (weeks 5-12; figure 5F).

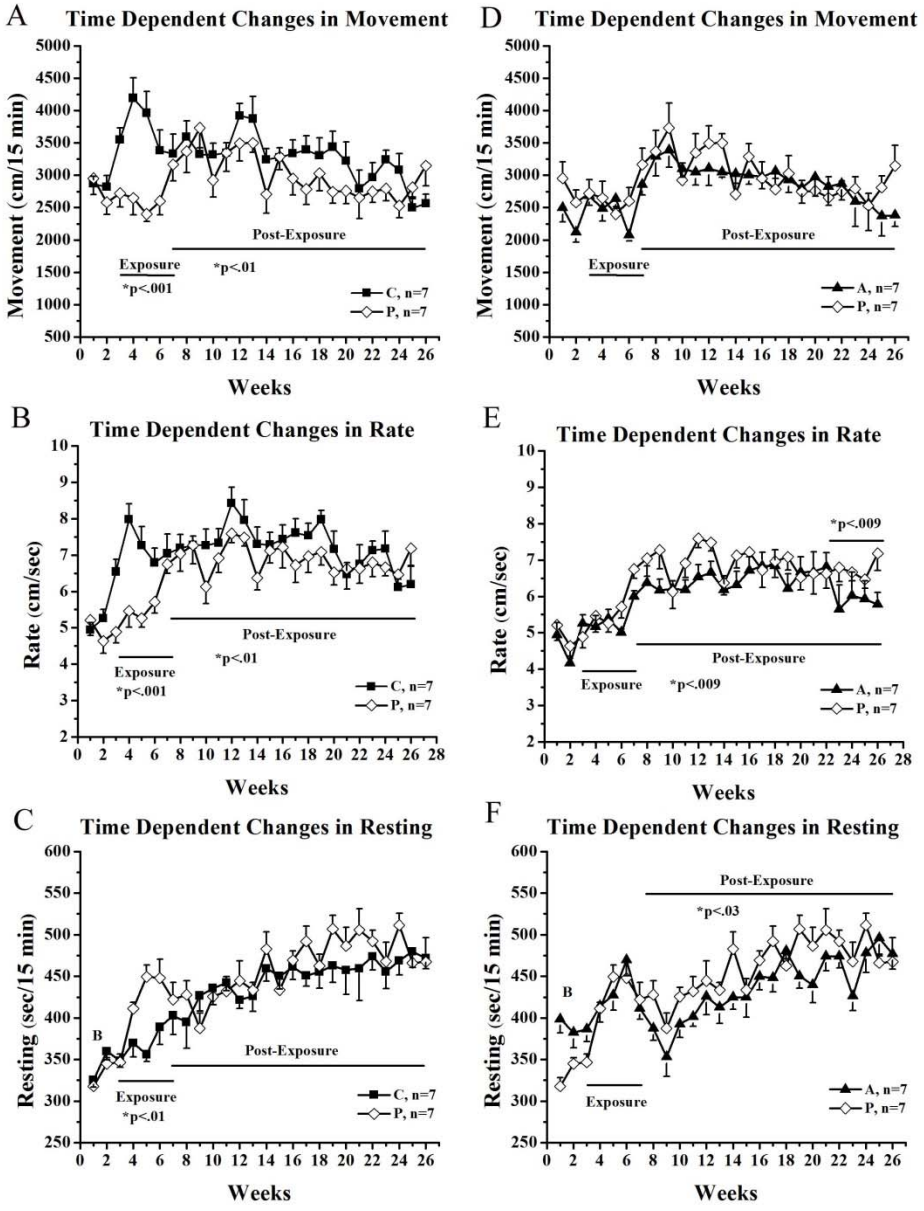


Figure 5. Excluding Permethrin from the Exposure Protocol Blocked of Pain Behavior Persistence. A) Significant movement distance effects were retained over post-exposure weeks 5-12 (F=6.79) despite the omission of permethrin, but blocked in weeks 17-20. **B)** Average movement rate scores were still reduced by GWI chemicals when permethrin was excluded from the exposure (weeks 5-12; F=6.80); rate shifts were lost at weeks 17-20 post exposure. **C)** Rest durations were unchanged at both early and late time periods. **D)** Movement distance scores were similar those with animals exposed to all 4 agents. **E)** Movement rate scores were shifted towards normal levels over the course of testing, including during the final 4 weeks (F=7.10 and F= 7.43; weeks 5-12 and 17-20, respectively). **F)** Resting scores were significantly higher in the absence of permethrin (F=5.17; weeks 5-12), but this transient effect did not persist into weeks 17-20. B: baseline testing; A: DEET, chlorpyrifos, permethrin, PB; C: (ethanol, corn oil, ethanol, water); P: DEET, chlorpyrifos, PB; Tests were not conducted on any measure 1-4 weeks post exposure.

Excerpted Discussion section from Flunker et al., attached:

We previously identified a protocol in which an 8 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 associated with the development of the GWI pain syndrome (syndrome 3; Haley and Kurt, 1997), we examined whether the addition of this repellant to our exposure protocol would promote the appearance of pain behaviors in our rat model. These 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. Rat ambulatory behaviors were suppressed as early as 5-12 weeks post-exposure and were still present out to the 21-24 week test period. Despite the powerful influence of DEET, the development and persistence of these pain signs was shown to be mainly dependent, albeit in a complex fashion, on the anticholinesterase components of the exposure protocol.

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

occurring during the exposure. Given this evidence, it appeared that the net amount of anticholinesterase activity in the 4 week exposure period drove the development of ambulation pain signs represented by decreased movement distance and average rate.

Finding that anticholinesterases played a fundamental role in these behavioral deficits fits well with our recent demonstration of maladaptations in mAChR signaling in muscle nociceptors harvested from rats that had undergone an 8 week exposure to chlorpyrifos, PB and permethrin (Nutter et al., 2015; Cooper et al., 2016). It also fits well with reports from multiple laboratories of disturbances in mAChR expression in the CNS after exposure to chlorpyrifos, PB and other agents, in various combinations (Nostrandt et al., 1997; Liu et al., 1999; Huff et al., 2001; Zhang et al., 2002; Abou-Donia et al., 2004; Padilla et al., 2005; Pung et al., 2006; see also Abou-Donia et al., 2003; Abdel Rahman et al., 2004a; Abdel Rahman et al., 2004b; Zou et al., 2006; Proskocil et al., 2010). A variety of motor and cognitive signs have been also been documented following extended exposure to these GW chemicals (Servatius et al., 1998; Servatius et al., 2000; Hoy et al., 1999; 2000; Abou-Donia et al., 2001; Abdel-Rahman et al., 2004a; Abou-Donia et al., 2004; Parihar et al., 2013).

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, whatsoever, seemed to be attributable to the inclusion of DEET in the exposure set. Previous attempts to create a chronic pain state with this same group of agents (chlorpyrifos, permethrin, PB), with a 4 week exposure, failed to produce any lasting deficits (Nutter et al., 2015). Expanding exposures to 8 weeks subsequently revealed

ambulation and resting deficits (Nutter et al., 2015) that were partially replicated in a subsequent study (Cooper et al., 2016). 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). As we have now shown, there was no evidence that DEET interacted directly with nociceptor ion channels believed to be important for the development of a chronic pain condition consequent to exposure to GWI chemicals.

We had reported previously that exposure to chlorpyrifos, permethrin and PB, sufficient to produce ambulatory and resting deficits, was paralleled by diminished conductance through muscle nociceptor, K_v7, ion channels (Nutter et al., 2014). While DEET clearly accelerated behavioral maladaptations, we could not demonstrate, in this report, any acute influences of DEET on K_v7 properties that would predict some further aggravation of the molecular effect. Nor did we observe that DEET might contribute to modulation of muscle or vascular nociceptor $Na_v1.8$, a known target of the pyrethroid insecticide permethrin (Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006), and potentially associated with reported changes in muscle nociceptor action potential properties (Nutter et al., 2015). Although an 8 week exposure to chlorpyrifos, permethrin and PB, was previously shown to modify activity of $Na_v1.9$, acute exposure to DEET had inconsistent and purely pharmacologic influences on the physiology of this ion channel (Nutter and Cooper, 2014). 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 preclude an indirect interaction through secondary pathways yet to be identified.

Functional synergisms that occur between several GW agents on behaviors and/or ion channel physiology could arise indirectly. When multiple xenobiotics are present in the bloodstream, they compete for entry into hepatic pathways that ultimately process them to inactive metabolites. Because there are often multiple pathways leading to several metabolites, the presence of other agents that utilize some these pathways will shift the pattern of metabolites produced (Abou-Donia et al., 1996; Usmani et al., 2002; Abu-Qare and Abou-Donia, 2008; see also Choi et al., 2004). The functional consequences of these interactions can be complex. Complexities arise from the delayed metabolism of agents competing for the same enzymatic pathway, and also due to the fact that some agents are first converted into more toxic derivatives. The metabolism of chlorpyrifos, DEET, and PB exemplify these issues.

Although CP is an AChE inhibitor, one of its hepatic metabolites, chlorpyrifos-oxon is 1,000 fold more potent in that role (Huff and Corcoran 1994). In the presence of DEET, the hepatic conversion of chlorpyrifos to its oxon form is increased 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 Abuo-Donia, 2008). Moreover, when PB is co-administered with DEET, it will slow 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 accelerated and prolonged ambulatory deficits.

Complex Influences of AChE Inhibitors on Pain Signs

The evidence that linked 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 deficits that occurred with 4 agents exposure 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 deficits (weeks 5-12). However, both anticholinesterases were required for the persistence of ambulation deficits out to 20 weeks post-exposure. Unexpectedly, we observed that the development of rest duration deficits 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 only permethrin, chlorpyrifos and DEET.

We could consider the ambulation and rest measures as points on a pain severity scale, in which reduction of movements (distance) and characteristics of movements (average rate) represent a lesser degree of pain than resting. Certainly, resting represents a cessation of movement altogether and is thereby an ultimate reduction of distance and rate. Accordingly, exposure to anticholinesterases CP and PB, in the presence of DEET, leads to development of a chronic pain that reduces and slows movement. When the protective effect of PB is absent, more extreme pain signs develop that are reflected in rest duration measures. The failure of this interpretation lies in the fact that this scenario predicts an even greater reduction of movement and rate scores in the absence of PB. This did not occur. Instead, movement distance and rate scores were rescued by removing PB from the exposure set. Therefore, the divergence between ambulation and resting must have another interpretation.

The disparate influence of the two anticholinesterases on rat activity measures could be due to distinct actions of neurotoxicants on various physiological targets. This could be as simple as the difference between the actions of a systemic anticholinesterase, such as chlorpyrifos, and one, such as PB, whose actions are restricted to the periphery (Newmark, 2005; Weinbroum, 2005; Weissman and Raveh, 2011). Alternately, an explanation might be found in the distinction between PB as a prophylactic against an irreversible anticholinesterase, versus its inability to oppose the extra-anticholinesterase activity of chlorpyrifos-oxon.

PB, a reversible inhibitor of AChE, was approved for use in the Gulf theater 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; Newmark, 2005; Weinbroum, 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 deficits. In partial support of this, we have shown that increasing the duration of exposure to CP to 8 weeks, also results in resting deficits (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, chlopryrifos-oxon, as well as some other organophophates 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). As noted above, 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 the PNS (Nutter and Cooper, 2015; Cooper et al., 2016). It is not clear whether the shift in receptor expression is the result of prolonged loss of AChE activity or due to the extra-anticholinesterase properties of chlorpyrifos-oxon.

PB is known to interfere with DEET metabolism, increasing DEET persistence, and thereby having the potential to accentuate the conversion of chlorpyrifos to its oxon form (Usmami et al., 2002; Abu-Qare and Abou-Donia, 2008). The presence of PB in the exposure set could have promoted the oxon dependent maladaptations (i.e., ambulation), and the absence of PB could have rescued them via the resulting increase in DEET metabolism during the critical exposure period. As we have shown, reducing the concentration of topical DEET (400 to 200 mg/kg; 50%-25%) powerfully diminished its capacity to accelerate the development of ambulation pain signs. We cannot confirm that the exclusion of PB would have resulted in a functional reduction of DEET to that extent. While a strong statistical association has been reported between the presence of pain, in veterans with GWI, and the use of DEET (75%) and pyridostigmine bromide (Haley and Kurt,

1997), it is by no means certain that this acceleration was due to a hepatic interaction between these agents. Nor can we exclude an alternative interpretation. It is also possible that the distinction between ambulation and resting deficits does not represent a divergence of pain signs, but rather represents a distinction between the sensory and motor manifestations of exposure to GWI chemicals.

Summary and Conclusions

DEET substantially accelerated and prolonged the pain signs that developed after a 4 week exposure to GW agents. 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 the anticholinesterases on their molecular targets.

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). The pain symptoms of GW veterans were highly diverse. They were manifested in variable patterns that included muscle, joint, back pain, abdominal pain and headache (Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). While extensive emphasis has been placed upon delayed emergence of the symptoms of GWI, about 25% of veterans developed GWI while still in theater (Kroenke et al., 1998).

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). The variations of 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 anticholinesterases as a primary risk factor for pain. Doubling the exposure duty cycle to PB and CP produced manifestations of pain-like behaviors and shifted muscle nociceptor physiology consistent with a chronic myalgia (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 pyridostigmine bromide.

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 a hepatic overload (Abou-Donia et al., 1996). Nevertheless, if the present data can be confirmed, routine administration of PB did afford a degree of protection against the physiological impact of some of the anticholinesterase insecticides to which soldiers were significantly 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 metabolites that asserted their deleterious actions through pathways that were independent of anticholinesterase activity but had the capacity to derange important components of the nervous system.

TASK 1.2: Targeting Maladapted Ion Channel Proteins with Systemic Treatments

TASK 1.3: Targeting Maladapted Ion Channel Proteins with Multiple Systemic Treatments

These Tasks are scheduled for year 2.

Specific Aim 2. Channel Protein Maladaptations in Myalgic and Arthralgic Rats

TASK 2.1: Assess K_v7 Physiology in Muscle and Vascular Nociceptors 12 and 16 Weeks After Exposure.

TASK 2.2 Assess $Na_v1.9$ Physiology in Nociceptors 12 and 16 weeks After Exposure

TASK 2.3 Assess Excitability and Spontaneous Activity in Nociceptors 12, 16 and 24 weeks After Exposure.

Time Line: Months 6-12

Behavioral studies indicated that the addition of DEET to the exposure protocol accelerated the development and shifted the pattern of chronic pain signs in rats (see above; figure 1). We had previously shown that activity of K_v7 and $Na_v1.9$ ion channels were affected by a 60 day exposure to permethrin, chlorpyrifos and PB (Nutter and Cooper, 2014; Nutter et al., 2015). In accordance with TASK 2.1 and 2.2 we examined the influence of a DEET augmented protocol on the activity of K_v7 and $Na_v1.9$ ion channels.

As in previous studies, rats were exposed to the GWI chemicals for 4 weeks. Control rats received only vehicle (corn oil, water, ethanol) exposures. Twelve weeks after exposures had ceased, rats were sacrificed and ganglionic cells were plated for electrophysiological studies. Additional studies were carried out on cells harvested 16 weeks after exposures. Those studies are still underway and will be reported at the end of the next grant period.

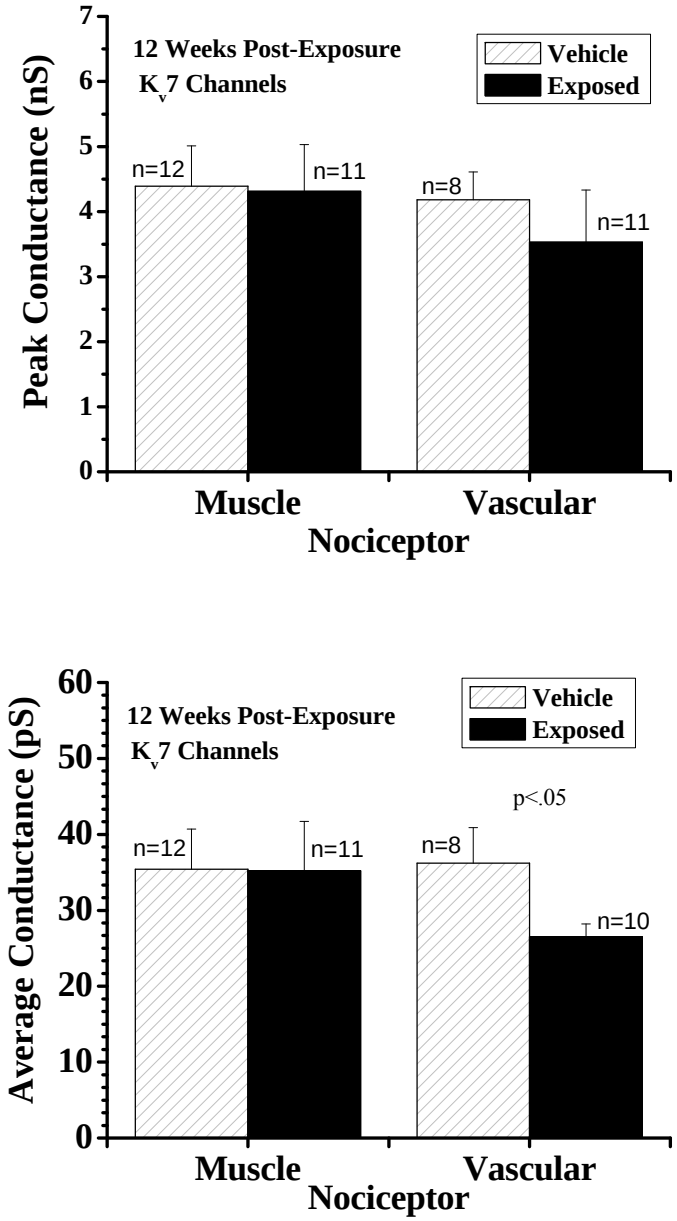


Figure 6. The Influence of 4 GWI Chemicals on K_v7 Activity. The addition of DEET to the protocol altered the influence on deep tissue nociceptors. Although there was little influence on muscle nociceptor K_v7 activity, the K_v7 average conductance was significantly decreased in vascular nociceptors (lower Panel).

The addition of DEET to the exposure protocol changed the impact of GWI chemicals on the physiology of deep tissue nociceptors. Twelve weeks following exposure to permethrin, chlorpyrifos, PB and DEET, muscle and vascular nociceptor physiology were altered in a manner consistent with increased excitability (figure 6. In a previous study in which rats were exposed over the same time period and identical doses to 3 GW chemicals (permethrin chlorpyrifos and PB), we did not observe any shifts in vascular K_v7 activity, and shifts in $Na_v1.9$ faded by the 12 week post exposure interval (Nutter et al., 2015). The addition of DEET to the exposure protocol resulted in the development of a persistent decrease in vascular nociceptor K_v7 activity and a significant increase in the activity of muscle nociceptor $Na_v1.9$ 12 weeks after exposures had ceased (figure 7). Both of these maladaptations suggested a hyperexcitable state in these nociceptor pools with potential impacts on autonomic function. Supporting experiments assessing vascular perfusion of the distal hindlimb (TASK 3) were consistent with these molecular findings of autonomic dysfunction in rats exposed to the DEET augmented protocol (see below; figure 11).

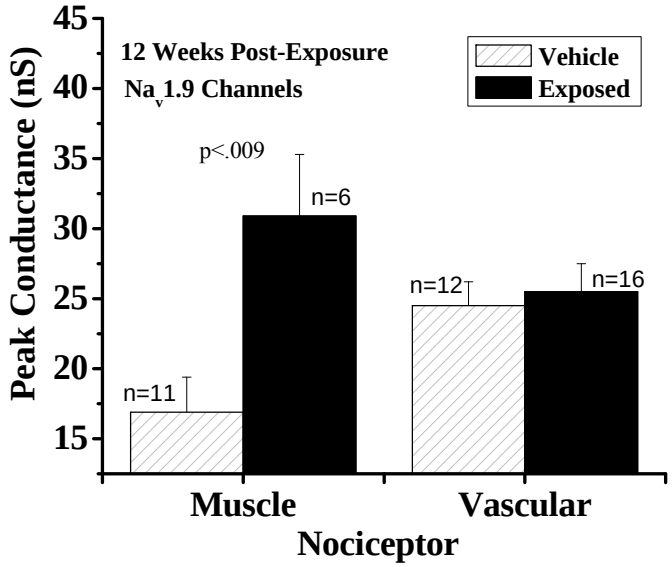


Figure 7. The DEET Augmented Protocol Altered $Na_v1.9$ Activity in Muscle Nociceptors.

TASK 2.4: Assess the Acute Influence of DEET on K_v7 and $Na_v1.9$ Physiology

Timeline: months 1-5

An abbreviated presentation of this material is presented below. A more complete presentation will be found in the attached manuscript.

We demonstrated that DEET accelerated the development of pain behaviors. Our previous studies have shown that exposure to permethrin, chlorpyrifos and PB can modify the activity of K_v7 and $Na_v1.9$ ion channels expressed in muscle and vascular nociceptors. We examine the hypothesis that the capacity of DEET to accelerate the development and lengthen the persistence of pain behaviors was due to its interaction with Na_v and/or K_v ion channels expressed in muscle and vascular nociceptors. In these studies we isolated membrane currents in muscle and vascular

nociceptor neurons that were harvested from young adult rats. Using whole cell patch clamp methodology, we exposed these neurons to DEET (10-100 μM). Brief results of these studies are presented below. A more complete presentation of methods can be found in the Appendix (p. 55).

There was no evidence that DEET modified the activity of K_v proteins (K_v7, K_{DR}; figure 8). Nor was there any evidence that DEET altered activity of Na_v ion channels at physiological dosages ($Na_v1.8$; $Na_v1.9$; figures 9 and 10 respectively). Although some shifts were apparent at a dose of 100 μM (figure 10), we did not consider this to be physiologically significant.

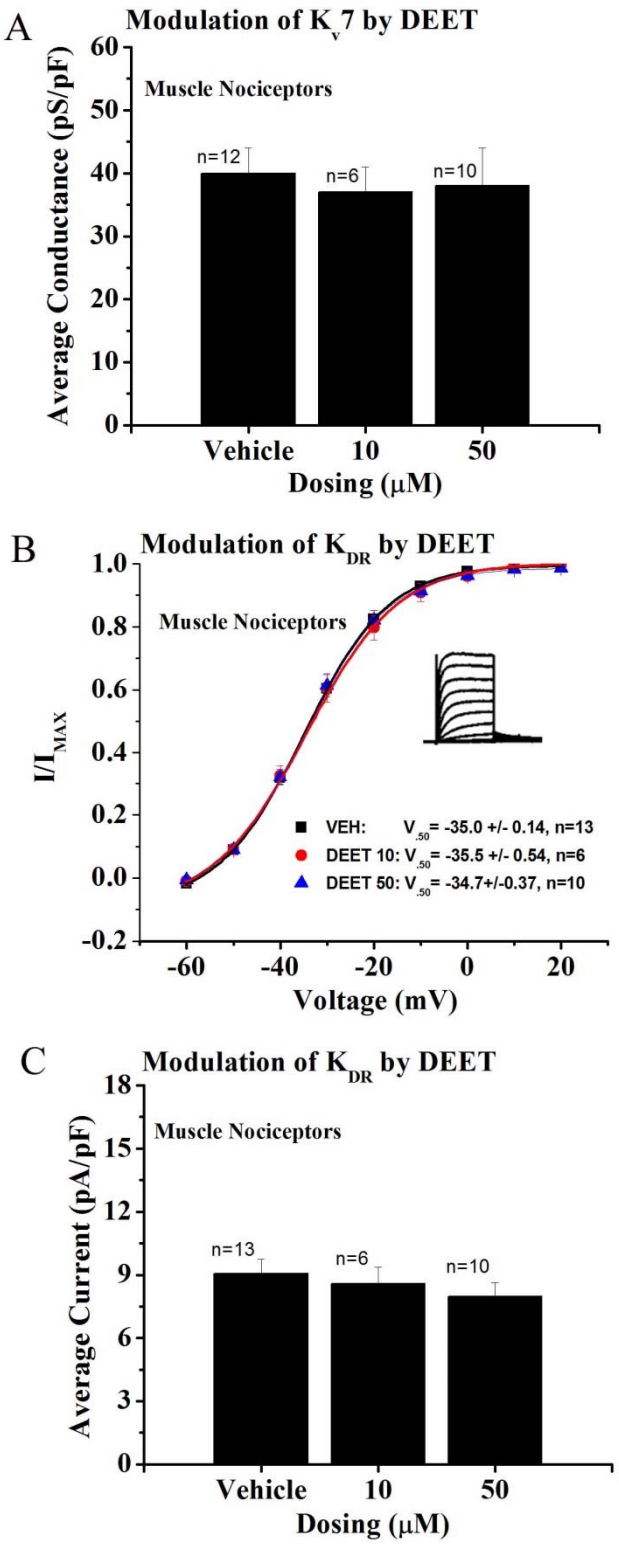


Figure 8. Voltage Activated K^+ Channels were Unaffected by Acute Exposure to DEET. A) The average conductance of muscle nociceptor K_v7 channels was not altered by DEET (10-50 μM); **B) and C)** Following a 12 minute exposure, there was no indication that either the voltage dependence or the average K_{DR} currents were modified by DEET (10-50 μM). 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.

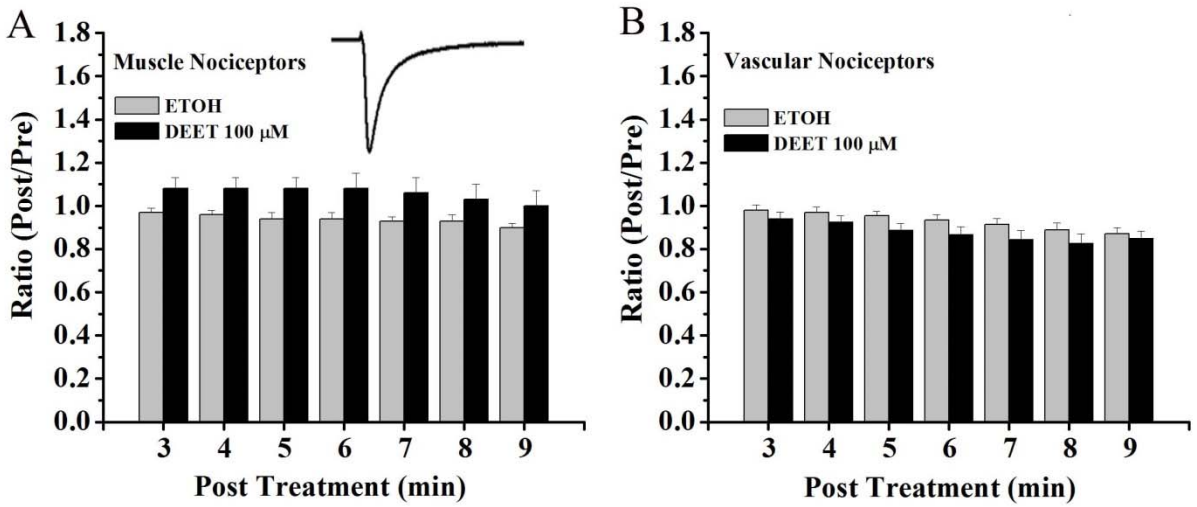


Figure 9. Time Dependent Modification of $Na_v1.8$ by DEET. A) The amplitude of muscle nociceptor $Na_v1.8$ was not changed by DEET (100 μM). A representative $Na_v1.8$ current is inserted. **B)** Vascular nociceptor $Na_v1.8$ amplitude was not altered by DEET (100 μM). Baseline records were taken prior 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.

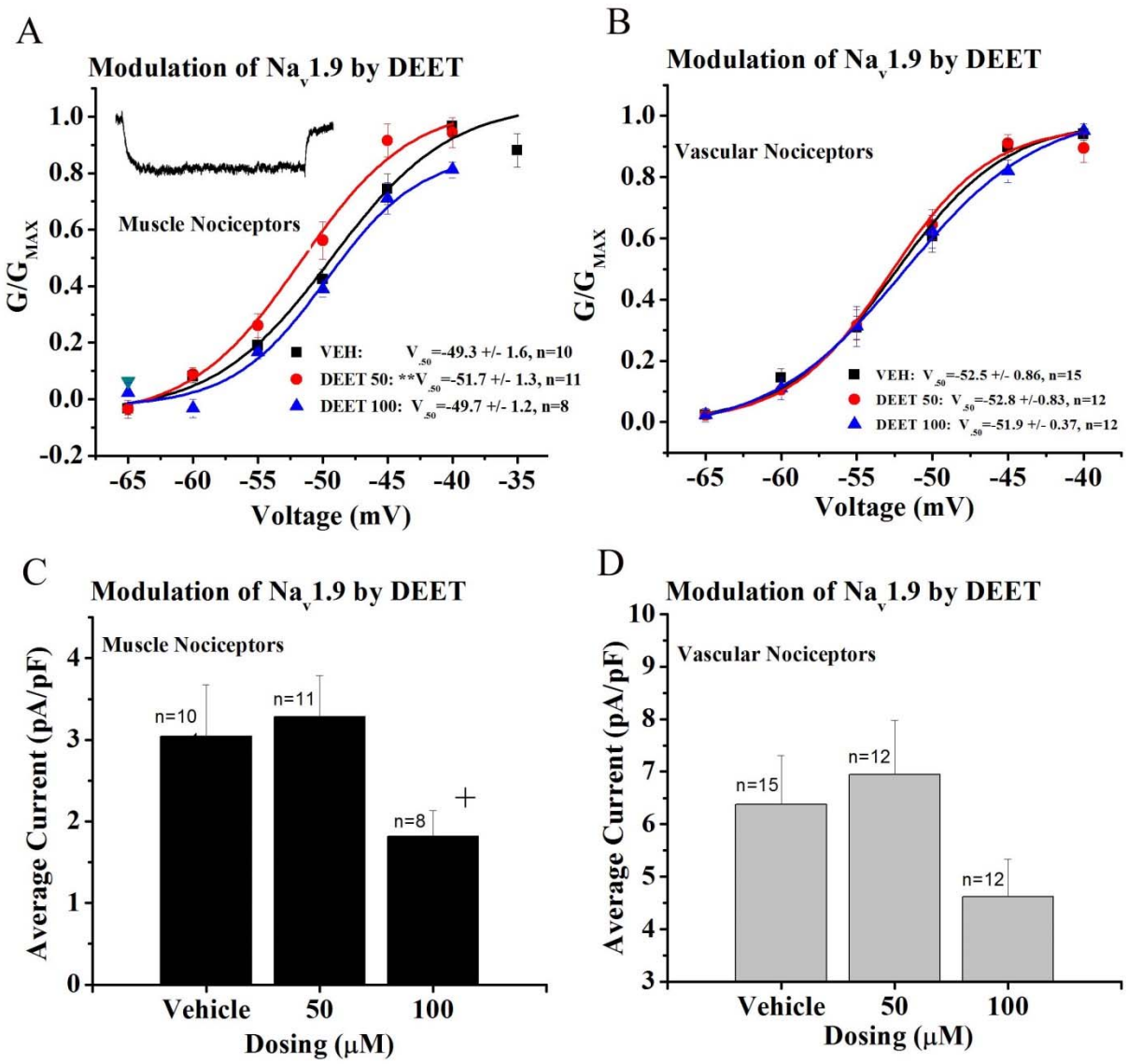


Figure 10. Voltage Activated, Na$_v$1.9, Channels were Weakly Modulated by Acute Exposure to DEET. A) The voltage dependent activation of muscle nociceptors was hyperpolarized at 50 but not at 100 μM DEET. A representative trace of a Na$_v$1.9 current (step to -50 mV; V_H=-120 mV) is included as an insert. **B)** The voltage dependent activation of vascular nociceptors was unaffected by DEET. **C)** Average currents of muscle nociceptors were unaffected by DEET ($p<.13$, Vehicle vs 100 μM; $p<.05$, 50 vs 100 μM); **D)** Vascular nociceptor average currents were unchanged ($p<.17$, Vehicle vs 100 μM; $p<.08$, 50 vs 100 μM). The voltage-activation curves were formed from the mean conductances of all cells averaged at a given voltage. Statistical tests were performed on the $V_{.50}$ 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 μM tests. Thirty-two rats contributed to these graphs.

Specific Aim 3. Autonomic Dysfunction resulting from GW-Chemical Exposure is Triggered by Hyperactivity in Vascular Nociceptors

TASK 3.1: *In vivo* assessments of changes in hindlimb autonomic vascular reflexes

Timeline: Months 6-15

In these experiments we examined whether vascular reflexes were modified by GW agents. Studies were conducted on rats that received all 4 GW chemicals and on rats that received only DEET, chlorpyrifos and permethrin (PB excluded). These studies are ongoing. Some of the results are presented below.

<u>TASK 3.1:</u> In vivo assessments of changes in hindlimb autonomic vascular reflexes

Description: Following optimized GW-Chemical exposure, measures of antidromic reflex vasodilation and ipsilateral decentralized nerve stimulation–elicited vasoconstriction will be performed in anesthetized, terminal preparations.

<u>METHODS:</u>

Animals: Rats in the "Exposed" group were treated with permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), pyridostigmine bromide (PB; 13 mg/kg), and insect repellant DEET (N,N-Diethyl-meta-toluamide, topical; 0-400 mg/kg; daily). Permethrin was applied with ETOH daily to a shaved region of the back between the forelimbs. Chlorpyrifos was injected subcutaneously with corn oil once every 7 days and PB was provided daily by oral gavage using tap water. PB dosages represented the standard military dose assuming a 70kg body weight. "Control" group rats were administered vehicle compounds

(ETOH, corn oil, water) with treatment timing identical to the Exposed group. All rats underwent blood flow testing prior to and after chemical treatments. A second exposed group of rats were treated in the same manner but with PB excluded from the exposure protocol. Other details of the methods used in these experiments are presented in the Appendix (p. 55).

Consistent with physiology studies that indicated a dysfunction in vascular nociceptors, animals treated with all GWI chemicals (permethrin, chlorpyrifos, PB and DEET (50%), displayed a significantly higher mean blood flow in the hindpaw heels (Fig. 11) at the measured time intervals of 2W, 4W, 6W, and 8W following the completion of chemical exposure treatment, stabilizing to control levels at 10W. Comparisons of corresponding individual perfusion measurements between exposed and control groups of left/right hindpaws were not significantly different which demonstrated there were no differences between left and right feet. Interestingly, the increased vasodilatation in chemically exposed animals was not due to an increase in autonomic cardiovascular measures during that period. Accordingly, no significant differences in exposed versus control measurements of systolic blood pressure, pulse rate, core body temperature, and paw skin temperature were observed during the post-exposure period (Fig. 12). This suggests the increased vasodilation may have been due to overactive vascular afferents releasing vasodilator transmitter like CGRP, supporting our hypothesis.

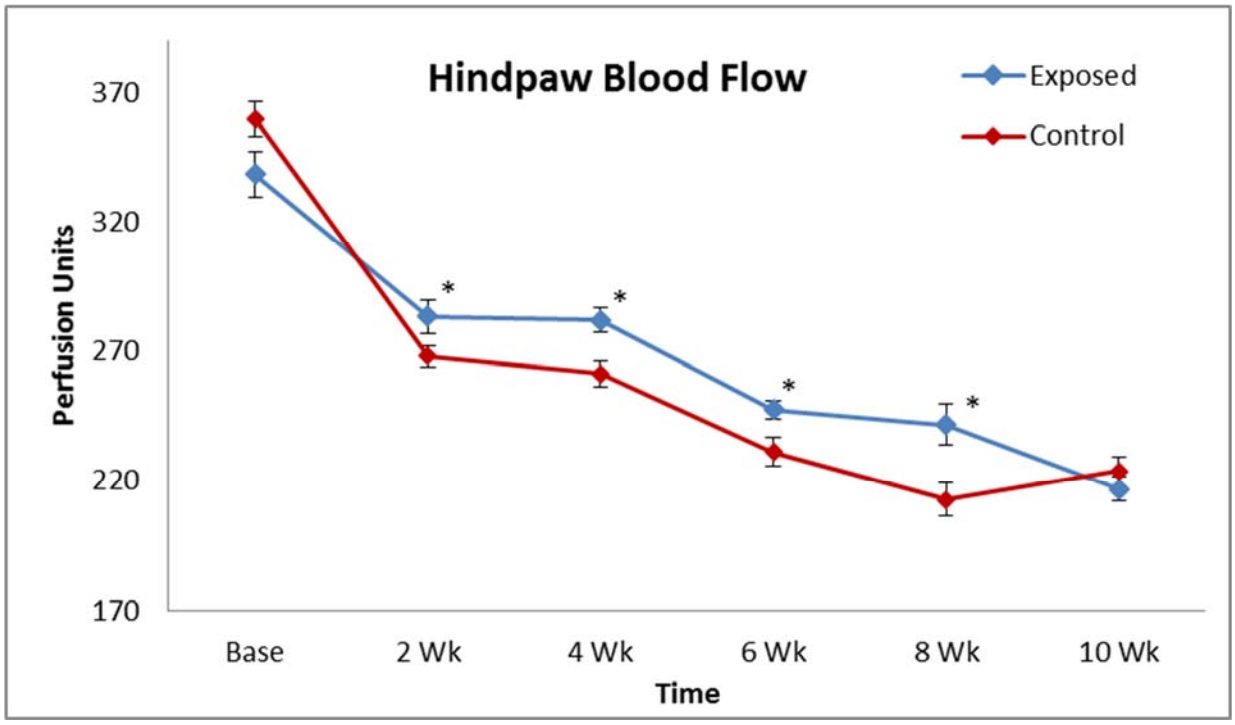


Fig 11. Exposure to 4 GWI chemicals produces increased vasodilatation up to 10W after end of exposure period. Mean ± SEM of hindpaw blood flow measured before (baseline) and every 2 weeks after the 4-week chemical exposure period. Time course (in post exposure weeks) of mean blood flow (in perfusion units) of the averaged heel region of the right and left hindpaws. Exposed animals (Group X rats, n=14) show significantly (*) greater perfusion post-exposure weeks 2, 4, 6, 8 compared to control animals (n=16).

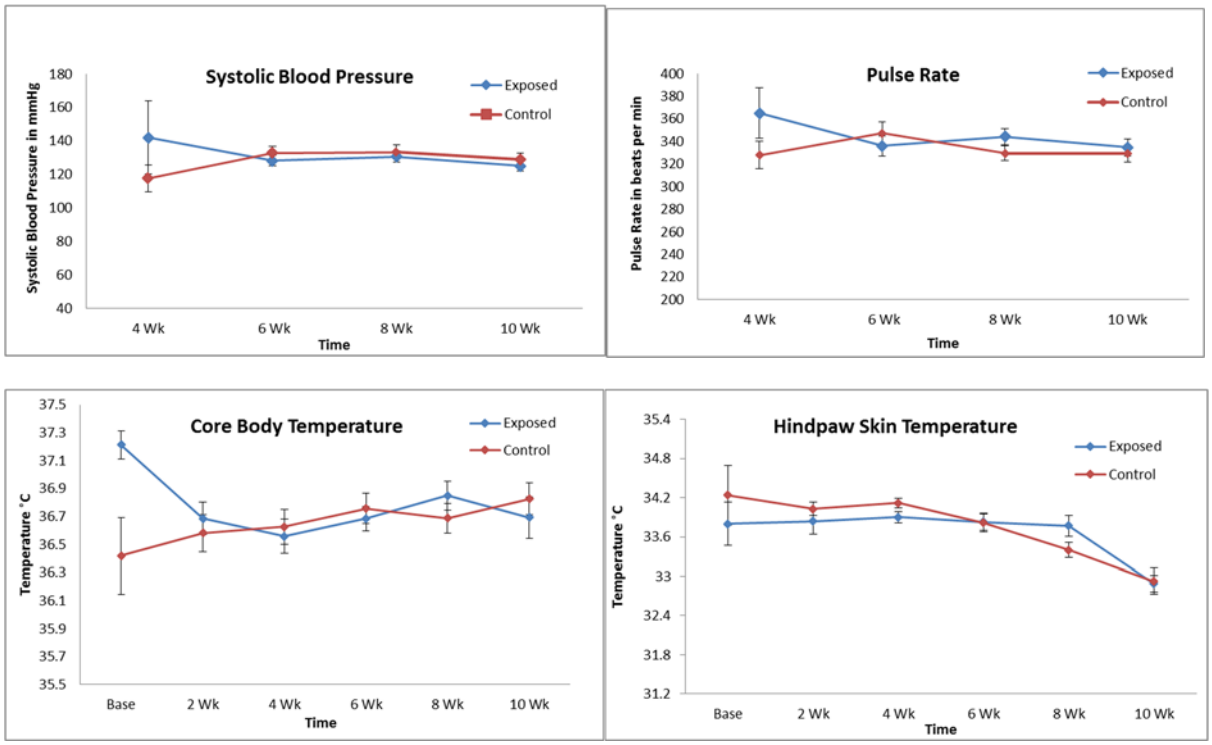


Fig. 12. Exposure to 4 GWI chemicals does not produce changes in autonomic vital signs parameters for up to 10W after end of exposure period. Mean ± SEM of systolic blood pressure, pulse rate, core body temperature, and hindpaw skin temperature measured before (baseline for temp data) and after the 4-week exposure period. There were no significant differences between exposed animals (Group X, n=14) compared to control animals (n=16).

In a separate group of animals exposed to only 3 GWI chemicals (Exposed minus PB), the increased vasodilatation in the hindpaws, seen with exposure to all 4 chemicals, did not occur (Fig. 13), suggesting an important role of PB in the development of blood flow alterations.

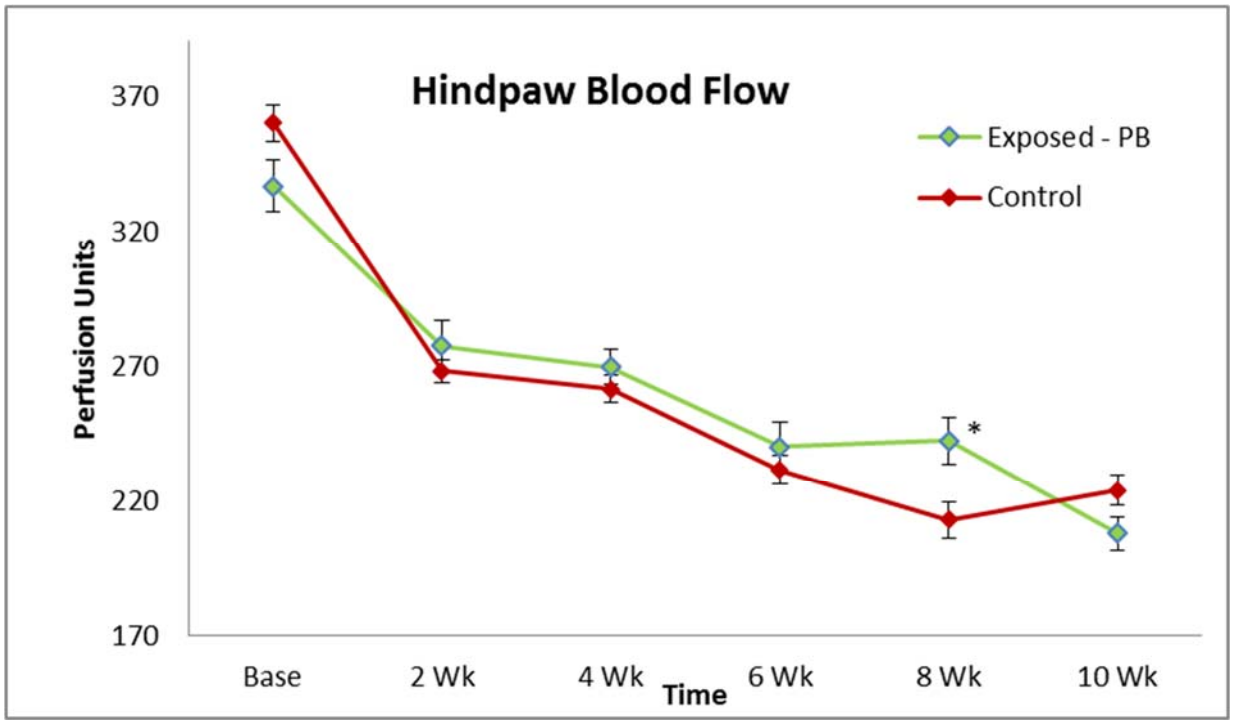


Fig 13. Exposure to 3 GWI chemicals (no PB) does not produce increased vasodilatation after the end of exposure period. Mean ± SEM of hindpaw blood flow measured before (baseline) and every 2 weeks after the 4-week chemical exposure period. Time course (in post exposure weeks) of mean blood flow (in perfusion units) of the averaged heel region of the right and left hindpaws. Exposed animals (n=8 rats) show significantly (*) greater perfusion only at post-exposure week 10 compared to control animals (n=16).

Stimulation of the decentralized distal stump of the left sciatic nerve of animals exposed to permethrin, chlorpyrifos, DEET, and PB produced higher blood flow (vasodilatation) in both the left gastrocnemius muscle and left hindpaw (Fig 14). Results to date show that final perfusion values observed subsequent to sciatic stimulation were significantly higher in both the left gastrocnemius muscle and hindpaw of post-exposure animals compared to controls. Initial perfusion values were higher in the left gastrocnemius muscle, however, no differences were observed in the initial blood flow of the left hindpaw of exposed and control animals, consistent with the time course of blood flow changes in the hindfeet measured throughout the post-exposure

period (Fig. 11). The right hindpaw and gastrocnemius muscle exhibited no change in blood flow in response to stimulation of the left distal sciatic nerve; similarly, distal sciatic stimulation did not produce any changes in systolic and diastolic blood pressures.

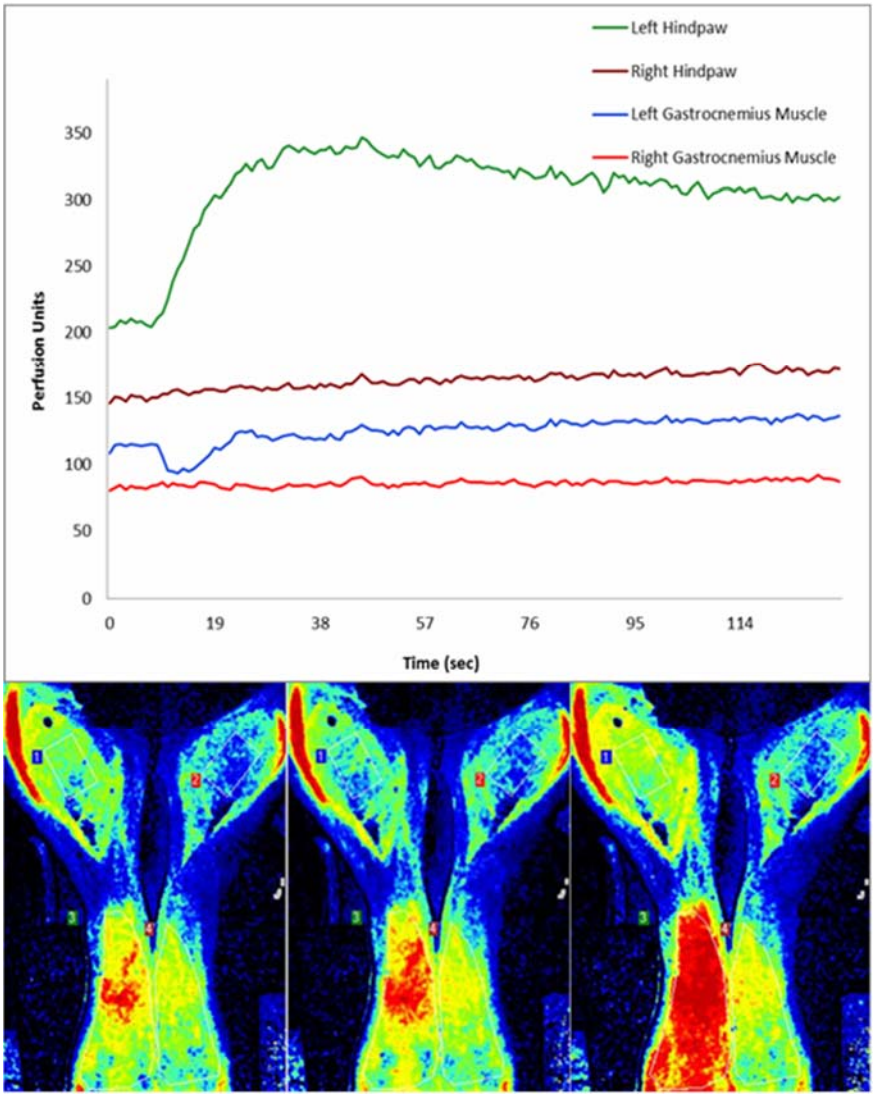


Fig. 14. Left Decentralized Distal Sciatic Nerve Stimulation. Top panel: Blood flow measurements with the LSCI of the distal left/right gastrocnemius muscles and left/right hindpaws (lower images) expressed graphically with perfusion values (in perfusion units) plotted on the y-axis and elapsed time along the x-axis. Left distal sciatic nerve stimulation (3 sec burst of 50Hz pulses) is presented at 5sec mark. Perfusion images in the bottom panels demonstrate time points before (left panel) and after sciatic stimulation (middle and right panels). Middle panel: Perfusion image at 12 sec showing maximal decreased blood flow in the left gastrocnemius muscle (vasoconstriction in left upper image) and increased blood flow in the left hindpaw (vasodilatation in left lower image). Right panel: Perfusion image peak blood flow in the left hindpaw (35 sec; vasodilatation). Blood flow levels are indicated by the "rainbow color map" where vasodilatation is yellow to red and vasoconstriction is blue. Note the lack of blood flow changes in the right gastrocnemius muscle and hindpaw because the stimulation is on the decentralized left sciatic nerve.

TASK 3.2: *In vivo* assessments of spontaneous activity in vascular nociceptors

TASK 3.3: *In vivo* assessments of the efficacy of treatment with CGRP blocker on autonomic measures

These studies were scheduled for year 2 (month 18).

4. Impact

Impact on Principal Disciplines

As a result of our behavioral studies, it has become clear that anticholinesterase insecticides, like chlorpyrifos, played a fundamental role in the development of GWI pain. Although DEET accelerated and lengthened the persistence of pain signs in rats, this outcome was likely to be due to its influence on the metabolism of chlorpyrifos (Usmani et al., 2002; Abou-Donia et al., 1996; Abu-Qare and Abuo-Donia, 2008; see above 'Discussion'). These interpretations are not definitive, as we have not provided direct evidence that DEET accelerated the conversion of chlorpyrifos to the considerably more toxic chlorpyrifos oxon; however, that effect has been demonstrated by other laboratories (Usmani et al., 2002). And while we could not demonstrate any direct influence of DEET on pain system neurons, we cannot rule out that some direct effects might eventually be identified.

Evidence was also presented, for the first time, that an interaction between chlorpyrifos and pyridostigmine bromide could alter the pattern of behavioral signs of pain. This interaction could explain the highly diverse pain symptoms exhibited by GW veterans whose exposure to anticholinesterases and pyridostigmine, in theater, were far more varied than the systematic exposures used in our laboratory. The self-administration of pyridostigmine, by deployed soldiers, was likely to have aggravated some symptoms while offering a degree of protection from others. Clarifying the distinct pathophysiology associated with GWI dependent, joint, muscle, back pain and headache should improve approaches to treatment.

We have also shown that our DEET augmented model not only extends sensory and physiological deficits, but also produces alterations in blood flow concomitant with changes in the physiology

of vascular nociceptors. Blood flow changes were only evident in rats exposed to all 4 agents. When PB was omitted from the exposure protocol, no changes in blood flow were observed. These autonomic maladaptations reproduced a portion of the autonomic symptoms that have been reported by GW veterans. Those included blood flow irregularities (Haley et al., 2009; Liu et al., 2011; Li et al., 2011). Up until this time, there has been little research on the pathophysiology of autonomic symptoms (Binns et al., 2008). The DEET augmented protocol should produce further insights into the pathophysiology of GWI.

There was no impact on technology transfer-nothing to report

There was no impact on society-nothing to report

5. Changes/Problems

Nothing to Report

6. Products

Journal Publications

The following manuscript was submitted for publication on August 10th, 2016. It is attached to this report (Appendix, p. 79).

Flunker[a], L.K., Nutter[a], T.J., Johnson[b], R.D. and Cooper[a,c], B.Y. DEET Amplifies Anticholinesterase Dependent Chronic Pain Signs in a Rat Model of Gulf War Illness Pain. Neurotoxicology, 2017.

Abstracts and Presentations

The following abstract was accepted for presentation at the Society for Neuroscience 2016. It is scheduled for presentation on November 13, 2016.

Cooper, B.Y., Nutter, T.J., Johnson, R.D and Flunker, L. Contributions of DEET to a Rat Model of Gulf War Illness Pain

Introduction. Veterans of the 1991 Gulf War commonly reported a delayed onset joint, muscle and other deep tissue pain. The Research Advisory Committee on Gulf War Illness (GWI) has determined that pesticides may have contributed to the development of the symptoms of GWI (Binns et al., 2008). We developed a rat model of GWI pain based upon a 60 day exposure to permethrin (P), chlorpyrifos (CP) and pyridostigmine bromide (PB; Nutter et al., 2015). In the present report, we combined behavioral and molecular approaches to examine the contribution of DEET to the development of the joint and muscle pain of GWI.

Methods. Juvenile male rats, weighing between 90 and 110 g, were exposed to various combinations of P (2.6 mg/kg; topical), CP (120 mg/kg; subcutaneous (SC)), PB (13 mg/kg; oral gavage), and DEET (400 mg/kg; topical) for 30 days. Using an identical administration schedule, control group rats received only vehicle exposures (topical ethanol, SC corn oil, water by gavage). All rats underwent behavioral testing before, during and after chemical exposures (hindlimb pressure withdrawal; open field activity (movement distance, movement rate and resting duration). Molecular studies were conducted to assess the influence of acute DEET on nociceptors. In molecular studies, young adult rats weighing 90-150 grams were anesthetized and decapitated.

Whole cell clamp experiments were conducted on excised dorsal root ganglion neurons that were identified as muscle or vascular nociceptors using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2002).

Results. When exposed to all 4 compounds, rats exhibited reduced open field activity (movement distance and rate) that resembled a myalgia or arthralgia 9-12 weeks after dosing had ceased ($p<.02$ and $p<.004$). When exposed to only 3 compounds, activity changes failed to materialize in the absence of PB or CP but persisted in the absence of permethrin (movement; $p<.05$); moreover, when PB was removed, rate decreases were significantly lessened relative to exposure to all 4 chemicals ($p<.05$). Molecular studies indicated that DEET significantly inhibited $Na_v1.9$ amplitude ($p<.04$; vascular nociceptors) but had no effect on K_v7 or $Na_v1.8$. The influence DEET on $Na_v1.9$ only occurred at relatively high doses that are not likely *in vivo* (100 μM).

Conclusions. DEET makes a significant contribution to a robust deep tissue pain syndrome in a rat model of GWI pain. PB was required for, and CP contributed to, motor activity changes while permethrin did not play a role at 12 weeks-post exposure. DEET might exert its influence through inhibition of $Na_v1.9$.

Inventions, patent applications, and/or licenses

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Personnel:

Name: Brian Y. Cooper, Ph.D., College of Dentistry

Project role: Principal Investigator,

Researcher Identifier (ORCID ID): 0000-0002-7592-588X

Nearest person month worked: 6

Contribution to Project:

Design, execution and analysis of physiology and behavior experiments (Tasks 1.1, 2.1, 2.4)

Preparation of manuscripts and abstracts

Preparation of IACUC protocols

Name: Richard D. Johnson, Ph.D., College of Veterinary Medicine

Project role: Co-Principal Investigator

Researcher Identifier: none

Person Months: 3

Contribution to the Project:

Design, execution and analysis of physiology experiments (Tasks 3.1)

Preparation of manuscripts and abstracts

Preparation of IACUC protocols

Thomas J. Nutter, Ph.D., College of Dentistry

Project Role: Biological Scientist

Researcher Identifier: none

Person Months: 12

Contribution to Project:

Execution of physiology experiments (Tasks 2.2, 2.4)

Linda Flunker, MS, College of Dentistry

Role on Project: Biological Scientist

Research Identifier: none

Person Months: 11

Contribution to Project:

Execution of Behavioral Studies (Tasks 1.1, 2.1, 2.4; dosing; activity measures; data collection and storage)

Funding Support: Ms Flunker is assigned 90% to the project and 10% to departmental projects

Name: Victoria Dugan, College of Veterinary Medicine

Role on Project: Biological Scientist

Researcher Identifier: none

Person Months: 6

Contribution to Project:

Execution of physiology experiments (Tasks 3.1)

Funding Support: Ms Dugan is assigned 50% to the project and 50% to NIH projects

Change in the Support for the PI:

Nothing to report

Partner Organizations:

Nothing to report

Changes/Problems**:**

Nothing to report

8. Special Reporting Requirements

Neurovascular and Autonomic Dysfunction Associated with Gulf War Illness Pain

GW140066 / W81XWH-15-1-0515

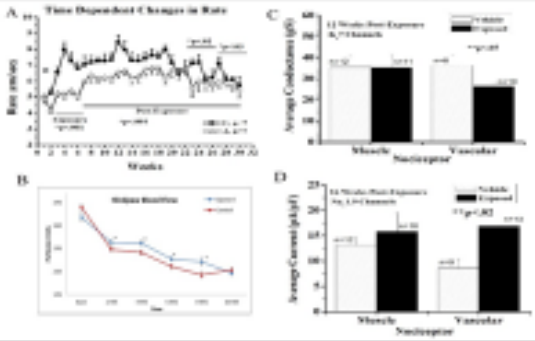
PI: Dr. Brian Cooper **Org:** University of Florida **Award Amount:** $ 1,023,883

Specific Aims

1) Reversing Signs of GWI Pain Behaviors Maintained by Vascular Nociceptors
2) Channel Protein Maladaptations in Myalgic and Arthralgic Rats
3) Autonomic Dysfunction from GW-Chemical Exposure Triggers Hyperactivity in Vascular Nociceptors

Approach

Gulf War (GW) pesticide neurotoxicants (permethrin, chlorpyrifos, DEET and pyridostigmine bromide) induce a maladaptive molecular imbalance between autonomic neural membrane proteins Nav1.9 and Kv7, leading to release of vasoactive peptides CGRP and SP and subsequent neurogenic inflammation that results in the widespread chronic pain of GWI. Here we examine how the physiology of rat vascular nociceptors and autonomic nerves are modified by exposure to GW chemicals and test whether treatment with CGRP blockers can reverse the established chronic pain condition.



A) Movement rate reduced 24 weeks post-exposure; B) Hindlimb blood flow increased; C) and D) Kv7 activity decreased and Nav1.9 activity increased in vascular nociceptors 12 and 16 weeks post-exposure predicting increased excitability.

Timeline and Cost

Activities FY	15	16	17
Specific Aim 1			
Specific Aim 2			
Specific Aim 3			
Data Analysis & Dissemination			
Estimated Budget	$ 501,392	$ 522,491	$ 0

Updated: 14 October 2016

Goals/Milestones (Award issued 30 September 2015)

FY15 Goals – ☑ IACUC/ACURO approval for animal studies, 30 Sept 2015
☐ Optimize Chemical Exposure Protocol to produce persistent pain behavior and autonomic dysfunction complex
☐ Confirm Kv7 and Nav1.9 amplitude is significantly impacted relative to controls

FY16 Goals – Assess activity/changes in hindlimb reflexes in vivo
☐ Initiate treatments to modulate ion channel protein targets; behavioral assessments to confirm
☐ Action potential excitability and activity is significantly increased

FY17 Goal – Data analysis and prepare dissemination of findings

Comments/Challenges/Issues/Concerns
None to date

Budget Expenditure to Date
Expenditures on track

9. Appendices

Statement of Work

Research TASKS	Rats	Months	University of Florida
Specific Aim 1. Reversing Signs of GWI Pain Behaviors Maintained by Vascular Nociceptors			
TASK 1.1: Optimize the Chemical Exposure Protocol	50	1 to 5	Beh: Dr. Cooper
Description: We will determine whether all 4 GWI chemicals (Permethrin, Chlorpyrifos, PB and DEET) are required			Autonomics: Dr. Johnson
to produce the persistent pain behavior and autonomic dysfunction complex			
Methods: Rats are exposed to 3 or 4 GWI chemicals for 4 weeks (see Table 1, project narrative).			
Behavioral measures of pain and autonomic functions are performed (pain measures weekly;			
autonomic measures bi-monthly).			
Milestone: Necessary conditions are established for a pain and autonomic disorder (reduced muscle pain threshold,			
decreased motor activity, increased resting, LSCI score, see TASK 3.1).			
TASK 1.2: Targeting Maladapted Ion Channel Proteins with Systemic Treatments			
Description: Once ion channel protein treatment targets are identified (Specific Aim 2), we will use agents	72	14 to 19	Beh: Dr. Cooper
that modulate these proteins (e.g., retigabine, riluzole, BMS-927711) to reverse signs of an			Autonomics: Dr. Johnson
established pain and autonomic disorder that are present 4-8 weeks after the chemical exposure			
has ended. Dose effects are examined within the 4-8 week window. The time course of			
successful treatments will be characterized. The side effects of successful treatments are			
evaluated. Male and female rats are used during tests			
Milestone A. Measures of pain and autonomic disorders are reduced significantly 2 hours following			
treatment (normalized muscle pain threshold, motor activity, resting, LSCI score, see TASK 3.1).			
Milestone B. Reduced pain and autonomic measures are maintained for 4 weeks following treatment			
TASK 1.3: Targeting Maladapted Ion Channel Proteins with Multiple Systemic Treatments	48	20 to 24	Beh: Dr. Cooper
Description: If single agent treatments fail to resolve the pain and autonomic behavior complex,			Autonomics: Dr. Johnson
we will examine the efficacy of multiple agents that target different proteins and may differentially influence			
pain versus autonomic signs.			
Milestone A. Measures of pain and autonomic disorders are reduced significantly 2 hours following			
treatment (normalized muscle pain threshold, motor activity, resting, LSCI score, see TASK 3.1).			
Milestone B. Reduced pain and autonomic measures are maintained for 4 weeks following treatment.			

Research TASKS	Rats	Months	University of Florida
Specific Aim 2. Channel Protein Maladaptations in Myalgic and Arthralgic Rats	**Rats**	**Months**	**University of Florida**
TASK 2.1: Assess K_v7 Physiology in Muscle and Vascular Nociceptors 12 and 16 Weeks After Exposure.	29 + 15	6 to 12	Phys: Dr. Cooper
Description: Rats are exposed to the optimized GW chemical protocol for 4 weeks. Behavioral measures of			Beh: Dr. Cooper
pain and autonomic functions are performed (pain measures weekly; autonomic measures bi-			Autonomics: Dr. Johnson
monthly). Cells are harvested from exposed rats 12 and 16 weeks after exposure (24 rats).			Phys: Dr. Johnson
Studies are conducted on vascular and muscle nociceptors. Whole cell voltage clamp			
electrophysiology is performed. Measures of voltage dependence, current amplitude and			
kinetics are assessed. Behavioral testing continues out to 24 weeks (20 rats).			
Milestone A: K_v7 amplitude is significantly decreased relative to vehicle treated controls			
12 and 16 weeks after exposure			
Milestone B: Pain behavior and autonomic signs are maintained for 24 weeks			
TASK 2.2 Assess $Na_v1.9$ Physiology in Nociceptors 12 and 16 weeks After Exposure	29 + 15	6 to 12	Phys: Dr. Cooper
Description: Rats are exposed to the optimized GW chemical protocol for 4 weeks. Behavioral measures of			Beh: Dr. Cooper
pain and autonomic function are performed (pain measures weekly; autonomic measures bi-monthly).			Autonomics: Dr. Johnson
Cells are harvested from exposed and vehicle treated rats 12 and 16 weeks (24 rats)			Phys: Dr. Johnson
following exposure. Whole cell voltage clamp electrophysiology is performed. Studies are			
conducted on vascular and muscle nociceptors. Measures of voltage dependence, current			
amplitude and kinetics are assessed. Behavioral testing continues out to 24 weeks (20 rats).			
Milestone A: $Na_v1.9$ amplitude is significantly increased relative to vehicle treated controls 12 and 16			
weeks after exposure.			
Milestone B: Pain behavior and autonomic signs are maintained for 24 weeks.			
TASK 2.3 Assess Excitability and Spontaneous Activity in Nociceptors 12, 16 and 24 weeks After Exposure.	36	14 to 20	Phys: Dr. Cooper
Description: Rats are exposed to the optimized GW chemical protocol for 4 weeks. Behavioral measures of			Beh: Dr. Cooper
pain and autonomic function are performed (pain measures weekly; autonomic measures			Autonomics: Dr. Johnson
biweekly). Cells are harvested from exposed and vehicle treated rats 12, 16 and 24 weeks			
following exposure. Whole cell current clamp electrophysiological studies are performed			
Studies are conducted on vascular nociceptors. Measures of spontaneous activity			
and excitability are assessed at room temperature and 35° C.			
Milestone A: Action potential excitability is significantly increased relative to vehicle treated controls			
Milestone B: Action potential spontaneous activity is significantly increased relative to vehicle treated controls			
TASK 2.4: Assess the Acute Influence of DEET on K_v7 and $Na_v1.9$ Physiology	48	1 to 5	Phys: Dr. Cooper
Description: The addition of DEET to the exposure protocol is essential to establishing a persistent pain			
behavior complex. The pathway to this outcome is unknown. We will assess the acute			
influence of DEET (100-600 μM) on the physiology of $Na_v1.9$ and K_v7 channel proteins. Whole			
cell voltage clamp electrophysiology is performed on young adult rats. Studies are conducted on			
vascular and muscle nociceptors. Measures of voltage dependence, current amplitude and			
kinetics are assessed			
Milestone A: K_v7 current amplitude is significantly decreased by DEET.			
Milestone B: Nav1.9 current amplitude is significantly increased by DEET			

Research TASKS	Rats	Months	University of Florida
Specific Aim 3. Autonomic Dysfunction resulting from GW-Chemical Exposure is Triggered by Hyperactivity in Vascular Nociceptors	**Rats**	**Months**	**University of Florida**
TASK 3.1: *In vivo* assessments of changes in hindlimb autonomic vascular reflexes	30	6 to 15	Phys: Dr. Johnson
Description: Following optimized GW-Chemical exposure (Task 2.1) and behavioral evidence of myalgia,	same as 2.1		
LCSI measures of antidromic reflex vasodilation and ipsilateral decentralized sympathetic trunk			
stimulation–elicited vasoconstriction will be taken in anesthetized,			
terminal preparations. Post-exposure periods will be 12W (n=12) and			
16W (n=12) along with 6 saline-controls at each time point			
Milestone A: GWI-Chemical exposure increases antidromic reflex vasodilatation in gastrocnemius muscle.			
Milestone B: GWI-Chemical exposure decreases sympathetic stimulation-induced vasoconstriction			
TASK 3.2: *In vivo* assessments of spontaneous activity in vascular nociceptors	30	6 to 15	Phys: Dr. Johnson
Description: Following optimized GW-Chemical exposure (Task 2.2) and behavioral evidence of myalgia,	same as 2.2		
levels of spontaneous activity in single vascular nociceptive			
afferent fibers will be measured in anesthetized, terminal preparations.			
Post-exposure periods will be 12W (n=12) and 16W (n=12) along with 6 saline-controls at each time point			
Milestone A: GWI-Chemical exposure increases spontaneous activity in vascular nociceptors			
TASK 3.3: *In vivo* assessments of the efficacy of treatment with CGRP blocker on autonomic measures	12	14 to 19	Phys: Dr. Johnson
Description: Following optimized GW-Chemical exposure (Task 1.2) and 4 weeks after treatment with CGRP blockers,	same as 1.2		
assessments of spontaneous activity in single vascular nociceptive afferent fibers and hindlimb autonomic			
vascular reflexes will be measured in anesthetized, terminal preparations, to determine if autonomic vascular reflexes			
and spontaneous activity measures are normalized. Post-exposure periods will be 4W (n=6) along with 6 saline-controls			
Milestone A: GWI-Chemical exposure induced changes in autonomic and vascular afferent-mediated reflexes are			
reduced or normalized after CGRP blocker treatment.			

Research TASKS	Preparation Grant Month	Submission Grant Month	University of Florid
TASK 4: Data Reduction and Dissemination			
TASK 4.1: Optimized Protocol	6	Combined with Physiology	Cooper Laboratory
TASK 4.2: Kv7 Measures (TASK 2.1)	12	15	Cooper Laboratory
TASK 4.3: Nav1.9 Measures (TASK 2.2)	12	15	Cooper Laboratory
TASK 4.4: Acute DEET on Kv7 and Nav1.9 (TASK 2.4)	6	9	Cooper Laboratory
TASK 4.5: Spontaneous Activity and Excitability (TASK 2.3)	18	24	Cooper Laboratory
TASK 4.6: Targeted Treatments (TASKs 1.2 and 1.3)	18	24	Cooper Laboratory
TASK 4.7: In vivo autonomic vascular reflexes (TASK 3.1)	18	24	Johnson Laboratory
TASK 4.8: In vivo spontaneous activity in vascular nociceptors (TASK 3.2)	18	24	Johnson Laboratory
TASK 4.9: In vivo efficacy of CGRP blockers on autonomic measures (TASK 3.3)	18	24	Johnson Laboratory

Methods

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. Chlorpyifos was administered by a subcutaneous injection (corn oil) once every 7 days. The dose of chlorpyrifos was intended to represent a net exposure to the potentially large and varied anticholinesterases that soldiers were exposed to in the Gulf theater (Binns et al., 2008).

Chlorpyrifos was administered in a corn oil formulation that released the agent over a couple of days (Smith et al., 2009). DEET was administered topically in ethanol at one of two concentrations (25% or 50%). PB was administered daily by oral gavage (tap water) based upon a standard military dose that was adjusted to account for faster pharmacokinetics in rodents (Birtley et al 1966; Husain et al., 1968; Aquilonius et al., 1980; Breyer-Pfaff et al., 1985). Rats were weighed once per week throughout the studies and doses were adjusted accordingly. Control rats received only vehicle exposures over the identical time course.

Five distinct groups of rats (n=10) were formed (see Table 1). One group received all 4 agents (Group A). Three groups were exposed to DEET at 50% concentration (400 mg/kg; ETOH) while a fourth group received all 4 agents with DEET reduced to half concentration (Group HD; 200 mg/kg; 25% in ETOH). Two groups received only 3 agents: Group PB (PB excluded) and Group CP (chlorpyrifos excluded). Group C served as the control group. There was little indication that any combination of chemical exposures affected final body weight (Table 1).

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

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.

Whole Cell Patch Clamp 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 $MgCl_2$, 2 $CaCl_2$, 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

boroscilicate 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 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 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).

Isolation of $Na_v1.8$ and $Na_v1.9$ 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. $Na_v1.9$ currents were recorded using the 70 mM Na_{iso} solution while $Na_v1.8$ 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 $Na_v1.9$

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 $Na_v1.9$ were measured 250 msec from the start of the voltage step to avoid contamination by $Na_v1.8$. The slow desensitizing $Na_v1.8$ 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: $G=I_{peak}/(V_m-V_{rev})$, 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: $G=G_{max}/(1+\exp((V_{.50}-V_m)/K))$, 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 $Na_v1.8$

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 $Na_v1.8$ 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 $Na_v1.8$ 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 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 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 (-40 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 μM; 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 on Behavior and Whole Cell Patch Studies

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 addition, due to substantial variability inherent in rat behavior measures, the highest and lowest score of each group was excluded from the analyses. Therefore, for the analysis, the final number of rats per group was reduced to 7.

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.

Autonomic Nervous System Studies

Longitudinal measure of autonomic parameters and blood flow: To measure autonomic responses in blood flow in the hindpaw (plantar foot), rats were briefly anesthetized with isoflurane (10-15min as described below for terminal experiment) and placed in sternal recumbency. Using a noninvasive laser speckle contrast imager (LSCI PeriCamPSI, PeriMed, Inc.) blood flow measurements were recorded for five minutes with the laser generator probe positioned 14.9-15.1cm above the tissue and the LSCI sample rate was 53 samples/sec. LSCI technology uses a laser that illuminates the area measured with scattered light and produces a speckled pattern based on the red blood cell movement. This pattern is then captured by a built-in camera and digitized to produce an image with different corresponding colors that represent multiple interference patterns.

Increased red blood cell movement results in a subsequent increased blurred speckled/interference pattern which then translates into increased blood flow. The analysis area on the hindpaw is set by the programmable software based on spatial landmarks and was used for all animals to validate inter-animal and within-animal comparisons. In longitudinal measures, after blood flow data was acquired, body core (rectal probe) and hindfoot (skin thermistors) temperatures were measured. Measurements were taken prior to chemical treatment (baseline), and every two weeks following treatment (2, 4, 6, 8, and 10 weeks) to examine chronic effects of GWI chemicals. Once blood flow measurements were completed, blood pressure and pulse rate were recorded using a computerized blood pressure system, Visitech BP2000 Series II Blood Pressure System, with the animal still anesthetized to prevent movement artifact. The blood pressure cuff was positioned at the base of the animal's tail and 3 consecutive measurements were recorded with a 10 second interval between pressure measurements. Averages of the three recordings were then used for data purposes. Anesthesia was then discontinued and animal returned to the cage for anesthetic recovery.

Surgical Preparation for Terminal in vivo Studies: In initial studies on exposed (all chemicals) and control rats 20-24 weeks after the end of exposure period, anesthesia was induced briefly with isoflurane from a calibrated vaporizer in a calibrated oxygen ventilator circuit interfaced with a rodent induction chamber with an approved scavenging system. Once the animal's movement stopped, it was transferred to the procedure table and fitted with an isoflurane equipped inhalant nose-cone to maintain anesthesia. Baseline blood flow measurements of the hindpaws were recorded with the same methods as mentioned above. Prior to surgery, proper plane of anesthesia was ensured and indicated by loss of the withdrawal reflex, palpebral reflex, and pinna reflex. Using an esophageal probe, body temperature was maintained at 37ºC using a custom circulating

water heating pads under the animal's core and hindlimbs. The trachea, common carotid artery, and jugular vein were intubated to assist with respiration, monitor blood pressure and heart rate, and provide IV access. Ventilation was monitored with an end tidal pCO2 monitor and artificial ventilation was used if necessary with a rodent ventilator supplied with oxygen. Baseline blood pressure was maintained at 75mmHg or above throughout the experiment. The animal was placed in the prone position and the left sciatic nerve was isolated within the ischiorectal fossa and surrounded by a silicon microelectrode cuff containing two silver stimulating electrodes.

Autonomic Measures in Terminal Experiments: After a short acting paralytic agent was administered to eliminate movement (atracurium besylate), LSCI measures of blood flow were obtained in the plantar hindfeet and surgically exposed surface of the distal half of the gastrocnemius muscles. Antidromic or reflex vasodilatation/vasoconstriction of the feet and muscle by a 3sec, 50Hz train burst stimulation (0.8ms pulse duration) of the (i) left distal sciatic nerve stump stimulation of ipsilateral nociceptors, (ii) left whole sciatic nerve stimulation, and (iii) proximal left sciatic nerve stump, were measured with LSCI.

Data Analysis: Blood flow recordings were analyzed using four regions of interest (ROI) that each outlined the left/right hindfeet and left/right heels. The ROI outlining the hindfeet incorporated the entire plantar surface excluding the digits; whereas ROI of the heels were standardized by measuring 1.6cm from the edge of the heel towards the distal midline. Mean blood perfusion values (perfusion units) obtained during the five minute blood flow recordings were documented for statistical analysis. All longitudinal blood flow values were statistically analyzed using MINITAB Statistical Software (MINITAB release 7; Minitab, State College, PA) measuring normality and significant differences between mean perfusion units of exposed and control groups. Terminal experiment blood flow values of the left hindpaw and gastrocnemius muscle, following

left distal sciatic nerve stimulation, were analyzed to determine significant differences between initial perfusion, measured at the end of the latent period of sciatic stimulation, and final (peak) perfusion of exposed and control rats. Normality of mean perfusion values were plotted using a Ryan-Joiner (similar to Shapiro-Wilk) test with a p-value < 0.05 considered a normal distribution. Based on distribution normality or non-normality, parametric (Two-sample t-tests) or non-parametric (Mann-Whitney rank sum tests) analyses were performed comparing exposed and control groups of identical time periods. All statistical tests used an alpha level of 0.05.

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DEET Accelerates Anticholinesterase Dependent Chronic Pain Signs in a Rat Model of Gulf War Illness Pain

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Abstract

Veterans returning from the 1991 Gulf War often developed chronic joint, muscle and other deep tissue pains. In a rat model of GWI (Gulf War Illness) pain, we observed that an 8 week, but not a 4 week, exposure to permethrin, chlorpyrifos (CP) and pyridostigmine bromide (PB) induced a delayed myalgia-like condition 9-12 weeks following cessation of the exposure (Nutter et al., 2015). DEET (N,N-diethyl-meta-toluamide) has been implicated in the development of the symptoms GWI. We examined how the addition of DEET to the exposure protocol influenced the development and persistence of pain-like behaviors. Three groups of rats (n=10) were exposed to permethrin (2.6 mg/kg; topical), CP (120 mg/kg; subcutaneous (s.c.), PB (13 mg/kg; oral gavage), and DEET (200 or 400 mg/kg; topical) or vehicle control (C) (topical ethanol, corn oil s.c., water by gavage) for 4 weeks. Two additional groups received 3 of the 4 GW agents. All rats underwent behavioral testing before, during and after chemical exposures: 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 μM) on muscle and vascular nociceptor K_v7, K_{DR}, $Na_v1.8$ and $Na_v1.9$. We report that DEET significantly enhanced the development and persistence of pain-signs. Rats exposed to all 4 compounds, for 4 weeks, 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 modified nociceptor K_v7, K_{DR}, $Na_v1.8$ or $Na_v1.9$ activity. We concluded that DEET enhanced the development and persistence of pain behaviors. Anticholinesterases CP and PB played a determinant role. Although PB was required for the development of certain pain signs, it also prevented the development of others.

1. 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 repellant 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 week 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.

2. Methods

2.1. Behavioral Studies

2.1.1. 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.

2.1.2. 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. Chlorpyifos was administered by a subcutaneous injection (corn oil) once every 7 days. The dose of chlorpyrifos was intended to represent a net exposure to the potentially large and varied anticholinesterases that soldiers were exposed to in the Gulf theater (Binns et al., 2008). Chlorpyrifos was administered in a corn oil formulation that released the agent over a couple of days (Smith et al., 2009). DEET was administered topically in ethanol at one of two concentrations (25% or 50%). PB was administered daily by oral gavage (tap water) based upon a standard

military dose that was adjusted to account for faster pharmacokinetics in rodents (Birtley et al 1966; Husain et al., 1968; Aquilonius et al., 1980; Breyer-Pfaff et al., 1985). Rats were weighed once per week throughout the studies and doses were adjusted accordingly. Control rats received only vehicle exposures over the identical time course.

Five distinct groups of rats (n=10) were formed (see Table 1). One group received all 4 agents (Group A). Three groups were exposed to DEET at 50% concentration (400 mg/kg; ETOH) while a fourth group received all 4 agents with DEET reduced to half concentration (Group HD; 200 mg/kg; 25% in ETOH). Two groups received only 3 agents: Group PB (PB excluded) and Group CP (chlorpyrifos excluded). Group C served as the control group. There was little indication that any combination of chemical exposures affected final body weight (Table 1).

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

2.1.3. 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.

2.2. Electrophysiological Studies

2.2.1. 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 $MgCl_2$, 2 $CaCl_2$, 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 boroscilicate 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 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 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.

2.2.2. 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 μM 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 μM 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).

2.2.3. Isolation of $Na_v1.8$ and $Na_v1.9$ 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. $Na_v1.9$ currents were recorded using the 70 mM Na_{iso} solution while $Na_v1.8$ 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.

2.2.4. Evocation and Characterization of $Na_v1.9$

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 $Na_v1.9$ were measured 250 msec from the start of the voltage step to avoid contamination by $Na_v1.8$. The slow desensitizing $Na_v1.8$ 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: $G=I_{peak}/(V_m-V_{rev})$, 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: $G=G_{max}/(1+\exp((V_{.50}-V_m)/K))$, 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).

2.2.5. Evocation and Characterization of $Na_v1.8$

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

$Na_v1.8$ 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 $Na_v1.8$ current was measured from the peak current to a point (2500 msec) following the voltage step to 0 mV.

2.2.6. 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 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH.

2.2.7. 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 (-40 to -70 mV) to obtain a mean normalized conductance. The peak conductance was determined by inspection.

2.2.8. 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 μM; 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.

2.3. 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.

3. Results

3.1. Behavior

After a period of acclimation and baseline behavioral testing, rats were divided into 5 groups (n=50). One group 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 second group (HD) received all 4 agents, but DEET was administered at half the concentration (200 mg/kg; 25% in ETOH). 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. All experimental groups were exposed to DEET and permethrin. 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 four 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 shown that an 8 week, but not a 4 week, treatment with identical doses of permethrin, chlorpyrifos and PB produced a delayed emergence of pain-like behaviors that were manifested as an increase in resting and a decrease in movement distance 9-12 weeks following the cessation of dosing (Nutter et al., 2015). Groups of rats exposed to the same concentrations of GW chemicals, but for only 4 weeks, exhibited paradoxical increases in movement rate with only transient increases of rest time scores during a 5 to 12 post-exposure assessment (figure 1D, E and F; reprinted from 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 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), this seemed to be mainly due to variance in the control group rather than recovery in exposed groups (figure 1A). Despite similar sliding of movement rate scores in the final month of control group testing, the slowing of movement rate, due to GWI agents, was still significant 21-24 weeks post-exposure (figure 1B).

Although 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 (figure 1E). As in all our

investigations, we failed to find that these GW agents produced any change in the semitendinosus muscle pressure-pain withdrawal test (PAM; figure 2D; Nutter et al., 2014; Nutter et al., 2015; Cooper et al., 2016). GW veterans do not report mechanical allodynia.

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).

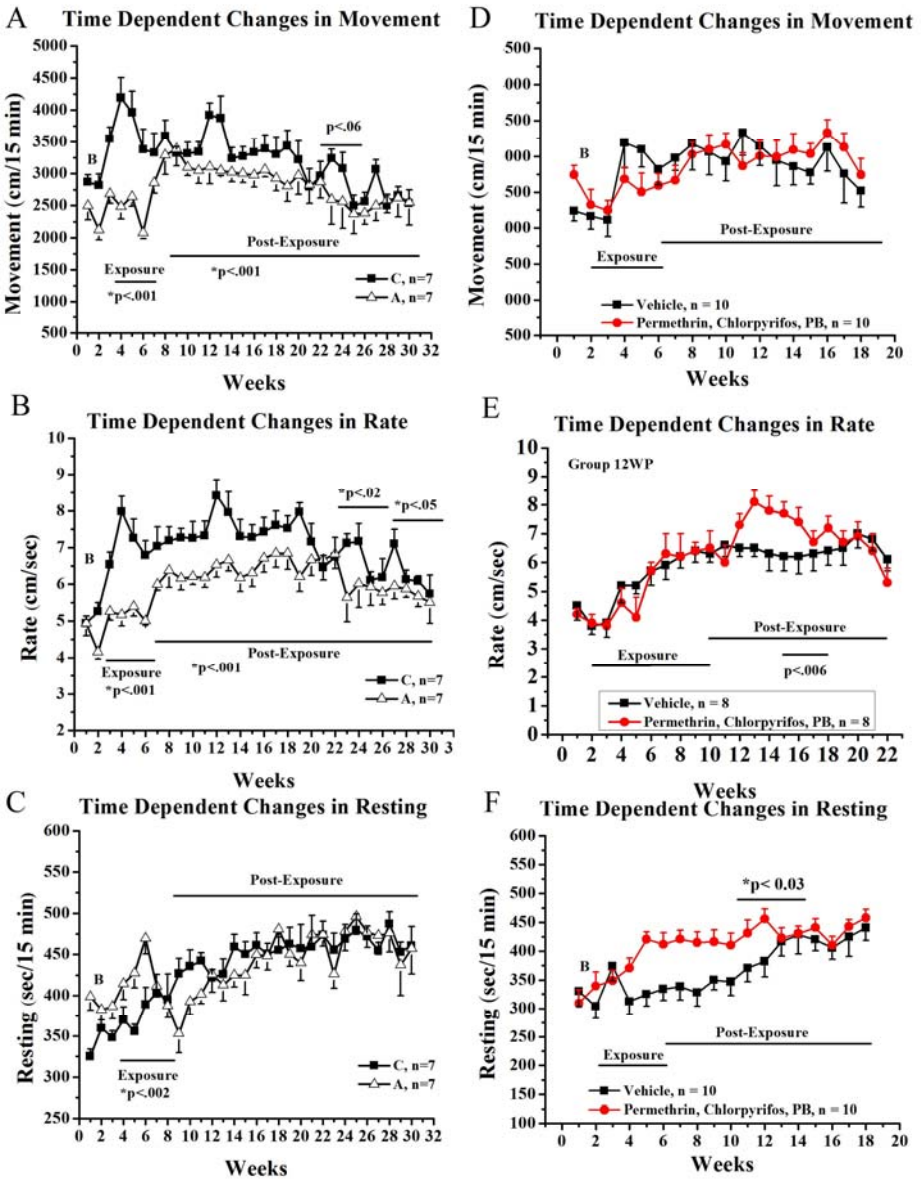

Figure 1. The Inclusion of DEET in the Exposure Protocol Produced Long Lasting Pain-Like Behaviors. A) Movement distance was significantly decreased at 5-12 weeks post exposure ($F=19.47$; $p<.001$). Movement distance pain signs approached significance 17-20 weeks post exposure ($F=3.72$; $p<.06$), but 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. **D and E)** In the absence of DEET, a 4 week exposure to chlorpyrifos, permethrin and PB did not produce any lasting pain-like behaviors. Movement distance was unchanged while average movement rate was paradoxically increased at post 9-12 weeks ($F= 7.23$; $p<.009$). **F)** Resting times were transiently increased during the post-exposure period (5-8 weeks; $F=4.70$; $p<.03$), but these shifts did not persist as far as post exposure weeks 9-12. 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). Panels D, E and F were reprinted from Nutter et al., 2015, and are presented as they were originally analyzed.

3.1.1 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.

3.1.1.1 DEET Concentration was Critical to the Development of Pain Behaviors

When the concentration of DEET was halved (Group HD; 200 mg/kg; 25% DEET), we failed to find any instances in which pain signs developed after a 4 week exposure (figure 2A, B and C). Movement distance and resting scores were paradoxically increased (movement distance) or decreased (resting duration) during the final 4 weeks of testing (post-exposure weeks 17-20). This outcome was partly similar to our previous report that paradoxical shifts were observed when rats were exposed to just the three GWI chemicals at identical concentrations and durations for 4 weeks. In that study, the activity shifts were different in pattern (increased movement rate; figure 1D), but the implications are essentially the same. The higher concentration of DEET (50%) was required to accelerate the development of pain-like signs similar to those that could be observed from an 8 week exposure to only three chemicals (Nutter et al., 2015). 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').

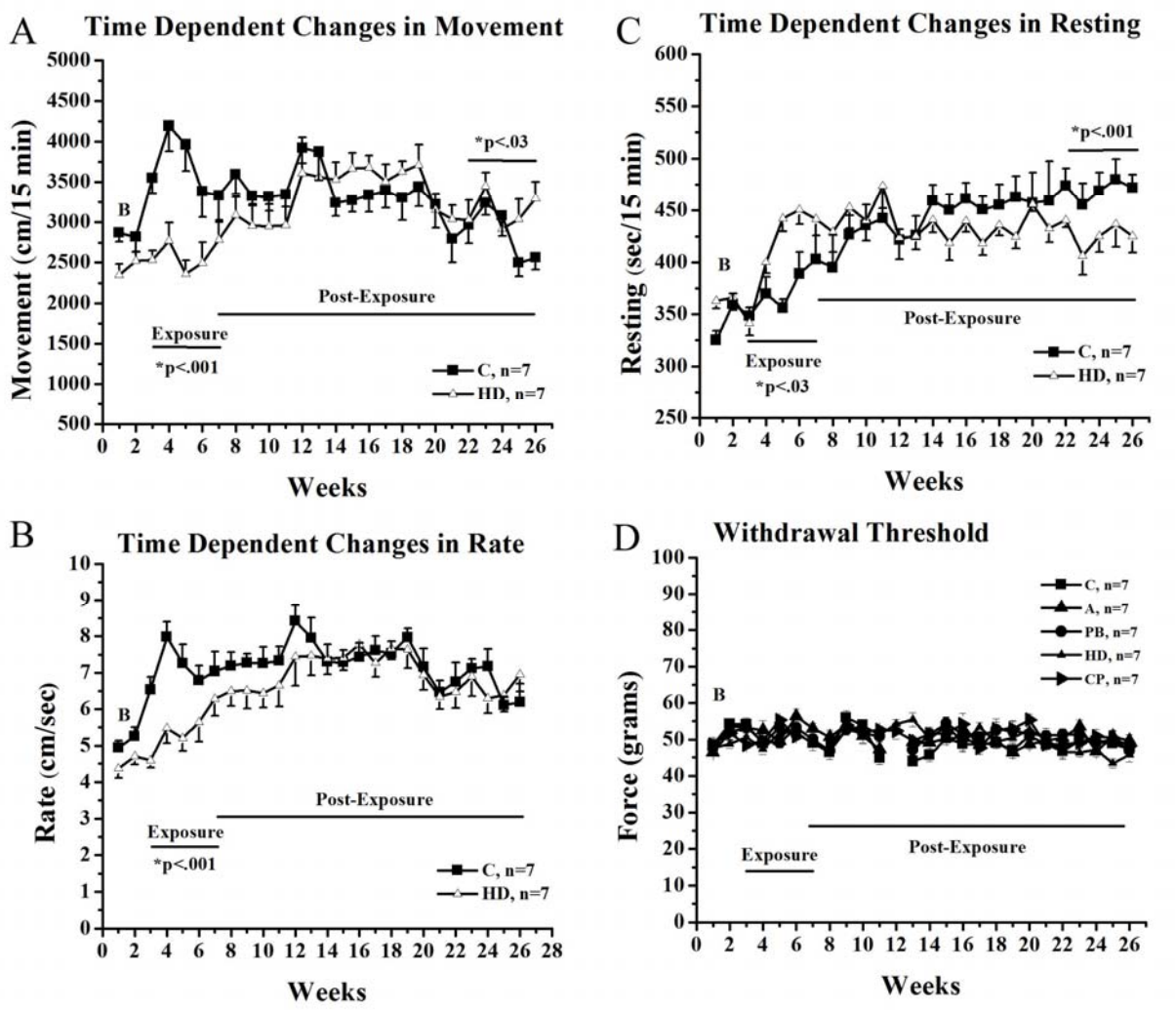

Figure 2. Pain-Like Behaviors Did Not Appear When DEET Concentration was Halved. A) Movement distance was paradoxically increased in during weeks 17-20 (F=5.50). **B)** Average movement rate was unchanged. **C)** Resting duration was paradoxically decreased (weeks 17-20; F=19.51). **D)** PAM withdrawal threshold tests were unchanged in all conditions of the experiment. B: baseline testing; A: DEET (400 mg/kg; 50%), PB, chlorpyrifos, permethrin; C/Vehicle: (ethanol, corn oil, ethanol, water); CP: DEET, PB, permethrin; HD: DEET (200 mg/kg; 25%), chlorpyrifos, PB, permethrin; PB: DEET (400 mg/kg), chlorpyrifos, permethrin.

3.1.1.2 The Contribution of Chlorpyrifos

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

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

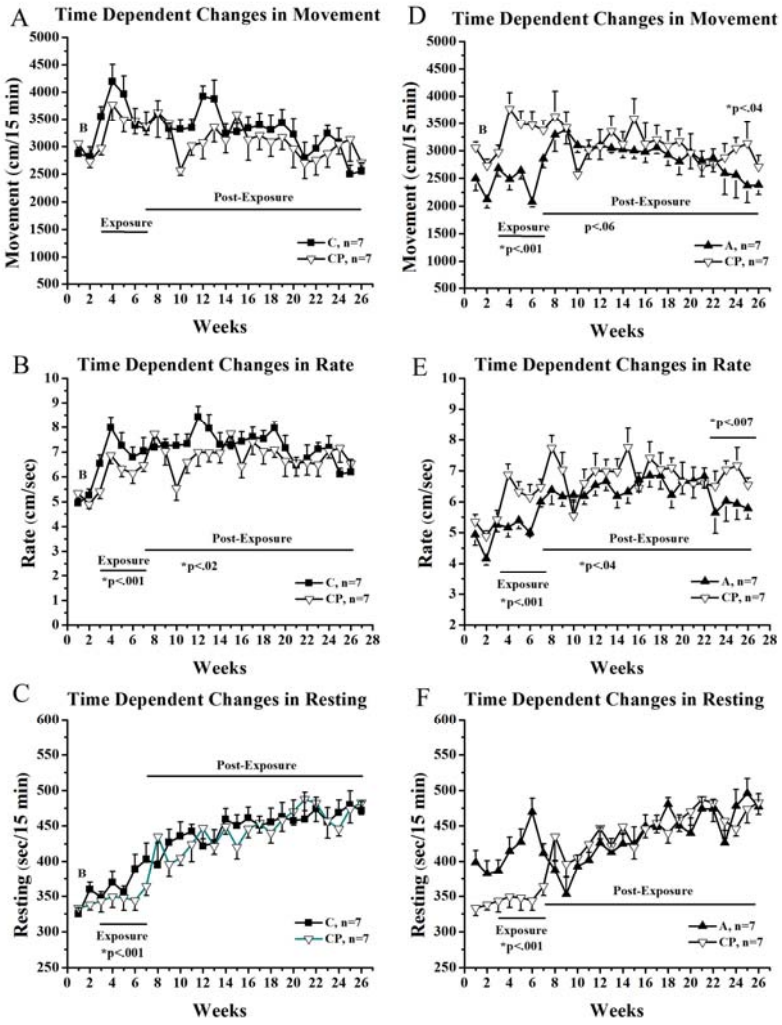

Figure 3. Excluding the AChE Inhibitor, Chlorpyrifos, from the Exposure Protocol Prevented Development of Persistent Pain Behaviors. A) The omission of CP prevented the suppression of movement (distance) by GW chemicals. **B)** Average movement rate was still significantly reduced in the early post-exposure 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.

3.1.1.3. 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.

3.2. 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).

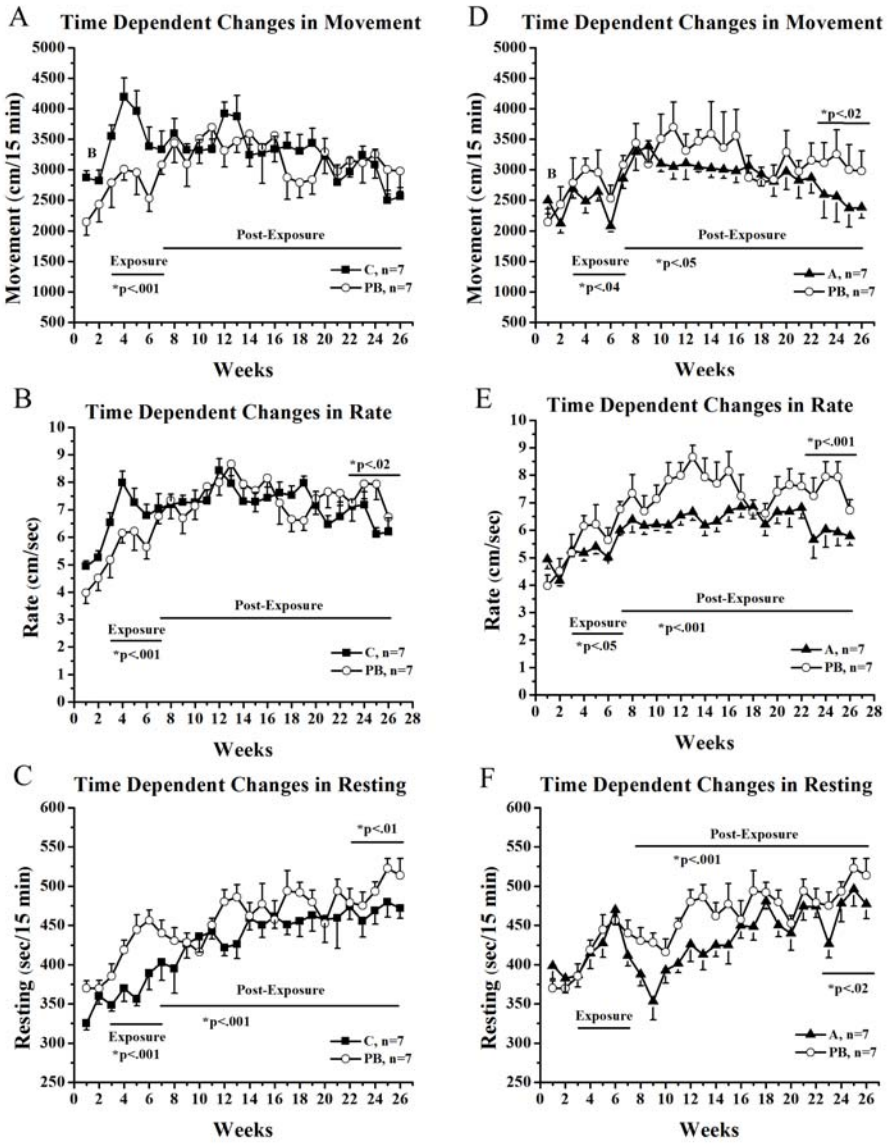

Figure 4. Excluding Pyridostigmine Bromide from the Exposure Protocol Differentially Contributed to the Development and Persistence of Pain Behaviors. A) Movement distance was unaffected in the absence of PB. **B)** Except for a paradoxical increase in weeks 17-20 (F=5.05), the average movement rate 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.

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 μM) 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).

3.2.1. 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 μM). 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 μM) 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.

3.2.2. 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). $Na_v1.8$ is expressed in a high proportion of nociceptors and is the principle Na_v that forms action potentials in these neurons (Djouhri et al., 2003; Jiang and Cooper, 2011). Prolonged exposure to permethrin, chlorpyrifos and PB changes inactivation characteristics of $Na_v1.8$ and prolongs action potential duration (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al., 2015). Given that high concentrations of DEET (>500 μM) 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).

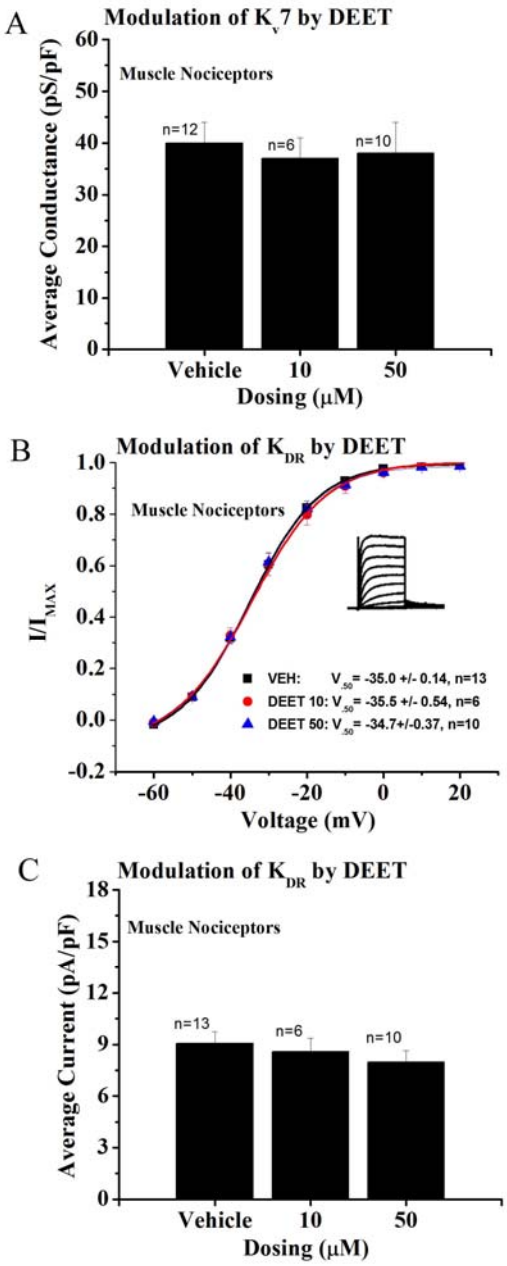

Figure 5. Voltage Activated K^+ Channels were Unaffected by Acute Exposure to DEET. A) The average conductance of muscle nociceptor K_v7 channels was not altered by DEET (10-50 μM); **B) and C)** Following a 12 minute exposure, there was no indication that either the voltage dependence or the average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

3.2.2.1 The Influence of DEET on $Na_v1.8$

Cells were plated as described above. Following identification of a neuron as a muscle or vascular nociceptor, $TTX_{insensitive}$ Na_v were isolated from other voltage activated currents using the Na_{iso} solution ($[Na^+]$ = 20 mM) and 500 nM TTX (described in 'Methods'). Time dependent changes to $Na_v1.8$, 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 μM) 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 $Na_v1.8$ 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 $Na_v1.8$ in either muscle or vascular nociceptors (figure 6).

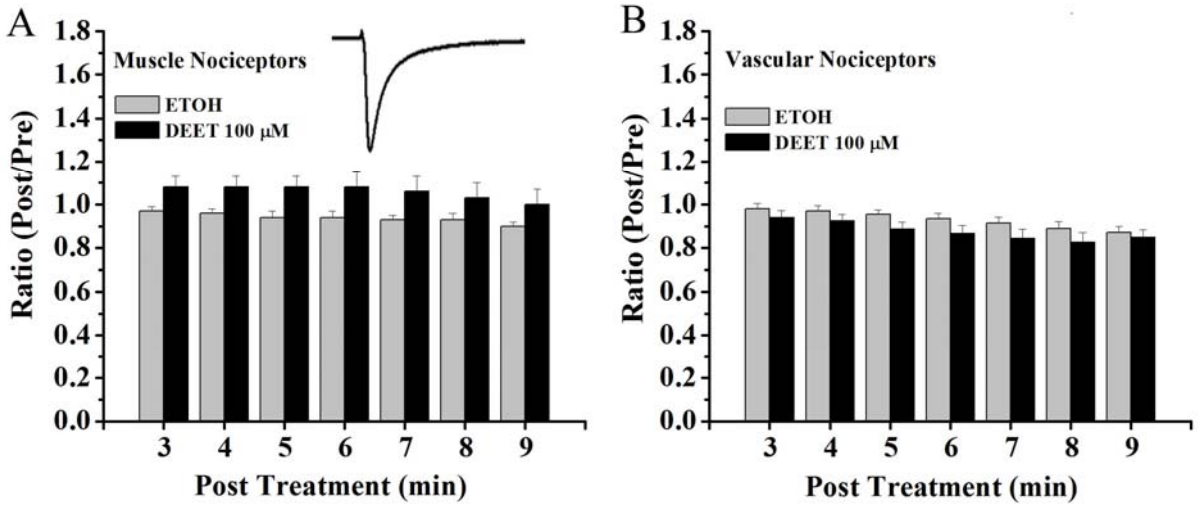

Figure 6. Time Dependent Modification of $Na_v1.8$ by DEET. A) The amplitude of muscle nociceptor $Na_v1.8$ was not changed by DEET (100 μM). A representative Nav1.8 current is inserted. **B)** Vascular nociceptor $Na_v1.8$ amplitude was not altered by DEET (100 μM). Baseline records were taken prior 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.

3.2.2.2. The Influence of DEET on $Na_v1.9$

Following an 8 week exposure to three GWI chemicals, the amplitude of $TTX_{insensitive}$, $Na_v1.9$ was significantly increased (Nutter et al., 2014). Although $Na_v1.9$ 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, $Na_v1.9$ 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 $Na_v1.9$ during exposure, it might amplify the post-exposure influence of GWI chemicals (permethrin, chlorpyrifos and PB) on the physiology of $Na_v1.9$. Accordingly, we examined whether an acute presentation of DEET would modify this unique $TTX_{insensitive}$ Na_v channel.

Muscle and vascular nociceptors were isolated as described above. A Na_{iso} solution ($[Na^+]$ = 70 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 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 $Na_v1.9$ ($V_{.50}$; 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.

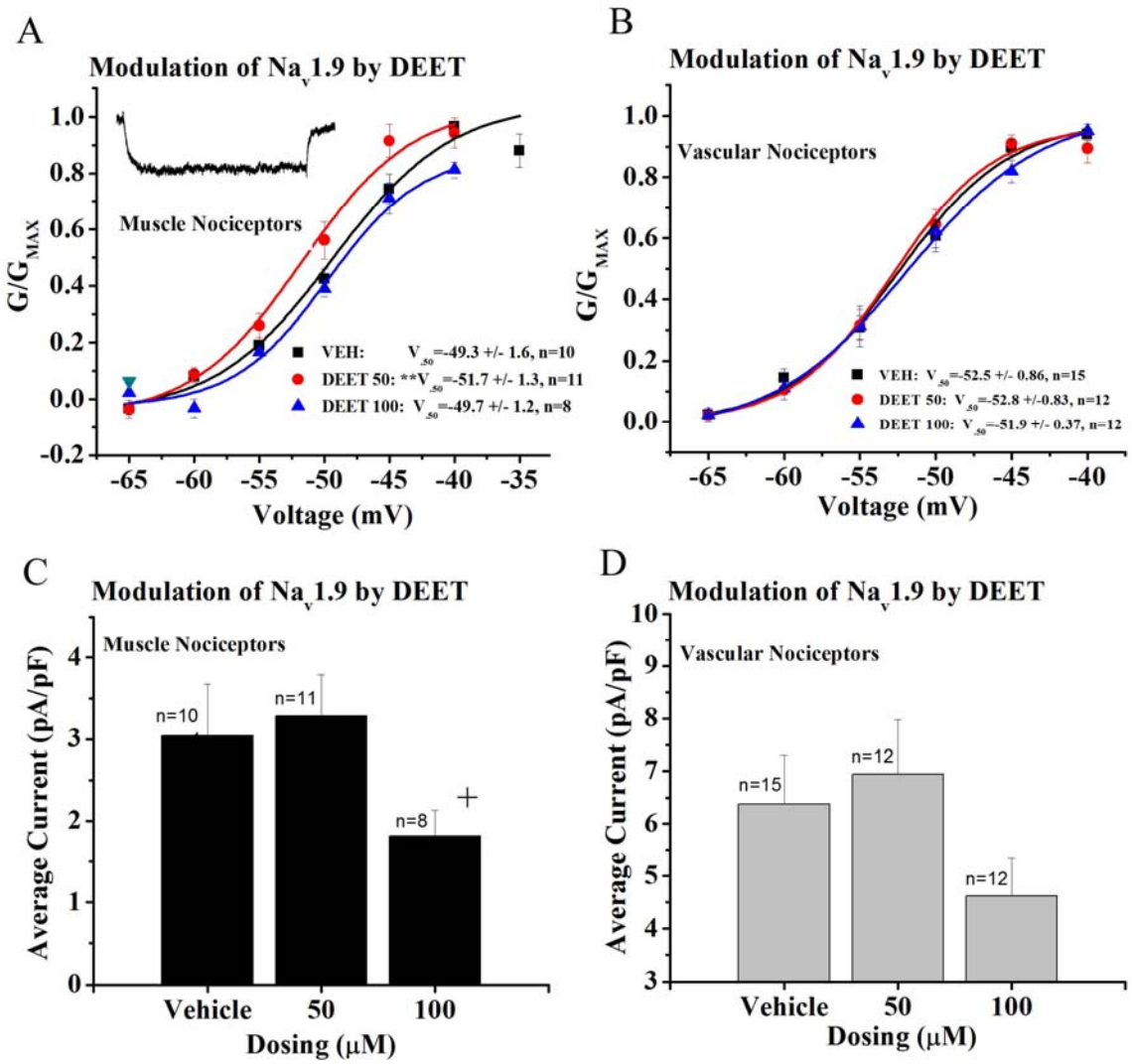

Figure 7. Voltage Activated, Na$_v$1.9, Channels were Weakly Modulated by Acute Exposure to DEET. A) The voltage dependent activation of muscle nociceptors was hyperpolarized at 50 but not at 100 μM DEET. A representative trace of a Na$_v$1.9 current (step to -50 mV; V_H=-120 mV) is included as an insert. **B)** The voltage dependent activation of vascular nociceptors was unaffected by DEET. **C)** Average currents of muscle nociceptors were unaffected by DEET ($p<.13$, Vehicle vs 100 μM; $p<.05$, 50 vs 100 μM); **D)** Vascular nociceptor average currents were unchanged ($p<.17$, Vehicle vs 100 μM; $p<.08$, 50 vs 100 μM). 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 μM tests. Thirty-two rats contributed to these graphs.

4. 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 repellant 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. Rat ambulatory behaviors were suppressed as early as 5-12 weeks post-exposure and remained suppressed out to the 21-24 week test period. Despite the powerful influence of DEET, the development and persistence of these pain signs was shown to be mainly dependent, albeit in a complex fashion, on the anticholinesterase components of the exposure protocol.

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

occurring during the exposure. Given this evidence, it appeared that the net amount of anticholinesterase activity in the 4 week exposure period drove the development of ambulation pain signs represented by decreased movement distance and average rate.

Finding that anticholinesterases played a fundamental role in these behavioral deficits fits well with our recent demonstration of maladaptations in mAChR signaling in muscle nociceptors harvested from rats that had undergone an 8 week exposure to chlorpyrifos, PB and permethrin (Nutter et al., 2015; Cooper et al., 2016). It also fits well with reports from multiple laboratories of disturbances in mAChR expression in the CNS after exposure to chlorpyrifos, PB and other agents, in various combinations (Nostrandt et al., 1997; Liu et al., 1999; Huff et al., 2001; Zhang et al., 2002; Abou-Donia et al., 2004; Padilla et al., 2005; Pung et al., 2006; see also Abou-Donia et al., 2003; Abdel Rahman et al., 2004a; Abdel Rahman et al., 2004b; Zou et al., 2006; Proskocil et al., 2010). A variety of motor and cognitive signs have been also been documented following extended exposure to these GW chemicals; although it is not clear that these are related to maladapted mAChR expression (Servatius et al., 1998; Servatius et al., 2000; Hoy et al., 1999; 2000; Abou-Donia et al., 2001; Abdel-Rahman et al., 2004a; Abou-Donia et al., 2004; Parihar et al., 2013).

4.1. 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, whatsoever, seemed to be attributable to the inclusion of DEET in the exposure set. Previous attempts to create a chronic pain state with

this same group of agents (chlorpyrifos, permethrin, PB), with a 4 week exposure, failed to produce any lasting pain signs (Nutter et al., 2015). Expanding exposures to 8 weeks subsequently revealed ambulation and resting pain signs (Nutter et al., 2015) that were partially replicated in a subsequent study (Cooper et al., 2016). 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). As we have now shown, there was no evidence that DEET interacted directly with nociceptor ion channels believed to be important for the development of a chronic pain condition consequent to exposure to GWI chemicals.

We had reported previously that exposure to chlorpyrifos, permethrin and PB, sufficient to produce ambulatory and resting pain signs, was paralleled by diminished conductance through muscle nociceptor, K_v7, ion channels (Nutter et al., 2014). While DEET clearly accelerated behavioral maladaptations, we could not demonstrate, in this report, any acute influences of DEET on K_v7 properties that would predict some further aggravation of the molecular effect. Nor did we observe that DEET might contribute to modulation of muscle or vascular nociceptor $Na_v1.8$; a known target of the pyrethroid insecticide permethrin (Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006), and potentially associated with reported changes in muscle nociceptor action potential properties (Nutter et al., 2015). Although an 8 week exposure to chlorpyrifos, permethrin and PB, was previously shown to modify activity of $Na_v1.9$, acute exposure to DEET had inconsistent and purely pharmacologic influences on the physiology of this ion channel (Nutter and Cooper, 2014). 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.

Functional synergisms that occur between several GW agents on behaviors and/or ion channel physiology could arise indirectly. When multiple xenobiotics are present in the bloodstream, they compete for entry into hepatic pathways that ultimately process them to inactive metabolites. Because there are often multiple pathways leading to several metabolites, the presence of other agents that utilize some these pathways will shift the pattern of metabolites produced (Abou-Donia et al., 1996; Usmani et al., 2002; Abu-Qare and Abou-Donia, 2008; see also Choi et al., 2004). The functional consequences of these interactions can be complex. Complexities arise from the delayed metabolism of agents competing for the same enzymatic pathway, and also due to the fact that some agents are first converted into more toxic derivatives. The metabolism of chlorpyrifos, DEET, and PB exemplify these issues.

Although CP is an AChE inhibitor, one of its hepatic metabolites, chlorpyrifos-oxon is 1,000 fold more potent in that role (Huff and Corcoran, 1994). In the presence of DEET, the hepatic conversion of chlorpyrifos to its oxon form is increased 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 Abuo-Donia, 2008). Moreover, when PB is co-administered with DEET, it will slow 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 accelerated and prolonged ambulatory deficits.

4.2. Complex Influences of AChE Inhibitors on Pain Signs

The evidence that linked 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 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.

We could consider the ambulation and rest measures as points on a pain severity scale, in which reduction of movements (distance) and characteristics of movements (average rate) represent a lesser degree of pain than resting. Certainly, resting represents a cessation of movement altogether and is thereby an ultimate reduction of distance and rate. Accordingly, exposure to anticholinesterases CP and PB, in the presence of DEET, leads to development of a chronic pain that reduces and slows movement. When the protective effect of PB is absent, more extreme pain signs develop that are reflected in rest duration measures. The failure of this interpretation lies in the fact that this scenario predicts an even greater reduction of movement and rate scores in the absence of PB. This did not occur. Instead, movement and rate scores were

rescued by removing PB from the exposure set. Therefore the divergence between ambulation and resting must have another interpretation.

The disparate influence of the two anticholinesterases on rat activity measures could be due to distinct actions of neurotoxicants on various physiological targets. This could be as simple as the difference between the actions of a systemic anticholinesterase, such as chlorpyrifos, and one, such as PB, whose actions are restricted to the periphery (Weinbroum, 2004; Newmark, 2005; Weissman and Raveh, 2011). Alternately, an explanation might be found in the distinction between PB as a prophylactic against an irreversible anticholinesterase, versus its inability to oppose the extra-anticholinesterase activity of chlorpyrifos-oxon.

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, chlopryrifos-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). As noted above, 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 the PNS (Nutter and Cooper, 2015; Cooper et al., 2016). It is not clear whether the shift in receptor expression is the result of prolonged loss of AChE activity or due to the extra-anticholinesterase properties of chlorpyrifos-oxon.

PB is known to interfere with DEET metabolism, increasing DEET persistence, and thereby having the potential to accentuate the conversion of chlorpyrifos to its oxon form (Usmani et al., 2002; Abu-Qare and Abou-Donia, 2008). The presence of PB in the exposure set could have promoted the oxon dependent maladaptations (i.e., ambulation), and the absence of PB could have rescued them via the resulting increase in DEET metabolism during the critical exposure period. As we have shown, reducing the concentration of topical DEET (400 to 200 mg/kg; 50%-25%) powerfully diminished its capacity to accelerate the development of ambulation pain signs. We cannot confirm that the exclusion of PB would have resulted in a functional reduction of DEET to

that extent. While a strong statistical association has been reported between the presence of pain, in veterans with GWI, and the use of DEET (75%) and pyridostigmine bromide (Haley and Kurt, 1997), it is by no means certain that this acceleration was due to a hepatic interaction between these agents. Nor can we exclude an alternative interpretation. It is also possible that the distinction between ambulation and resting deficits does not represent a divergence of pain signs, but rather represents a distinction between the sensory and motor manifestations of exposure to GWI chemicals.

4.3. Summary and Conclusions

DEET substantially accelerated and prolonged the pain signs that developed after a 4 week exposure to GW agents. 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 the anticholinesterases on their molecular targets.

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). The pain symptoms of GW veterans were highly diverse. They were manifested in variable patterns that included muscle, joint, back pain, abdominal pain and headache (Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). While extensive emphasis has been placed upon delayed emergence of the symptoms of GWI, about 25% of veterans developed GWI while still in theater (Kroenke et al., 1998).

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). The variations of 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 anticholinesterases as a primary risk factor for pain. Doubling the exposure duty cycle to PB and CP produced manifestations of pain-like behaviors and shifted muscle nociceptor physiology consistent with a chronic myalgia (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 pyridostigmine bromide.

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

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