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14. ABSTRACT

It has been reported that exposure to DEET was highly correlated with the development of GWI pain. We examined the consequences of adding DEET to an exposure protocol composed of permethrin, chlorpyrifos and pyridostigmine bromide (PB) on the development of pain behaviors and autonomic dysfunction in a rat model of GWI. It was demonstrated that the development and degree of pain behaviors were greatly accelerated and magnified in the DEET augmented protocol. These pain behavior signs were accompanied by peripheral chronic vasodilation. However, the appearance of any pain behaviors or chronic vasodilation were wholly dependent on the presence of anticholinesterases (PB, chlorpyrifos) in the exposure set. A series of cellular and molecular studies indicated that the pathophysiology of this pain was due to maladaptations to ion channel $Na_v1.9$ and muscarinic receptors linkages to K_v7 and TRPA1 in vascular and muscle nociceptors. The K_v7 opener, Retigabine, significantly reversed pain signs in rats 9-13 weeks following exposure. The capacity to reverse an established chronic myalgia in a rat model suggests that K_v7 openers could be a promising treatment for GW veterans suffering from chronic pain.

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 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).

It had been reported that application of high concentrations of, the insect repellant, DEET was uniquely associated the pain symptoms of GWI (Haley and Kurt, 1997). In the project covered by this report, we examined the contribution of DEET to the development and persistence of post-exposure pain behaviors and the molecular maladaptations that accompany them. 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, TRPA1, Nav1.9, MDBD

3. Accomplishments

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 79).

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, pattern 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 cellular changes (e.g., excitability) in deep tissue nociceptors following exposure to the DEET augmented protocol
7) Characterize alterations of vascular nociceptor function, in vivo, in GW chemical exposed rats.
8) Determine whether FDA approved and experimental drugs can reverse pain behaviors of rats in our model of GWI pain.

A summary of accomplishments over the 2 year grant period (see below for details):

1. The addition of DEET to the exposure protocol (permethrin, chlorpyrifos and PB) accelerated and prolonged the pain signs that developed after a 4 week exposure.
2. DEET alone had no direct impact on the function of two molecular species previously shown to be modified by chronic exposure to permethrin, chlorpyrifos and PB (K_v7, $Na_v1.9$).
3. The appearance of pain-like signs was variously dependent on the anticholinesterases (PB and chlorpyrifos) or permethrin. Removal of any of these from the chronic exposure period prevented development of one or more ambulation deficits. Resting had a unique relationship to PB.
4. Consistent with a 3 agent exposure, K_v7 activity was decreased by the DEET augmented exposure protocol.
5. Consistent with a 3 agent exposure, Nav1.9 activity was increased by the DEET augmented exposure protocol.
6. Exclusion of PB from the 4 agent exposure group, blocked the development of pain signs but did not alter the appearance of K_v7 deficits.
7. Exclusion of PB from the 4 agent exposure group, blocked the development of pain signs and reduced the activity of $Na_v1.9$.
8. Treatment with the K_v7 opener, Retigabine, significantly improved pain signs that developed 9-12 weeks following exposure to the 4 agent protocol.
9. Deep tissue nociceptor excitability was significantly greater in rats exposed to 4 agents.
10. The increased nociceptor excitability was due to greater muscarinic receptor evoked TRPA1.

11. Exposure to the 4 agent protocol produced chronic vasodilation.
12. The development of chronic vasodilation was prevented by exclusion of PB from the exposure set.

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

This aim was designed to determine the specific contribution of various components of the exposure protocol. Once those aspects were identified, outcome-linked treatments could 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.

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).

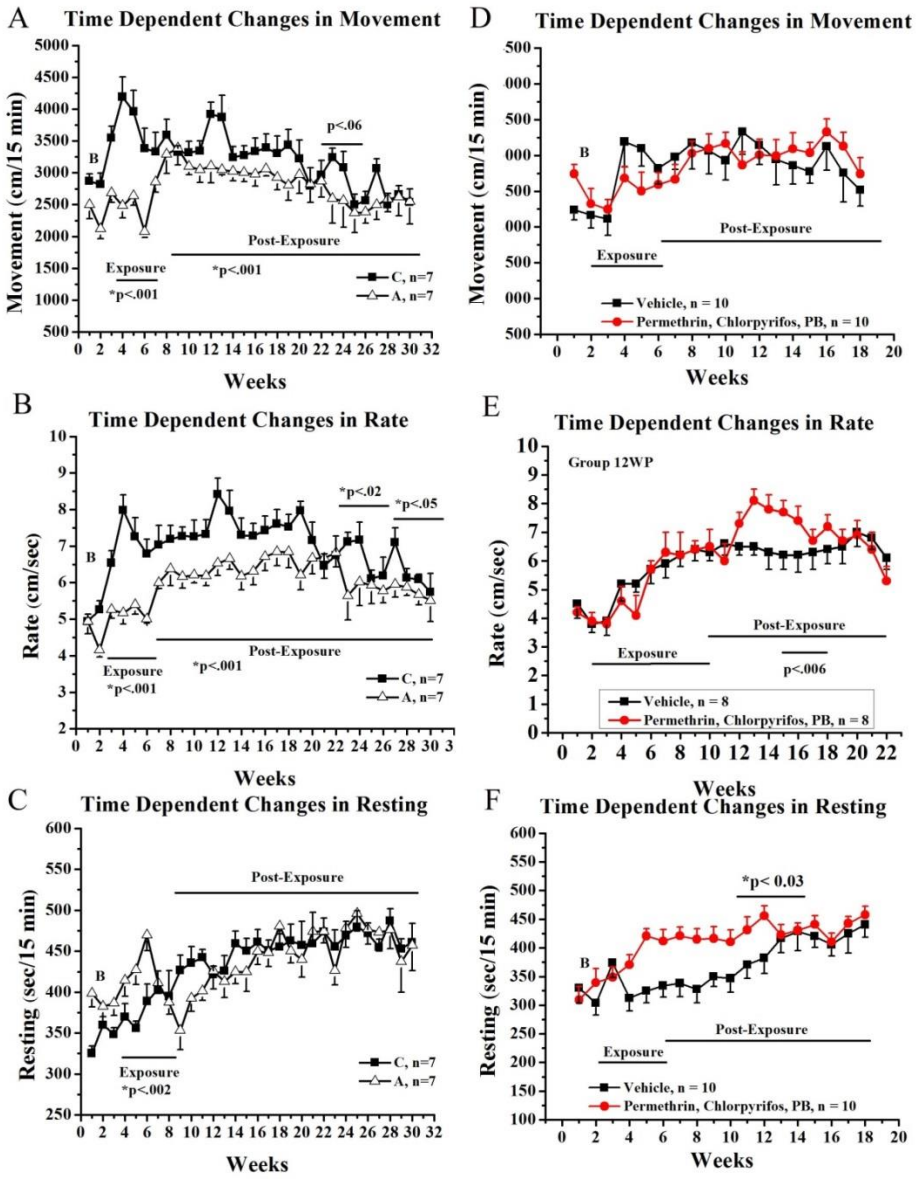

Figure 1. The Inclusion of DEET in the Exposure Protocol Produced Long Lasting Pain-Like Behaviors. A) Movement distance was significantly decreased at 5-12 weeks post exposure ($F=19.47$; $p<.001$). Movement distance pain signs approached significance 17-20 weeks post exposure ($F=3.72$; $p<.06$), but 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.

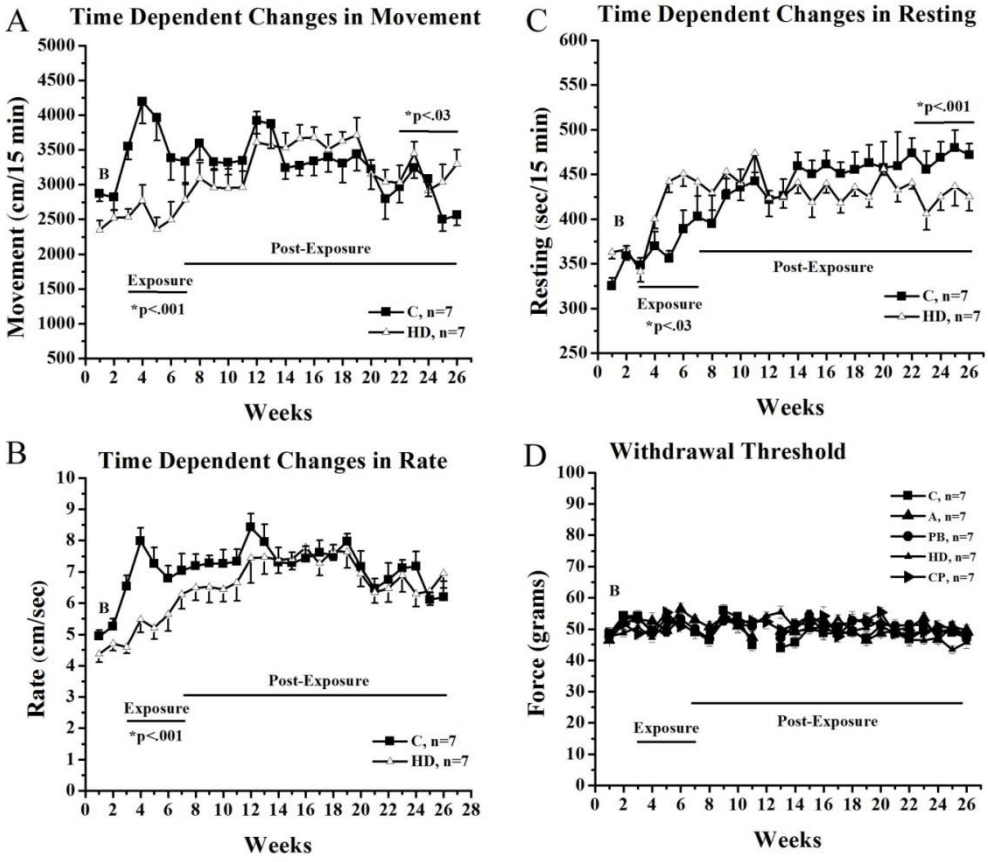

Figure 2. Pain-Like Behaviors Did Not Appear When DEET Concentration was Halved. A) Movement distance was paradoxically increased in during weeks 17-20 (F=5.50). **B)** Average movement rate was unchanged. **C)** Resting duration was paradoxically decreased (weeks 17-20; F=19.51). **D)** PAM withdrawal 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.

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.

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.

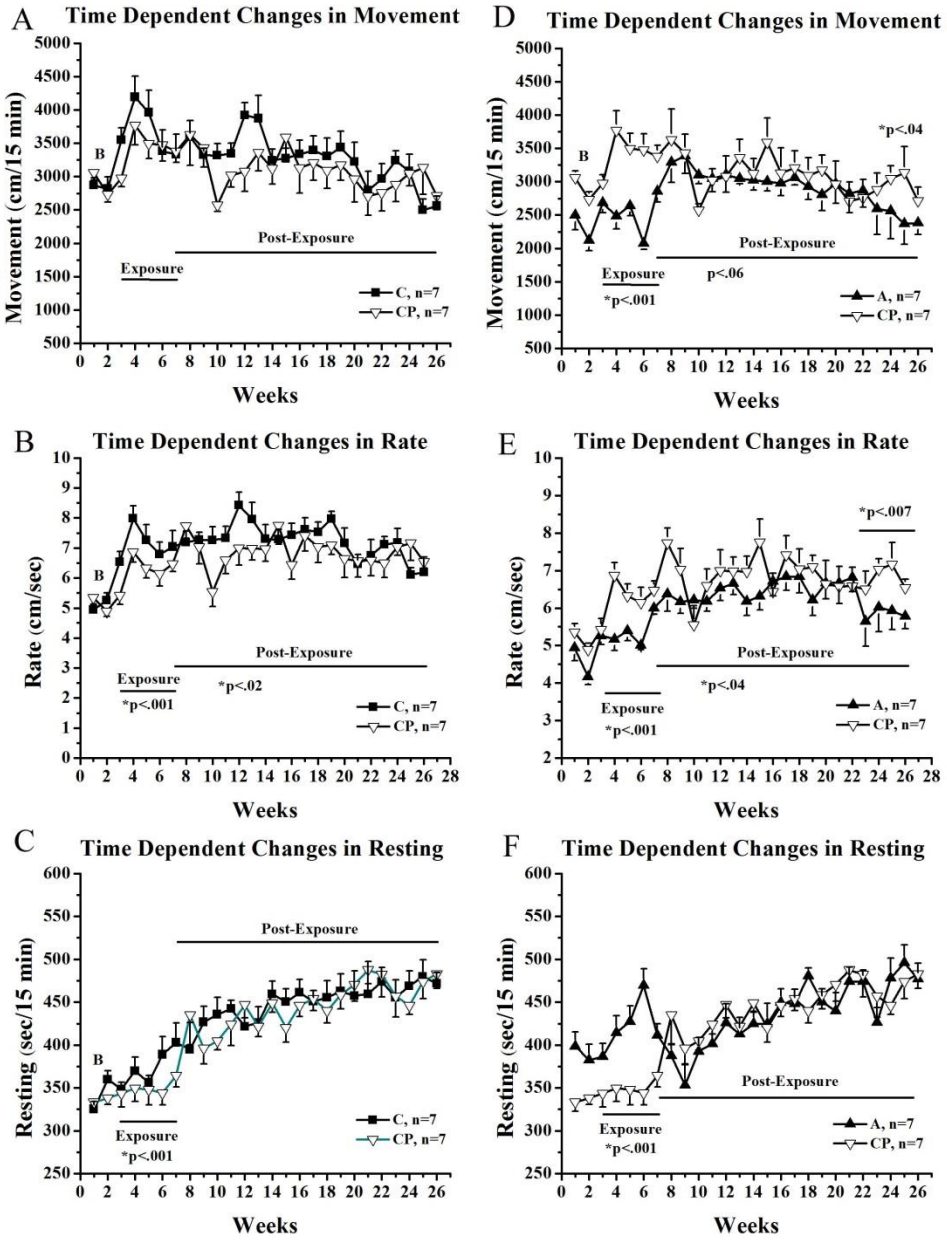

Figure 3. Excluding the AChE Inhibitor, Chlorpyrifos, from the Exposure Protocol Prevented Development of Persistent Pain Behaviors. A) The omission of CP prevented the suppression of movement (distance) by GW chemicals. **B)** Average movement rate was still significantly reduced in the early post-exposure 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 Flunker et al., 2017 (Discussion; Appendix, p. 146).

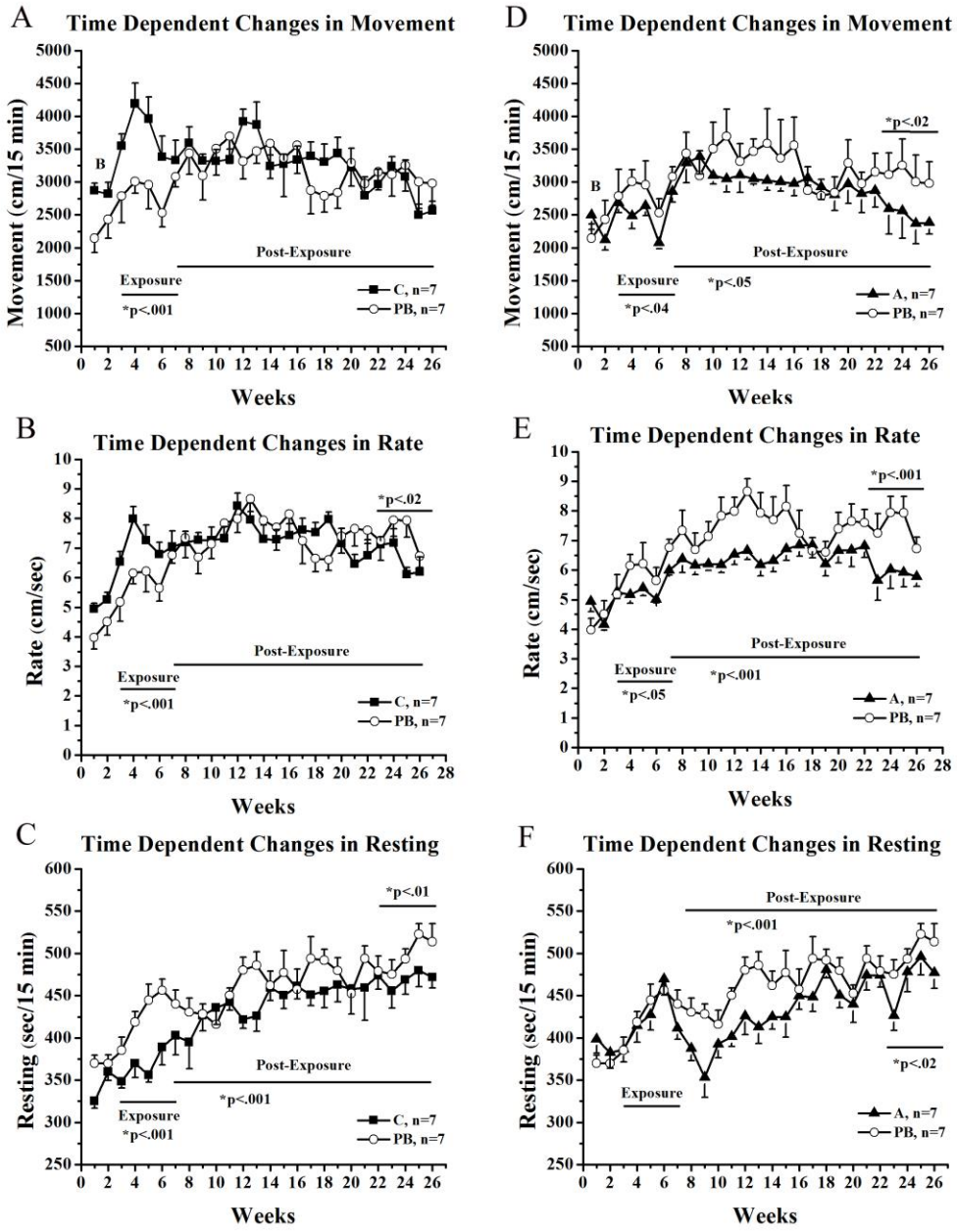

Figure 4. Excluding Pyridostigmine Bromide from the Exposure Protocol Differentially Contributed to the Development and Persistence of Pain Behaviors. A) Movement distance was unaffected in the absence of PB. **B)** Except for a paradoxical increase in weeks 17-20 (F=5.05), the average movement rate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

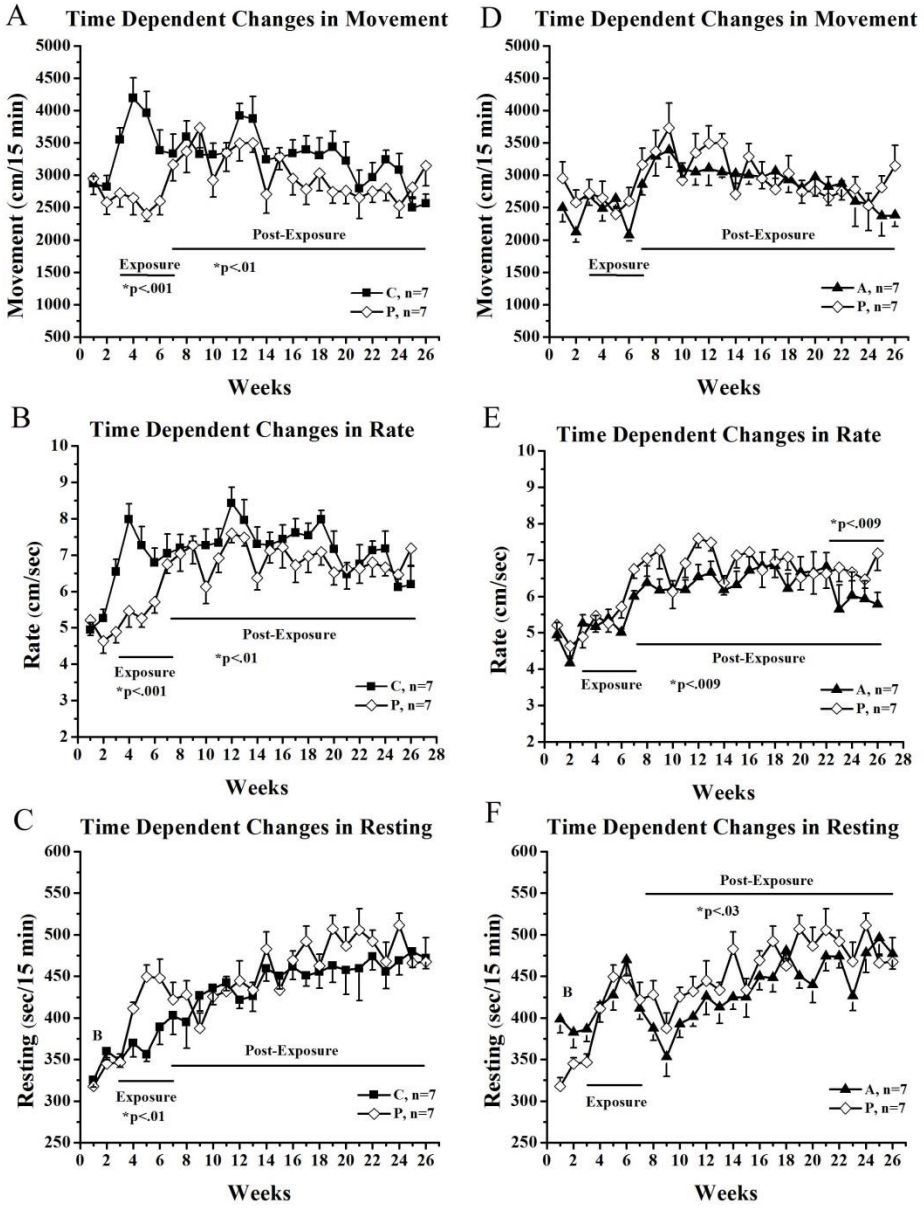

Figure 5. Excluding Permethrin from the Exposure Protocol Blocked of Pain Behavior Persistence. A) Significant movement distance effects were retained over post-exposure weeks 5-12 (F=6.79) despite the omission of permethrin, but blocked in weeks 17-20. **B)** Average movement rate scores were still 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.

Summary and Conclusions for Task 1.1

DEET substantially accelerated and prolonged the pain signs that developed after a 4 week exposure to GW agents. The pattern of pain signs associated with a DEET augmented exposure set diverged from those observed after an 8 week exposure to the same GW agents in the absence of DEET (Nutter et al., 2015; Cooper et al., 2016). 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). When DEET was included in the exposure set, pain signs developed more rapidly, suggesting that DEET exposure was important for those veterans that experienced early symptoms. However the inclusion of DEET in the exposure set did not invariabley result in rapid pain signs observed in figure 1 (and figure 9, below). As will be shown below, other groups exposed to the 4 GW chemicals exhibited delayed development of ambulation signs (see figures 6, 7 and 8 below), and in one instance developed resting signs that were not present in the experiments of Task 1 (see figure 9).

Soldiers deployed to the Persian Gulf were 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. Our data indicated that different patterns of behavioral signs covaried with inclusion/exclusion of PB and permethrin.

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).

TASK 1.2: Targeting Maladapted Ion Channel Proteins with Systemic Treatments

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

We exposed groups of rats to the optimized protocol (DEET, chlorpyrifos, PB, permethrin) in order to determine whether drug treatments that targeted previously demonstrated molecular maladaptations could acutely or permanently reverse pain signs that persisted following the exposure to GW chemicals. We had demonstrated, in year 1, that the DEET augmented exposure produced rapid, robust and persistent pain signs that were suitable for drug treatment studies. Based upon our findings in TASK 2, the molecular targets for treatment drugs were K_v7 and $Na_v1.9$. We also had a strong interest in targeting CGRP (calcitonin gene related peptide) receptors, because activity in vascular nociceptors would release CGRP, induce vasodilation and generate a host of pro-inflammatory consequences in both the CNS and PNS (Li et al., 2008; De Corato et al., 2011; Malon et al., 2011). As shown below, persistent vasodilation was part of the symptom complex that developed in out model.

We had previously shown that K_v7 was down regulated following exposure to either 3 GW chemicals (permethrin, chlorpyrifos and PB; 8 week exposure; Nutter et al., 2015) or 4 GW chemicals (DEET, permethrin, chlorpyrifos and PB; 4 week exposure; year 1 report; Flunker et al., 2017). Down regulation of this ion channel increases nociceptor excitability and could contribute to a chronic pain condition (Brown and Passmore, 2009).

We used the established and clinically effective K_v7 opener, Retigabine, in order to examine whether rat pain behaviors could be temporarily or permanently reversed. K_v7 openers have been

used to treat some forms of chronic pain with success (Flupirtine; Devulder et al., 2010). In the studies below, we examined whether a 4 week treatment with Retigabine could acutely and/or permanently reverse the pain-like symptoms that developed in our GWI model.

Retigabine Treatments Reverse Pain-Like Behaviors

Rats were prepared for behavioral studies (see Appendix, Methods, p. 84). GRP A2 was composed of rats that were exposed, for 4 weeks, to DEET, permethrin, chlorpyrifos and PB. GRP C2 rats were exposed only to the corresponding vehicles. Ambulation and resting behaviors were assessed once per week.

Retigabine treatments were initiated at 9WP (9 weeks post exposure) in half of the GRP A2 rats. These treated animals were designated as GRP R. Retigabine treatment continued daily for 4 weeks (9-12WP; 1200 mg/kg/day in DMSO; oral gavage). Half of GRP A2 and all GRP C2 rats received DMSO during the treatment phase. Behavior testing occurred daily within 2-3 hours of gavage. In order to determine the persistence of any significant Retigabine related recovery, testing continued for another month after the treatments ended (13-16WP).

Rats of GRP A2 developed pain-like behaviors. These were manifested as a significant decrease in ambulation. The onset of effects were delayed in a manner consistent with most GW veterans (~75%; Kroenke et al., 1998). Movement distance and average rate of movement were significantly decreased at 9-12WP following the 4 week exposure to GW chemicals (figure 6A and B; $p<.01$ and $p<.002$ respectively). Movement rate was also decreased at 16WP.

Treatment with Retigabine (GRP R) produced mixed, but generally good outcomes. Retigabine rescued the exposure-induced reduction in average movement rate. Average movement rate of GRP R rats was significantly increased relative to vehicle treated GRP A ($p<.006$), and was not significantly depressed relative to vehicle treated (GRP C2) animals (figure 6B). These outcomes are consistent with a reduction in chronic pain behaviors. Over the years in which we have used this GWI model, rate of movement has been the most consistent measure of persistent and delayed deficits that appear following GW chemical exposure (Nutter et al., 2015; Flunker et al., 2017).

Movement distance score outcomes were less definitive. Although Retigabine treated animals 'improved' to the extent that their scores were not significantly decreased relative to GRP C2 ($p<.10$), trends were still strongly towards reduced movement distance. Moreover, Retigabine failed to significantly improve movement distance scores relative to GRP A2 rats (figure 6A).

Resting scores were not significantly affected by GW chemical exposure ($p<.12$). This is consistent with our prior published reports (Flunker et al., 2017). Retigabine paradoxically increased resting scores over that of control subjects; suggesting some non-specific effects (GRP R vs GRP C2; figure 6C; $p<.002$). Resting scores returned to normal levels after Retigabine treatments ended. These non-specific effects of Retigabine are not likely to have falsely impacted rate and movement scores as those manifested increased activity with Retigabine treatment. Retigabine is known to produce drowsiness in humans (Ciliberto et al., 2012).

Although Retigabine significantly improved ambulation scores during the treatment phase, once treatment with Retigabine ceased, rate of movement and distance of movement measures indicated a return of pain-influenced movements (Figure 6B; 13-16WP; Post-Treatment). That is, the treatment was successful during administration of Retigabine, but once treatment ceased, pain

signs returned. Importantly, GRP A2 also continued to exhibit diminished ambulation rate relative to GRP C2 at 13-16WP ($p<.002$; Post-Treatment; figure 6B).

Summary and Conclusions for Task 1.2 and 1.3

Retigabine treatment produced a partial but transient rescue of pain-like ambulation behaviors associated with exposure to GW chemicals. As such, the data predicts that Retigabine could be an effective palliative, but not a cure for GWI pain. Potentially the administration of higher doses, the prolongation of treatment or the increase of treatment frequency within days would improve outcomes or even produce a lasting remission. In clinical applications, Retigabine is administered 3 times daily. Staff limitations and the fact that other studies (TASK 3) were being executed simultaneously, prevented the implementation of multiple daily dosing regimens. Alternately, other K_v7 openers, both established (Flutirpine) and experimental (Wang et al, 2017) could produce better outcomes. Such efforts should be pursued.

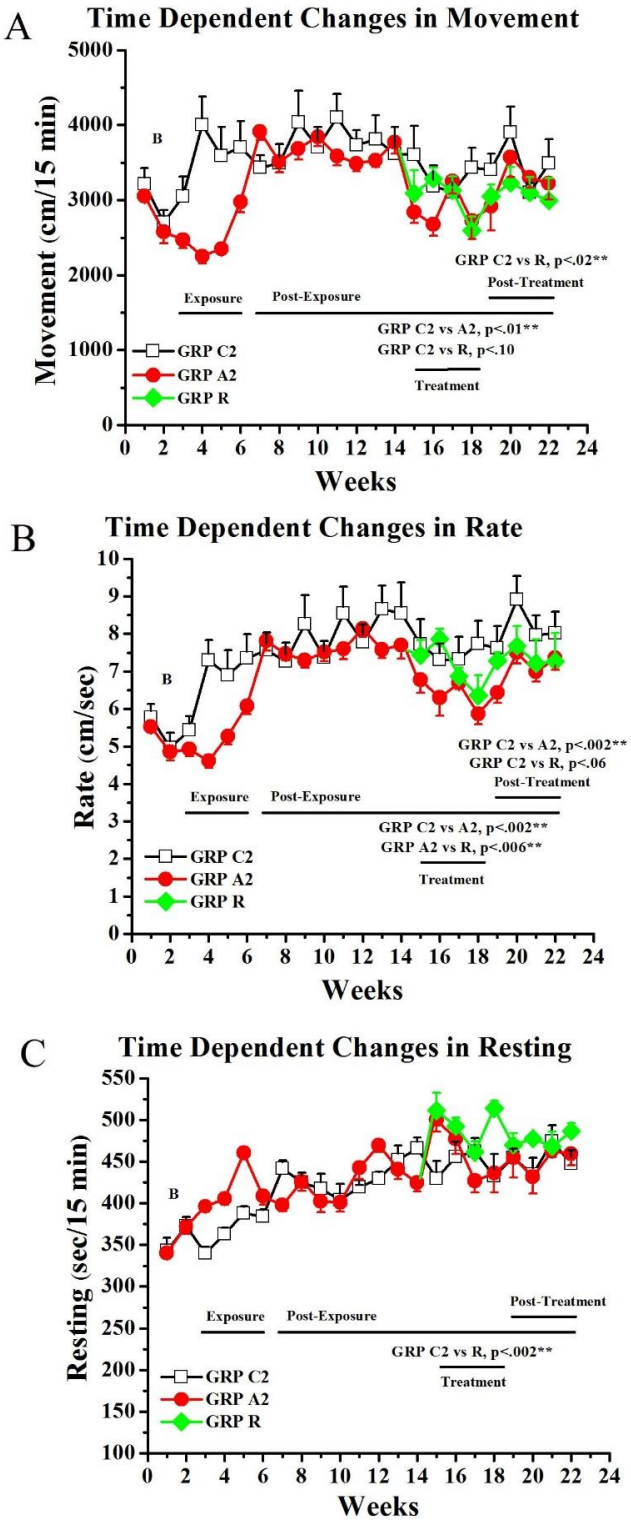

Figure 6. Treatment with Retigabine Produced a Transient Reduction of Pain-Like Behaviors. **A)** Exposure to all 4 GW agents (GRP A2) significantly decreased movement distance scores at 9-12 but not 13-16WP. Once daily Retigabine failed to significantly improved movement distance performance. **B)** Exposure to GW chemicals significantly decreased movement rate scores at both 9-12 and 13-16WP. Retigabine significantly improved rate scores relative to GRP A2 and rescued these scores relative to GRP C2 during treatment (9-12WP}. In the post-treatment phase, movement rates were not improved relative to exposed untreated rats (13-16WP; GRP A2). **C)** Resting duration was unchanged by exposure to GW agents at any observation period. Retigabine significantly increased resting scores at 9-12WP. B: baseline testing; GRP A2: DEET, chlorpyrifos, PB, permethrin; GRP C2 (n=6): (ethanol, corn oil, ethanol, water); GRP R (n=6): DEET, chlorpyrifos, PB, permethrin. Prior to treatment, GRP A2 was composed of 12 rats. During and after treatment GRP A2 was composed of 6 rats. **significantly different by ANOVA.

We had previously shown that $Na_v1.9$ was upregulated 8 weeks following exposure to the 3 agent protocol (Nutter and Cooper, 2014). We have now demonstrated the $Na_v1.9$ exhibited increased activity at 16WP when rats were exposed to the 4 agent protocol (see below; TASK 2). We used an FDA approved agent, Riluzole, to examine whether inhibition of this ion channel could improve pain signs in rats exposed to GWI agents. Our laboratory demonstrated that Riluzole can inhibit $Na_v1.9$ current in vascular nociceptors (see Appendix, figure A1. p. 80). In the studies below, we examined whether a 4 week treatment with Riluzole could acutely and/or permanently reverse the pain-like symptoms that developed in our model. As indicated in our SOW, we also examined whether combinations of two treatments (Riluzole and Retigabine) would produce a better outcome.

Riluzole Fails to Ameliorate Pain-Like Behaviors

Animals were prepared in a similar fashion to examine whether Riluzole (500 mg/kg/day; oral gavage) could also improve pain-like behaviors in our GWI model. Groups were formed and tests were conducted using procedures that were identical with the Retigabine studies presented above. An additional group was treated with combined doses of Riluzole and Retigabine.

Rats treated with Riluzole exhibited only greater reductions of movement distance and increases in resting relative to control rats (figure 7A and 7C). This pattern could have indicated increased pain behavior in these measures or more likely reflected substantial side effects associated with the Riluzole administration. In contrast, rate of movement was not affected by Riluzole (figure 7B). Given that the rate of movement has been our most reliable measure we conclude that there was no beneficial pain outcomes in Riluzole treated rats.

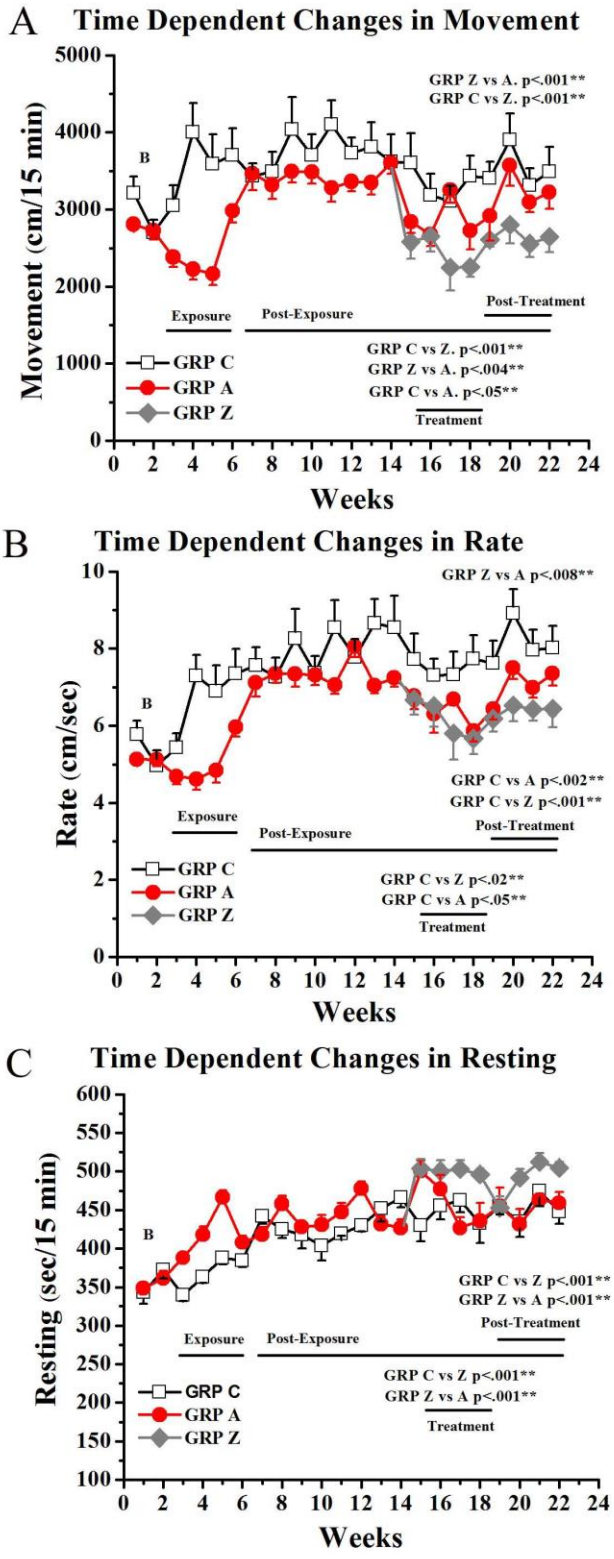

Figure 7. Treatment with Riluzole Depressed All Behavioral Measures. **A)** Exposure to all 4 GW agents (GRP A) significantly decreased movement distance scores at 9-12 but not 13-16WP. Once daily Riluzole (Z) reduced movement distance even further at 9-12WP. These scores remained depressed after Riluzole treatment ceased. **B)** Exposure to GW chemicals also significantly decreased movement rate scores (9-12 and 13-16WP). Riluzole treatment had no influence on rate scores either during treatment (9-12WP) or in the post-treatment phase (13-16WP). **C)** Resting duration was unchanged by exposure to GW agents at any observation period. Regardless, Riluzole significantly increased resting scores at 9-12WP. They remained elevated at 13-16WP. B: baseline testing; GRP A: DEET, chlorpyrifos, PB, permethrin; GRP C (n=6): (ethanol, corn oil, ethanol, water); GRP R (n=6): DEET, chlorpyrifos, PB, permethrin. Prior to treatment, GRP A was composed of 12 rats. During and after treatment GRP A was composed of 6 rats. **significantly different by ANOVA.

In accordance with the SOW, an additional group of rats were treated with a combination of Retigabine and Riluzole (Figure 8). These studies indicated only increases in pain measures (Figure 8). It was likely that the overall reduction in movement reflected non-specific influences of the combined drug treatment.

Although we originally proposed a treatment test series using a CGRP blocker (BMS-927711) we were unable to execute this portion of the SOW. When the project was submitted in 2014, the CGRP receptor blocker, BMS-927711, was in a stage II clinical trial for use as a migraine treatment. However, it was withdrawn from trials in 2016 due to side effects. We were unable to obtain the compound from Burroughs Welcome or any other vendor despite repeated attempts over a 4 month period. We could not identify another suitable CGRP blocker to use in these studies.

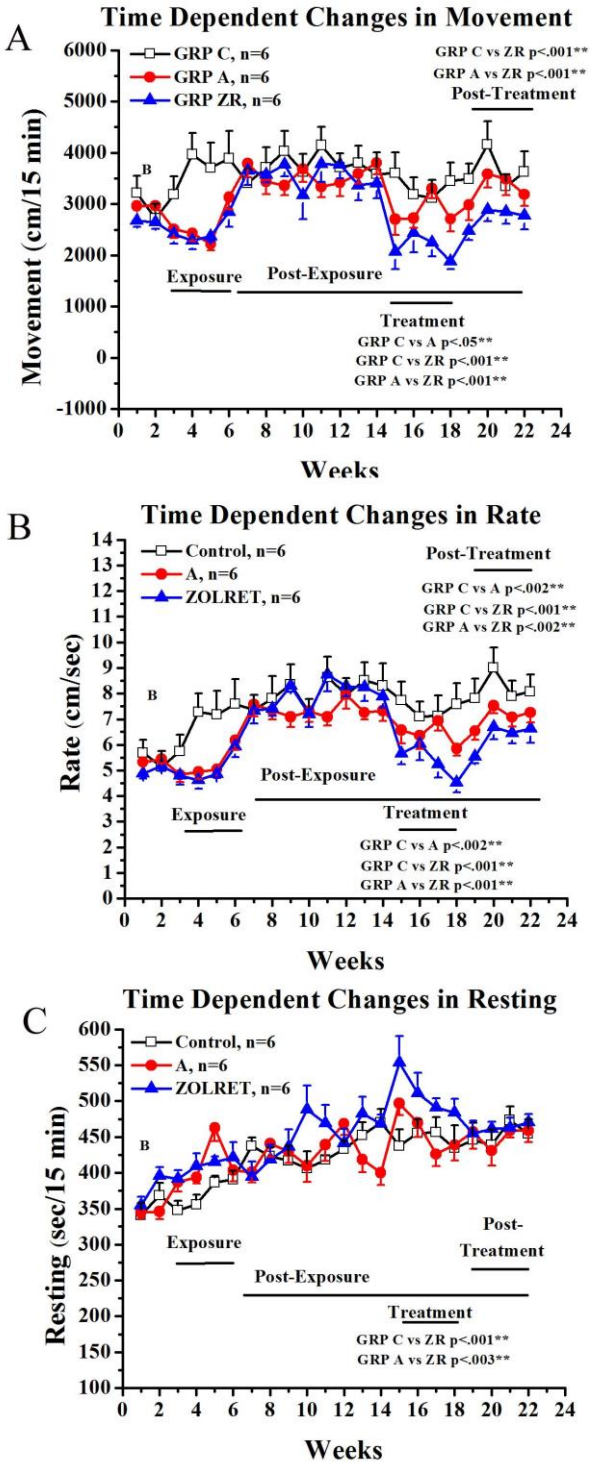

Figure 8. Treatment with Riluzole and Retigabine Depressed All Behavioral Measures. **A)** Exposure to all 4 GW agents (GRP A) significantly decreased movement distance scores at both 9-12WP. Once daily Riluzole and Retigabine (ZR) reduced movement distance even further at 9-12WP. GRP ZR scores remained depressed after ZR treatment ceased. **B)** Exposure to GW chemicals significantly decreased movement rate scores (9-12 and 13-16WP). ZR treatment further decreased rate scores during treatment (9-12WP) and in the post-treatment phase (13-16WP). **C)** Resting duration was unchanged by exposure to GW agents at any observation period. Regardless, ZR significantly increased resting scores at 9-12WP. Rest scored returned to normal levels at the end of the treatment period (13-16WP). B: baseline testing; GRP A: DEET, chlorpyrifos, PB, permethrin; GRP C (n=6): (ethanol, corn oil, ethanol, water); GRP ZR (n=6): DEET, chlorpyrifos, PB, permethrin. **significantly different by ANOVA.

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

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.

Time Line: Months 6-12

Rats were prepared and behaviors assessed for these experiments as described above (see also Appendix; Methods, p. 84). As in previous studies, rats were exposed to the 4 GWI chemicals for 4 weeks (optimized protocol). Control rats received only vehicle (corn oil, water, ethanol, ethanol) exposures. An additional group of rats was prepared that were exposed only to 3 GW chemicals (DEET, permethrin, chlorpyrifos). We had shown in year 1 that rats did not develop pain signs when PB was excluded from the exposure protocol. We used this analytical leverage to determine whether molecular maladaptations were also dependent on PB exposure and therefore related to the presence of pain-signs in rats.

We examined the ambulatory and resting behaviors of rats that were exposed to 3 or 4 GW chemicals (4 weeks; n=38). One group was exposed to DEET, permethrin, chlorpyrifos and PB (Grp A). A second group was exposed to DEET, permethrin, and chlorpyrifos, but PB was excluded (Grp PB). A third group served as a vehicle control (Grp C; water gavage, ethanol topical, corn oil s.c., and ethanol topical).

Behavioral testing was conducted once per week, lasted 15 minutes, and was fully automated by means of an array of infrared beams (AccuScan). AccuScan software converted beam interruptions into measures of ambulation (total distance moved, average rate of movement) and the total time at rest. Consistent with our previous published work, a repeated measures ANOVA was used to assess ambulation and resting scores captured in 4 week blocks (9-12 weeks and 13-16 weeks post-exposure; 9-12WP and 13-16WP).

Rats exposed to all 4 agents (Grp A) developed enduring pain-like behaviors. Movement distance was significantly reduced at both the 9-12 and 13-16 week post-exposure periods (figure 9A; Grp C vs A). Movement rate was also depressed, but emerged only at the late, 13-16WP, assessment period (figure 9B; Grp C vs A). Reduced ambulation was accompanied by a significant increase in rest duration at both test periods (figure 9C; Grp C vs A).

Exclusion of PB from the exposure set significantly altered the pattern of behavioral outcomes. In the absence of PB, ambulation deficits were 'rescued' at 9-12WP (movement only) and 13-16WP (figure 9A; Grp C vs Grp PB). This was evidenced as significant increases in movement distance and rate for GRP PB relative to GRP A. It is noteworthy, that, in most comparisons, movement distance and rate scores of Grp PB were not only increased relative to GRP A, but also paradoxically elevated over scores of vehicle-exposed rats (figure 9A and 9B; Grp C vs Grp PB).

The absence of PB from the exposure set failed to rescue resting deficits at the 9-12WP assessment period (figure 9C; Grp C vs PB); however, resting deficits were eventually rescued at 13-16WP (figure 9C). At no time were any of the resting scores paradoxically shifted relative to GRP C. This pattern is similar to our published findings of a complex relationship between resting behaviors and GW chemical exposure (Flunker et al., 2017; see also figure 1).

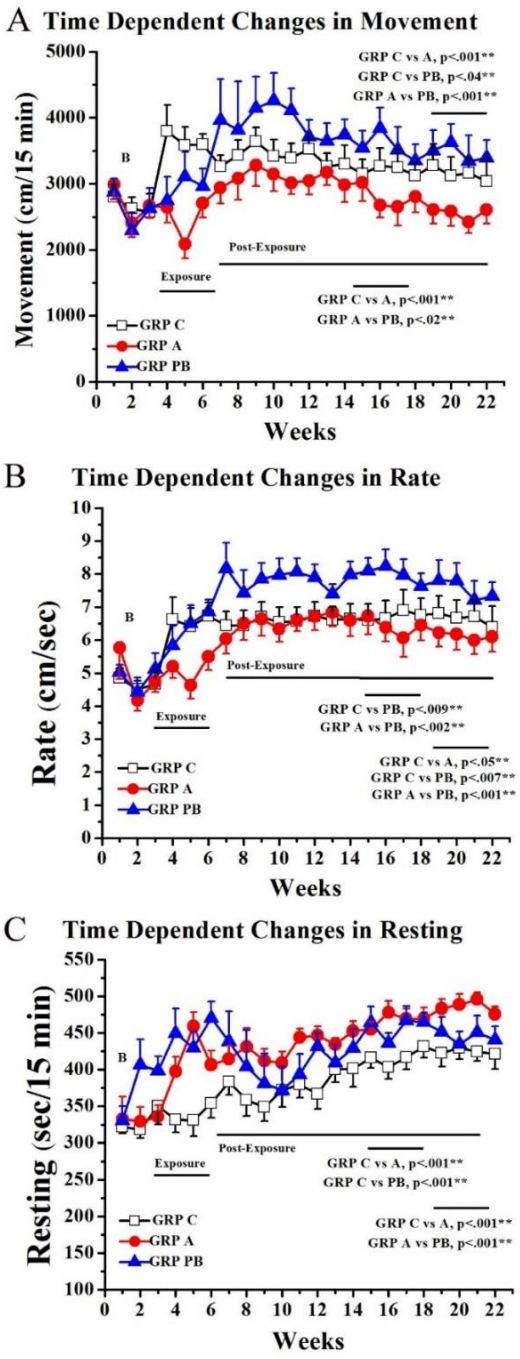

Figure 9. Exposure to 4 GW Chemicals Produces Persistent Pain-Like Behaviors that Required PB. **A)** Exposure to all 4 GW agents (GRP A) significantly decreased movement distance scores at both 9-12 and 13-16WP. When PB was excluded from the exposure set (GRP PB), movement distance was no longer suppressed. **B)** The 4 GW agents also significantly decreased movement rate scores, but declines in rate were delayed until 13-16WP. When PB was excluded from the exposure set, movement rate was significantly increased. **C)** Resting duration was significantly increased at both 9-12 and 13-16WP. In the absence of PB, resting scores were rescued only at 13-16WP. B: baseline testing; GRP A (n=10): DEET, chlorpyrifos, PB, permethrin; GRP C (n=10): (ethanol, corn oil, ethanol, water); GRP PB (n=10): DEET, chlorpyrifos, permethrin. **significantly different by ANOVA.

K_v7 Activity in Muscle and Vascular nociceptors Diverged from Pain Behaviors

We had previously shown that the 3 agent protocol produced a significant decline in K_v7 voltage dependent conductance that was consistent with increased cellular excitability and reduced ambulatory behavior changes that persisted at 12WP (Nutter et al., 2015). The addition of DEET to the exposure protocol extended the impact of GWI chemicals on the physiology of deep tissue nociceptors. Twelve weeks following exposure to permethrin, chlorpyrifos, PB and DEET, vascular nociceptor Kv7 physiology was still depressed (figure 10).

This finding was put to a further test by utilizing cells from groups of rats that had not been exposed to PB during the chronic exposure phase. As shown in figure 1, these 'GRP PB' rats do not develop ambulatory deficits despite having been exposed to permethrin, chlorpyrifos and DEET. Consistent with that finding, rats of GRP PB2 also failed to develop ambulatory deficits (figure 9A and B). Yet when the voltage-conductance of K_v7 deep tissue nociceptors of this group were examined, we observed that the decline of K_v7 activity had been retained (figure 10). That is, the decline in K_v7 activity appeared in cells harvested from rats exhibiting ambulation deficits, and in those whose ambulation deficits were rescued at 12WP by exclusion of PB. The divergence between the behavioral and physiological data indicates that the shifts in voltage-conductance of K_v7 were unrelated to behavioral changes in ambulation. Nevertheless, the decline in K_v7 activity could still be linked to resting deficits that were retained in GRP PB (figure 9C) or perhaps to other forms of pain that we were unable to measure in an activity box.

When a group of rats were euthanized at 16 WP, it was clear that the relationship between ambulation, resting and K_v7 deficits were severely strained. At 16WP, changes in K_v7 conductance of GRP A rats was significantly *higher* in muscle and vascular nociceptors, despite

the persistent decline in ambulation at this test interval in these rats (figure 9). Accordingly, it did not appear that shifts in K_v7 voltage dependent conductance were critical to either ambulation or resting deficits.

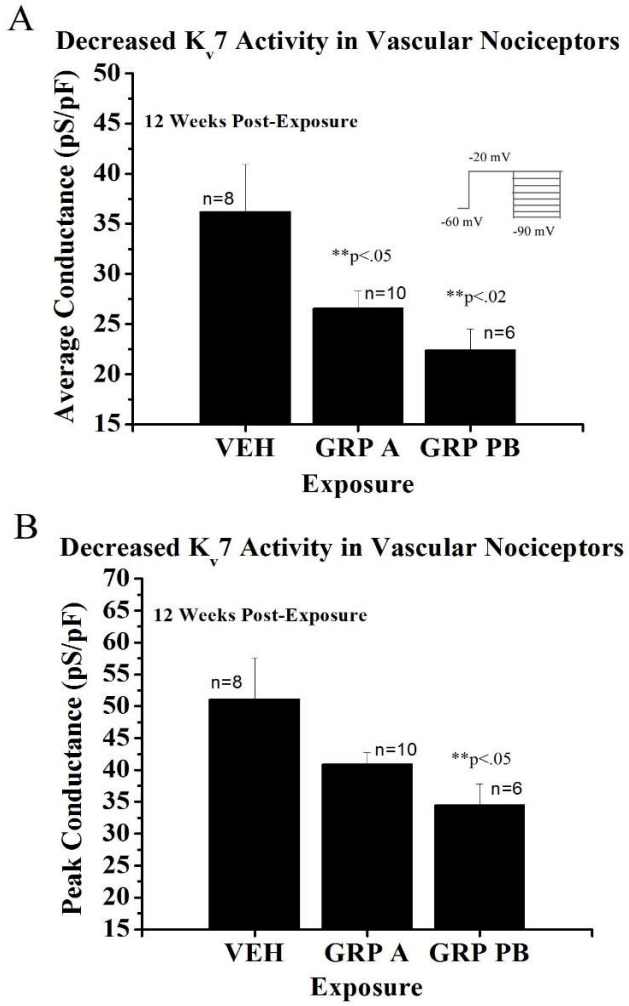

Figure 10. The Conductance of K_v7 Ion Channels Was Reduced in Vascular Nociceptors of Rats 12 Weeks Following Exposure to GW Chemicals. A) The average conductance (-30 to -70 mV) was significantly reduced in both Group A and Group PB nociceptors. **B)** The peak conductance was significantly reduced only in cells harvested from rats that were not exposed to PB during the 4 week period. Muscle nociceptor K_v7 was unchanged (not shown). GRP A: PB, permethrin, chlorpyrifos and DEET; GRP PB: permethrin, chlorpyrifos and DEET VEH: water, ETOH, corn oil, ETOH

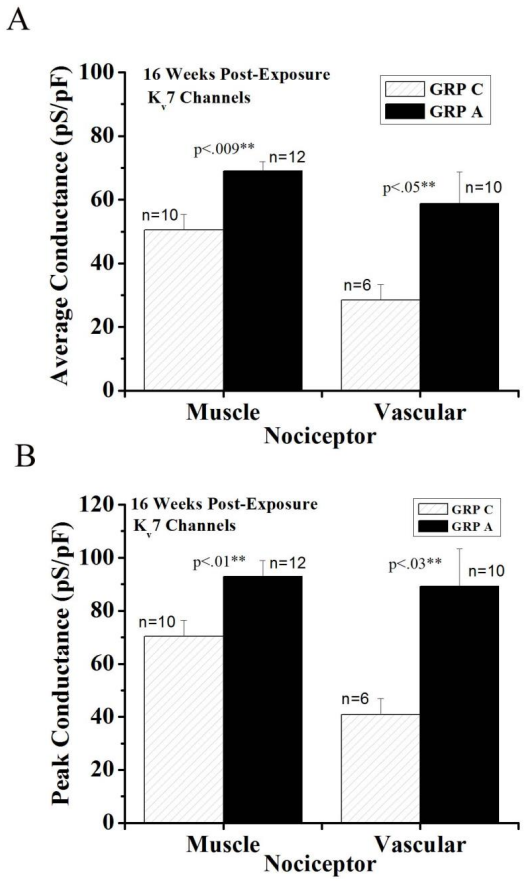

Figure 11. The Average and Peak Conductance of K_v7 ion Channels at 16 Weeks Post-Exposure. A) The average conductance was significantly increased in muscle and vascular nociceptors. **B)** The peak conductance was also significantly increased in muscle and vascular nociceptors at 16WP. The average conductance was computed from averaged tail current amplitudes that were evoked following a series of stepped repolarizations (-30 to -90 mV, 10 mV steps) from -20 mV. GRP A: DEET, permethrin, chlorpyrifos; GRP C: ETOH, ETOH, corn oil, water. Thirteen rats contributed to these experiments (7 GRP C; 6 GRP A).

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

In a previous study, in which rats that were exposed to 3 GW chemicals (permethrin chlorpyrifos and PB), our laboratory documented increases in nociceptor $Na_v1.9$ activity 8 weeks following the termination of exposures (Nutter and Cooper, 2014). As heightened activity of $Na_v1.9$ is associated with increased nociceptor excitability, and as $Na_v1.9$ is widely expressed in nociceptors, we examined whether the addition of DEET to the exposure protocol influenced the activity of $Na_v1.9$.

Over a period of 4 weeks, rats were exposed to 4 GWI agents (permethrin, chlorpyrifos, PB and DEET) or their corresponding vehicles. A third group of rats (GRP PB) were exposed to only 3 GW agents (permethrin, chlorpyrifos and DEET; see figure 9). Neurons were harvested from these animals at 12 and 16WP exposure. Following cell characterization and series resistance compensation, deep tissue nociceptors were exposed to a Na_{iso} solution for 2 minutes (see Appendix, Methods, p. 88). Voltage dependent activation was assessed by application of a series of voltage steps (-80 to -20 mV; 5 mV steps; V_H=-120 mV). Boltzmann functions were subsequently fit to the computed values of the peak conductance and plotted against the test voltage series. The voltage of half activation ($V_{.5}$) was determined for each individual cell recording. Cells that failed Boltzmann function fits were excluded from the study.

Comparison of computed $V_{.5}$s from control and exposed rats revealed hyperpolarizing shifts in the voltage dependence in vascular nociceptors (figure 12C and D). This outcome indicates that more $Na_v1.9$ current will be evoked as neurons are depolarized and is consistent with increased nociceptor excitability in those rats exhibiting pain-like behaviors. This shift in the $V_{.5}$s could

promote action potential firing during depolarizing events (Copel et al., 2009; Herzog et al., 2001; Maingret et al., 2008; Nutter and Cooper, 2014).

Moreover, when tests were conducted on nociceptors harvested from GRP PB rats (PB excluded from exposure), the $V_{.5}$ values of vascular nociceptors were similar to Grp C, vehicle controls, but significantly depolarized relative to GRP A neurons (figure 12E and F). Therefore, the removal of PB from the exposure protocol was associated with rescue of pain-like behaviors, and also rescued the voltage shifts associated with vascular nociceptor excitability (16WP).

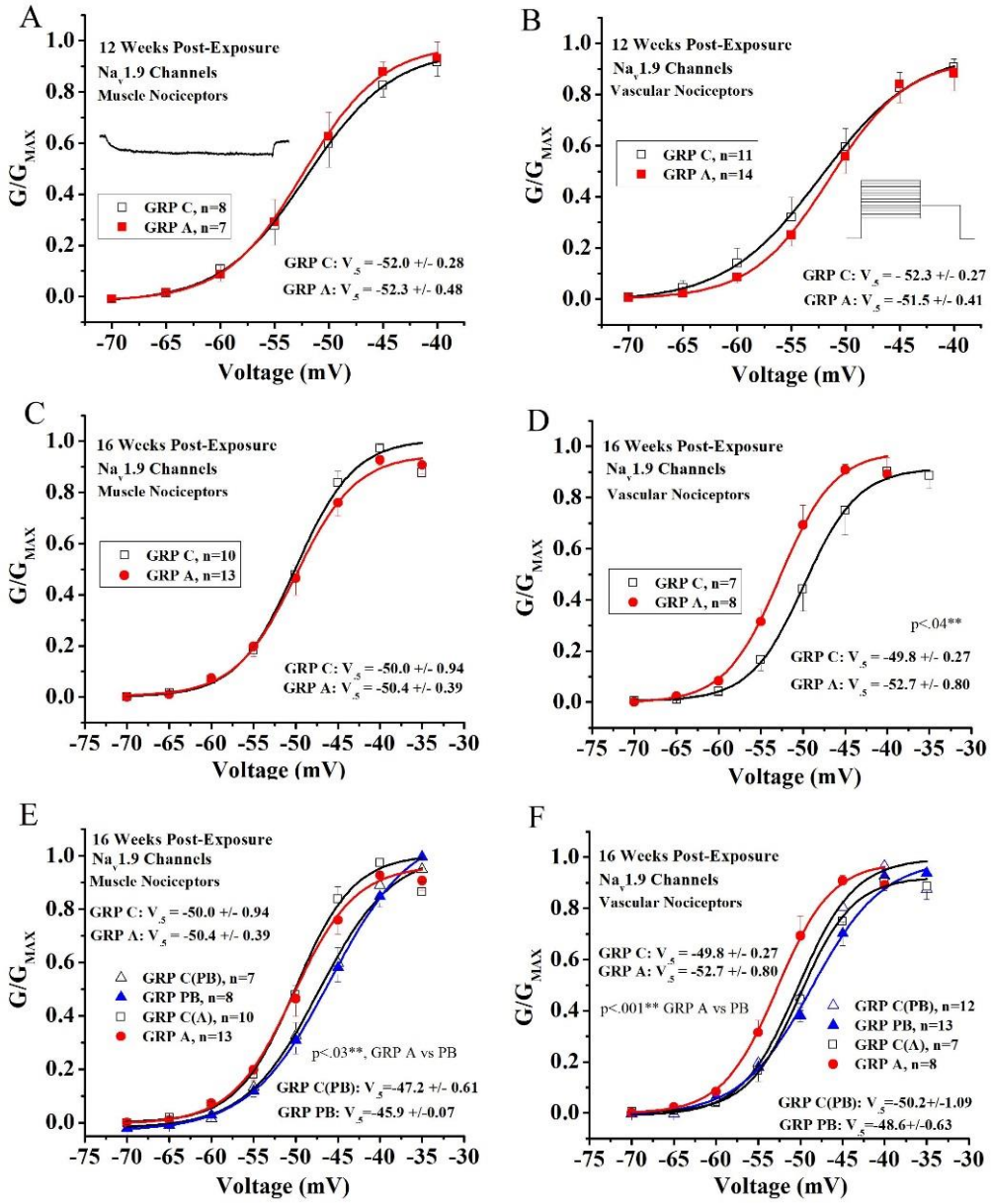

Figure 12. Voltage Dependent Activation of Na$_v$1.9 Shifts with Exposure to 4 GW Chemicals. A and B) Twelve weeks after exposure, the $V_{.5}$ of activation was similar for muscle and vascular nociceptors (GRP C vs GRP A). An insert, in panel A, presents a representative trace that was evoked at -50 mV from a vascular nociceptor (300 msec duration; 1997 pA amplitude). The voltage step protocol is presented as an insert in panel B. **C and D)** Sixteen weeks after exposure, the $V_{.5}$ of activation was hyperpolarized in vascular but not muscle nociceptors (GRP C vs GRP A). **E) and F)** In the absence of PB in the exposure set (GRP PB), the activation $V_{.5}$ was depolarized in both muscle and vascular nociceptors relative to GRP A, but non-different from experiment specific controls (GRP PB vs GRP C(PB)). GRP A: DEET, permethrin, chlorpyrifos, PB; GRP PB: DEET, permethrin, chlorpyrifos; GRP C: ETOH, ETOH, corn oil, water; GRP C(A): control cases for GRP A; GRP C(PB): control cases for GRP PB. Twenty-four rats contributed to these experiments.

A shift in voltage dependence ($V_{.5}$) predicts that more current will be evoked, but these currents could be independently reduced by other influences on $Na_v1.9$ physiology and result in no net change in excitability. The average evoked current was determined across the active range for each neuron (-65 to -45 mV) and then normalized to the cell dimension parameter capacitance (pF). Statistical comparisons indicated that the average evoked nomalized current amplitudes were significantly increased in vascular nociceptors at 16WP exposure (GRP C vs GRP A, 16 WP; figure 13B). When PB was absent from the exposure protocol, no difference in average currents were detected (GRP C vs GRP PB, 16WP; figure 13C), but the average amplitude of GRP PB currents were significantly reduced relative to GRP A ($p < .001$). These molecular outcomes were consistent with the behavioral outcomes for GRPs A and PB at 16WP and tend to support critical role of vascular nociceptor $Na_v1.9$ in the manifestation of pain-like behaviors that persisted following exposure to GW chemicals.

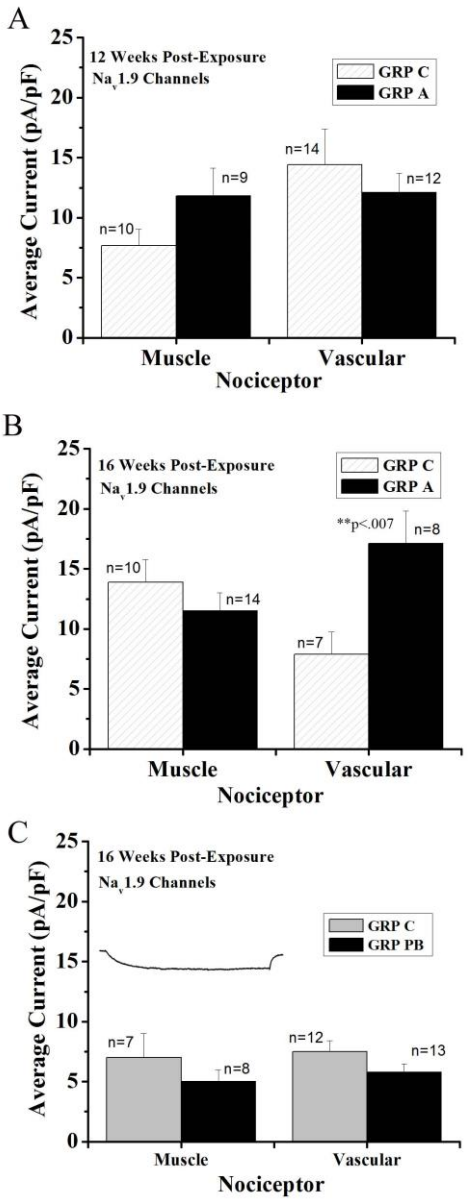

Figure 13. Exposure to GW Chemicals Raises the Average Evoked $Na_v1.9$ Current. A) Twelve weeks after exposure, the average $Na_v1.9$ current of muscle nociceptors was unchanged (GRP C vs GRP A). B) Sixteen weeks after exposure, the average $Na_v1.9$ current of vascular nociceptors was significantly higher (GRP C vs GRP A), but muscle nociceptors were unaffected. **C)** When PB was excluded from the exposure protocol (GRP PB), the average evoked currents were unchanged in either nociceptor class. An insert, in panel C, presents a representative trace that was evoked at -50 mV from a muscle nociceptor (300 msec duration; 961 pA amplitude). GRP A: DEET, permethrin, chlorpyrifos, PB; GRP PB: DEET, permethrin, chlorpyrifos; GRP C: ETOH, ETOH, corn oil, water. Twenty-four rats contributed to these experiments (see also figure 2; 8 GRP C; 10 GRP A; 6 GRP PB).

Summary and Conclusions Tasks 2.1 and 2.2

We were able to demonstrate, and replicate, GW chemical induced shifts in the molecular activity of K_v7 and $Na_v1.9$ that had accompanied toxicant exposures. Manipulation of the exposure set, by exclusion of PB, further demonstrated that shifts in K_v7 activity did not parallel behavioral assessments of ambulation deficits that appeared over the course of testing. The divergence of K_v7 voltage-conductance from the behavioral outcomes, in the absence of PB and at the 16WP tests indicated that voltage dependent status of this ion channel was not directly linked to the presence of chronic pain in our rat model. Therefore, the capacity of K_v7 openers to act as a palliative was likely due to the generalized capacity of K_v7 to retard cellular excitability. It is also possible that muscarinic coupling to K_v7 is actually the important factor, rather than the voltage dependent status of K_v7 itself. We have shown, in published reports, muscarinic coupling via the mAChR (muscarinic form of the cholinergic receptor) to K_v7 were amplified following exposure to 3 GW agents (permethrin, chlorpyrifos and PB; Cooper et al., 2016). In a circumstance in which both G protein coupled receptors (mAChR) and actuators of excitability (K_v7) are differentially altered, the net effect of the combined adaptation become most important. The net effect on excitability was examined in TASK 2.3, below.

In contrast, our examination of $Na_v1.9$ activity in relation to behavioral effects clearly indicated that activity of this ion channel covaried with the presence and absence of behavioral signs; particularly those pain signs that persisted 16 weeks post-exposure. The importance of this ion channel in the pathophysiology of the condition was amplified by these findings. The present lack of a recognized specific inhibitor of $Na_v1.9$ activity makes pre-clinical testing difficult.

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

Maladapted physiology of $Na_v1.9$ and K_v7 ion channels predict changes in nociceptor excitability that could underlie GWI pain. Accordingly, we examined both the general membrane excitability and specific changes in excitability to neurotransmitter/paracrine messenger ACh in populations of vascular and muscle nociceptors which are known to be influenced by chronic exposure to GW chemicals. Studies on mAChR were indicated because: 1) cholinesterase inhibitors directly influence extracellular ACh levels; 2) muscarinic ACh receptors (mAChR) are known to link to K_v7 and increase cellular excitability via G protein dependent inhibition; and 3) other laboratories, as well as our own laboratory, have documented that mAChR's, and their physiological influences, are modified following chronic exposure to anticholinesterases associated with 1991 Persian Gulf War (Abdel Rahman et al., 2004; Zou et al., 2006; Cooper et al., 2016). These studies were carried out in current clamp mode to allow for the generation of action potentials.

Animal were prepared in the usual manner. Rats were exposed to 4 GWI chemicals for 4 weeks (GRP A: DEET, permethrin, chlorpyrifos and PB). A second group of rats were exposed to 3GWI chemicals (GRP PB: DEET, permethrin, chlorpyrifos). Control rats received only vehicle exposures (GRP C: ethanol, ethanol, corn oil, water). Behavioral tests were conducted on a weekly basis. Patterns of ambulation and resting deficits were similar to above studies (figure 14; see also figures 1, 6, 7, 8 and 9). Movement Rate deficits emerged after a delay of about 12 weeks while movement distance and resting deficits were manifested much earlier. Rats were euthanized 16 weeks following the end of the exposure period, and cells were harvested for electrophysiological experiments.

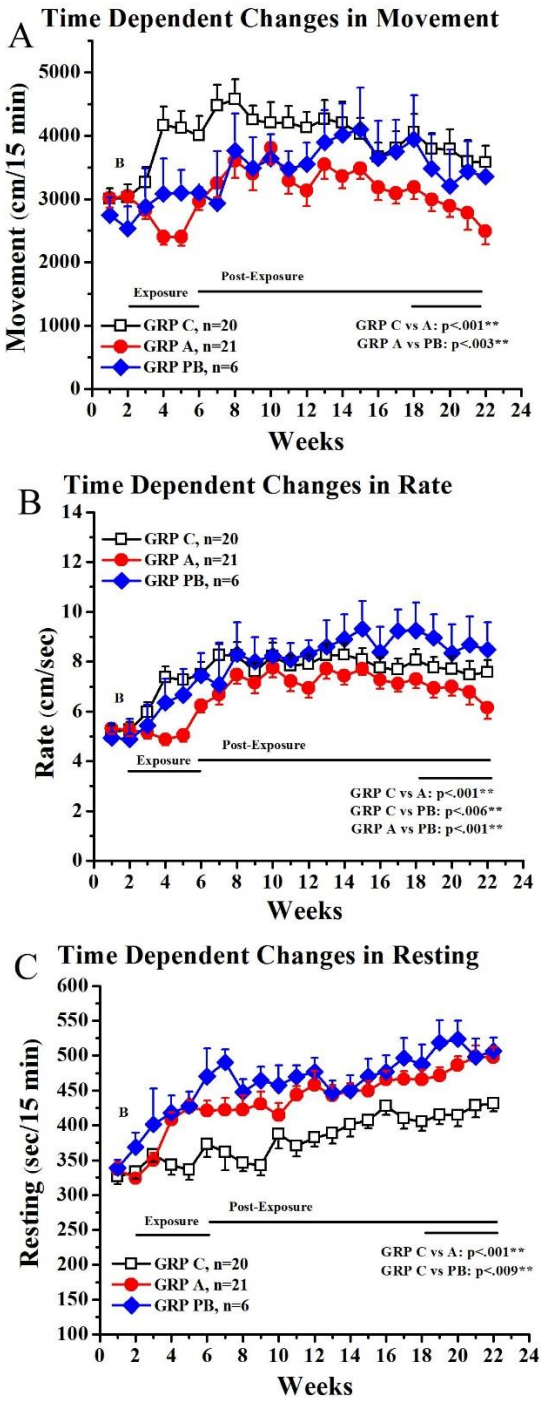

Figure 14. Exposure to 4 GW Chemicals Produced Persistent Pain-Like Behaviors at 16 Weeks Post-Exposure. **A)** Exposure to all 4 GW agents (GRP A) significantly decreased movement distance scores at 13-16WP. When PB was excluded, movement scores were significantly improved. **B)** Movement rate scores declined significantly at 13-16WP. In the absence of PB, rate scores improved significantly. **C)** Resting duration was significantly increased at 13-16WP. Despite the exclusion of PB resting behavior remained elevated. B: baseline testing; GRP A (n=21): DEET, chlorpyrifos, PB, permethrin; GRP PB (n=6): DEET, chlorpyrifos, permethrin; GRP C (n=20): ethanol, corn oil, ethanol, water. Statistical tests were not conducted at 9-12WP. Rats at 16WP were used in excitability experiments. **significantly different by ANOVA.

For excitability studies, deep tissue muscle and vascular nociceptors were brought into current clamp mode. Following a one minute observation period, during which cells were examined for spontaneous activity, current injection studies were initiated. Stepped, square wave injections ranged from 100 to 1200 pA (100 pA steps, ascending; 250 msec duration; 1 sec interstep interval). The threshold and total number of action potentials evoked were determined off-line. Cells whose original resting membrane potential fell out of a range of -65 to -55 mV were excluded from current injection studies. These tests were conducted at ambient temperature (19 °C). After a 1 minute recovery period, the temperature of the superfused solution was increased to 34.5 °C and maintained for 2 minutes. At this point, a 34.5 °C heated solution containing the highly specific muscarinic agonist, Oxotremorine-M, was applied to the cell by close superfusion (OXO; 10 μM; 1 minute).

Current inject studies revealed heightened membrane excitability in vascular nociceptors that were harvested from GRP A rats (figure 15A and B). This took the form of both decreased threshold and increase AP discharge ($p < .04$ and .02, respectively; Table 2). In contrast, membrane excitability of muscle nociceptors was unchanged by exposure to GW chemicals. No spontaneous activity was observed in either class of neurons in either experimental group.

The application of a muscarinic agonist, OXO, depolarized muscle and vascular nociceptors. These depolarizations were often accompanied by powerful action potential bursts (figure 15C and D; Table 3). The pattern of depolarization was complex and appeared to be composed of multiple components. Large auto-reversing depolarizations, with bursting AP discharges, were much more frequent in muscle nociceptors than vascular nociceptors (6/7 exposed and 5/8 control cases). An

additional 10 cases did not exhibit the auto-reversing current (7 exposed; 3 controls). All 25 muscle nociceptors exhibited a persistent depolarization that outlasted the OXO application by more than 1 minute (figure 15E; Table 3).

While all vascular nociceptors exhibited the persistent depolarization following OXO application (19/19 cases; figure 15E), only one of these nociceptors expressed an auto-reversing early depolarization with bursts of APs during the application (Table 3). Three additional cases also emitted burst discharges, but these cells did not survive the OXO challenge, and therefore the data could not be scored.

Table 2

Membrane Excitability									
				RMP		**Threshold**		**Total APs**	
				mV		**pA**			
	Vehicle Control Rats								
		Muscle		60.1 ± 1.0	16	387 ± 46	16	10.4 ± 1.1	16
		Vascular		60.0 ± 1.4	8	387 ± 55	8	10.3 ± 1.3	8
	GWIC Exposed Rats								
		Muscle		59.8 ± 1.4	10	410 ± 66	10	14.2 ± 3.5	10
		Vascular		62.0 ± 1.0	11	285 ± 66*	14	16.4 ± 1.7*	14
RMP: Resting Membrane Potential						* significantly different from vehicle control			
AP: Action Potential									
GWIC: Gulf War Illness Chemicals									

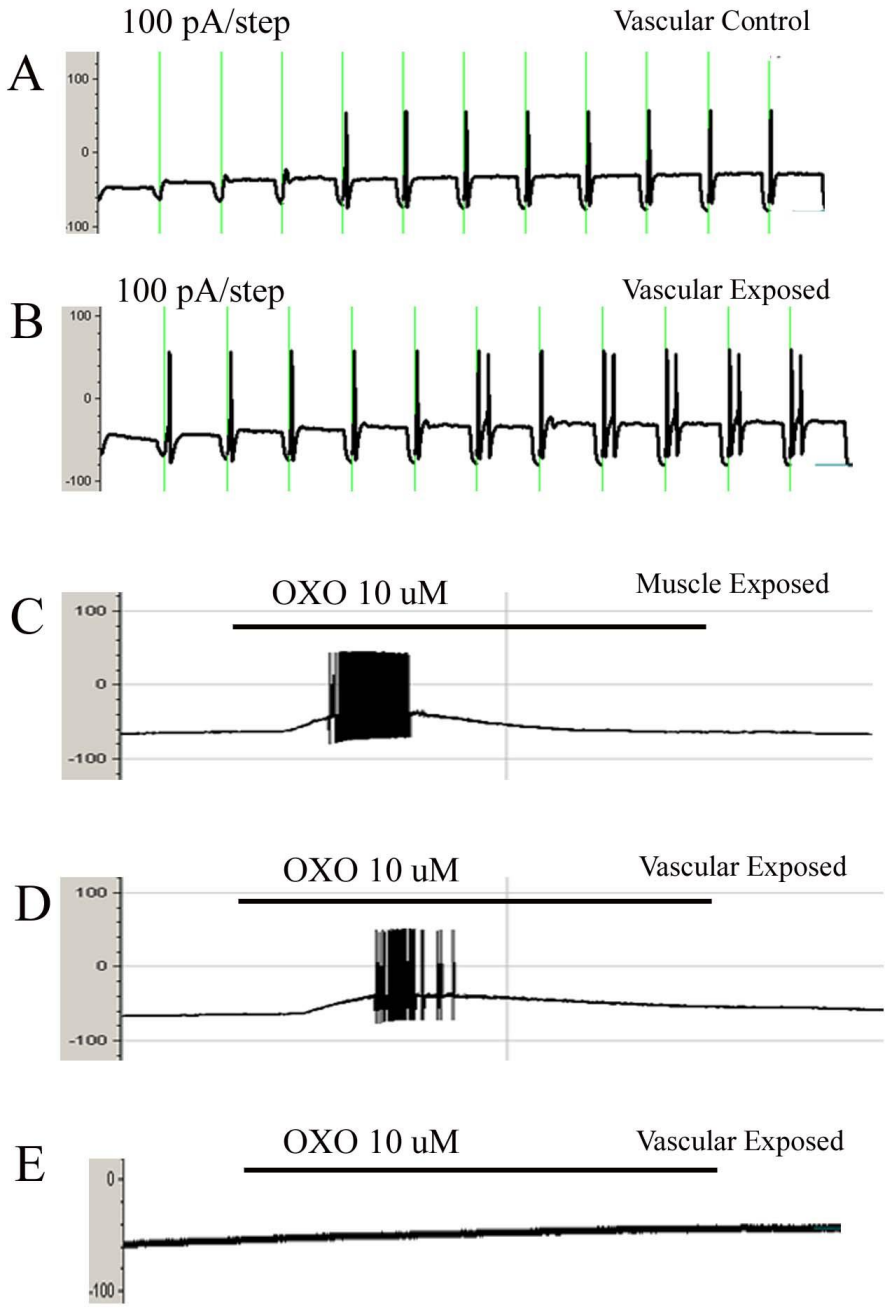

Figure 15. Deep Tissue Nociceptor Excitability Testing. A) Representative current injection excitability testing of a vascular nociceptor from a control (GRP C) rat. **B)** Representative current injection excitability testing of a vascular nociceptor from an exposed (GRP A) rat. **C)** Representative action potential burst from a muscle nociceptor from an exposed rat during OXO presentation (GRP A). The auto-reversing current is apparent. A persistent depolarization remains after a 2 minute wash (full 2 min are not shown). **D)** Representative action potential burst of a vascular nociceptor from an exposed rat during OXO presentation (GRP A). A persistent depolarization remains after a 2 minute wash (not shown). **E)** Representative slow depolarization of a vascular nociceptor from an exposed rat (GRP A). All cases are from 16WP rats. Bold line indicates the application of OXO-M (1 min). The traces were truncated from the full 200 sec to permit better resolution of the burst discharge.

TABLE 3

Muscarinic Reactivity																
			RMP		**Depolarization**		**Net Reversed**		**Total APs**		**Burst Duration**		**Frequency**		**Persistent Depolarization**	
			mV		**mV**		mV				**sec**		**APs/sec**		**mV**	
Vehicle Control Rats																
	Muscle		60.9 ± 1.2[1]	11	20.8 ± 0.8	8	14.6 ± 1.0[2]	8	117.2 ± 26.3	5	12.3 ± 1.3	5	9.9 ± 2.5	5	9.8 ± 1.1[3]	11
	Vascular		62.0 ± 1.9[1]	5	9.4 ± 0.6	5	NA	5	0	5	NA	5	NA	5	15.1 ± 0.44[3]	5
GWIC Exposed Rats																
	Muscle		62.4 ± 1.0[1]	14	24.0 ± 0.9*	7	19 ± 1.2*	7	86.2 ± 28	6	17.8 ± 3.1	5	5.2 ± 1.6	5	11.3 ± 1.1[3]	14
	Vascular		62.7 ± 1.9[1]	8	7.6 0.9	7	NA	7	29	1	9.03	1	3.2 ± 12.6	1	12.3 ± 0.9[3]*	8
Muscarinic Reactivity Following HC-03001																
			RMP		**Depolarization**		**Net Reversed**		**Total APs**		**Burst Duration**		**Frequency**		**Persistent Depolarization**	
			mV		**mV**		mV				**sec**		**APs/sec**		**mV**	
Vehicle Control Rats																
HC Treated	**Muscle**		62.6 ± 2.9	7	19.5 ± 1.2	7	5.6 ± 3.2**	7	33.2 ± 8.5**	6	18.7 ± 2.3**	5	2.3 ± 0.5**	5	8.8 ± 1.1	7
GWIC Exposed Rats																
HC Treated	**Muscle**		60.6 ± 3.6	8	14.7 ± 1.0***$^{\Delta}$	8	2.9 ± 1.4***	8	81.3 ± 36.4	3	18.0 ± 4.7	3	5.0 ± 2.1	3	13.0 ± 1.9	8

1. Includes only cases with RMP between -55 and -65 mV
2. Peak depolarization minus minimum post-OXO RMP
3. Includes cases without auto-reversing depolarization

RMP: Resting Membrane Potential
AP: Action Potential
GWIC: Gulf War Illness Chemicals

* significantly different from vehicle control
** significantly different from vehicle control
*** significantly different from GWIC exposed
Δ significantly different from HC Vehicle control

Exposure to the 4 agent exposure protocol produced a significant increase in muscle nociceptor excitability to a muscarinic agonist. Following application of the mAChR agonist OXO, the peak auto-reversing depolarization (that lead to bursting; figure 15B), as well as the total reversed depolarization, were significantly greater in muscle nociceptors harvested from GRP A vs those from GRP C rats ($p<.03$ and $p<.02$; figure 16A and Table 3). Action potential bursting properties (number of evoked APs, burst duration ($p<.14$), average burst frequency) did not differ for muscle nociceptors (GRP A vs GRP C; Table 3). In contrast, GRP PB muscle nociceptors, harvested from rats exposed only to DEET, permethrin and chlorpyrifos, did not manifest greater peak and reversed depolarization; moreover, in the absence of PB, the peak and reversed depolarizations were significantly less that nociceptors from GRP A (figure 16A and B). Therefore, the mAChR instigated depolarization co-varied with the presence and absence of PB in the exposure set, and appeared to be critically linked to the maintenance of pain behaviors in our rat model. The increased sensitivity to muscarinic depolarization could have contributed to the reversals of ambulation deficits observed in Retigabine treated rats (figure 6, p. 24). K_v7 channels are directly opened by Retigabine and would actively oppose membrane depolarization and any AP bursting that could result from that depolarization.

The enhanced depolarizations in muscle nociceptors excised from GRP A rats could be a basis for the chronic myalgia of GWI. Because of the importance of the auto-reversing depolarization to mAChR induced depolarization and nociceptor AP discharge, we examined the molecular basis of the auto-reversing depolarization in muscle nociceptors harvested from both exposed and control rats. We had previously shown, in non-exposed (naïve) rats, that the depolarization associated with OXO presentation were mediated, in part, by ion channel TRPA1 (Cooper et al., 2016; see attached

manuscript, p. 102). We have now shown that the OXO-induced depolarization was enhanced 16 weeks post-exposure to the four GWI chemicals. To determine whether this enhancement was due to a greater contribution of TRPA1 in exposed rats, we assessed the contribution of TRPA1 to the OXO-induced depolarization in muscle nociceptors harvested from GRP A and GRP C rats, 16 weeks after GW chemical exposures had ceased.

After preliminary cell characterizations, rat muscle nociceptors were exposed to the specific TRPA1 inhibitor (HC-030031; 3 min, 10 μM; 1 min at 19 °C and 2 minutes at 35 °C). Following pretreatment with HC-030031, we assessed depolarization and action potential discharge to OXO (1 min, close superfusion; 10 μM; 35 °C). From these experiments it was clear that the powerful auto-reversing depolarization in muscle nociceptors was mediated by TRPA1, and that this contribution was greatly enhanced in GW agent-exposed rat neurons. As illustrated in figure 16, HC-030031 significantly decreased OXO induced auto-reversing depolarization in muscle nociceptors from GWI chemical exposed rats ($p<.001$), but not in control rats (figure 16A and B; Table 3). Both the peak magnitude of the depolarization and the net reversing depolarization (peak depolarization minus minimum depolarization post-OXO) were significantly less in GRP A exposed rats treated with HC-030031 ($p<.01$). The net auto-reversed depolarization was nearly eliminated in exposed rat nociceptors ($p<.001$; figure 16B; Table 3).

In the presence of the TRPA1 inhibitor, only 3 of 8 exposed cases manifested AP burst discharges (vs 6/7 in GRP A). These were too few cases to analyze; however, 6 of 7 control cases still exhibited AP bursting in the presence of the TRPA1 inhibitor. In these cases, AP burst number, burst duration and average burst frequency were significantly decreased relative to cases not pre-

treated with the TRPA1 inhibitor ($p<.01$, $p<.04$ and $p<.02$ respectively; Table 3). Therefore, TRPA1 inhibitors reduced AP burst discharges in muscle nociceptors, and differentially decreased the auto-reversing depolarization that produced these discharges in GWI exposed rats.

The experiments of TASK 2.3 confirm that TRPA1 was the source of the auto-reversing depolarization and that these same depolarizations were enhanced in rats previously exposed to PB, chlorpyrifos, permethrin and DEET. It is not clear whether this enhancement was solely due to TRPA1 augmentation in GWI exposed rats or whether the coupling of this channel to muscarinic receptors was the dominant factor. Ultimately, both could be involved, and could have important implications for treatment of GWI pain. We used the TRPA1 specific agonist, cinnamaldehyde, to separate the contribution of the TRPA1 component from other factors contributing to muscarinic activation of the channel.

Muscle nociceptors were isolated from rats exposed to GWI chemicals or their respective vehicles (16 weeks post-exposure). Following preliminary characterizations, cells were exposed, by close superfusion, to a saturating dose of cinnamaldehyde (100 μM). Application continued until peak currents were achieved, as evidenced by desensitization, or a 4 minute maximal application period was surpassed. Studies were conducted at room temperature. Sixteen of 19 cases exhibited powerful currents upon application of cinnamaldehyde. There were no significant differences between the peak currents evoked in muscle nociceptors excised from exposed or control rats (figure 16D). This data indicated that, while TRPA1 was a critical effector in the production of mAChR-induced nociceptor discharge to OXO, it was the muscarinic linkage to TRPA1 that was fundamentally responsible for the enhanced TRPA1 depolarization.

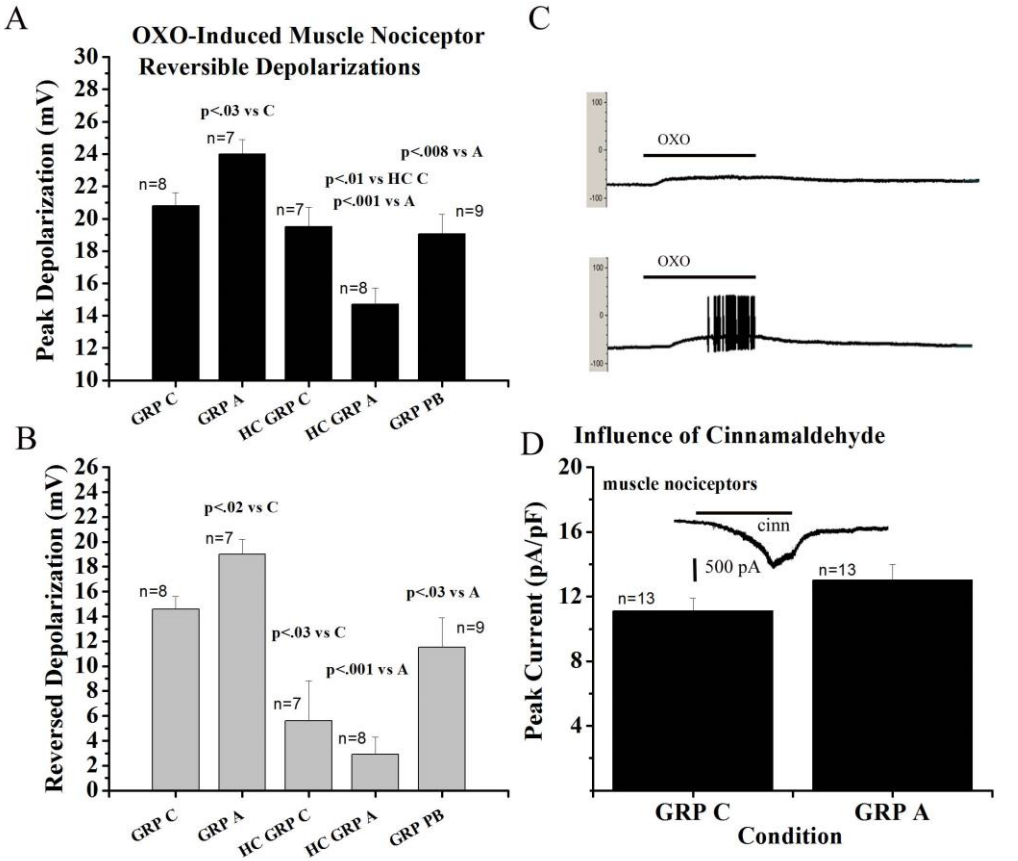

Figure 16. **TRPA1 Mediates Enhanced Reversible Depolarizations in Muscle Nociceptors. A)** The peak depolarization is greater in GW agent exposed nociceptors. The specific TRPA1 antagonist (HC-03001) inhibits the peak of the auto-reversing depolarization in GWI agent exposed muscle nociceptors, but not vehicle exposed. When PB was excluded, the effect of exposure is significantly less. **B)** The net auto-reversing depolarization is inhibited in both agent exposed and vehicle exposed neurons. When PB was excluded, the effect of exposure to GW agents is significantly less. **C)** Representative traces of HC-03001 pretreated OXO depolarization, with and without associated AP bursting. Note that reversing takes place after OXO presentation ended, rather than during the OXO application (see figure 15). **D)** The TRPA1 agonist cinnamaldehyde (100 μM) evokes similar amplitude currents in GW exposed (GRP A) and vehicle exposed (GRP C) muscle nociceptors. Insert presents a representative response to cinnamaldehyde in a GRP A muscle nociceptor. HC: HC-03001; GRP A: permethrin, chlorpyrifos, PB, DEET; GRP PB: permethrin, chlorpyrifos, DEET GRP C: ethanol, corn oil, water, ethanol. OXO: Oxotremorine-M, 10 μM, 1 min at 35° C.; cinn: cinnamaldehyde 100 μM.

TASK 2.3: Summary and Conclusions

Vascular and muscle nociceptors both exhibited excitability shifts 16 weeks post-exposure to GW chemicals. Muscle nociceptor excitability was specifically tied to an increase in TRPA1 mediated depolarization that was secondary to mAChR activation. The magnified TRPA1 effect varied with the presence or absence of PB in the exposure set; thereby, cementing its role as a critical component of the long lasting behavioral deficits. We had previously demonstrated that multiple nociceptor ion channels exhibited altered functionality to mAChR following exposure to GW chemicals (K_v7 and K_{DR}; Cooper et al., 2016, see attached, p. 102). OXO (Oxotremorine-M) is a non-specific mAChR agonist that activates 4 muscarinic receptors M1, M2, M3 and M4. DRG neurons mainly express M2 and M4 subtypes (Bernardini et al. 1999; Tata et al., 2000; Cai et al., 2009). It is not known how these mAChR are differentially distributed amongst muscle nociceptors, but given their role in the modulation of TRPA1 and K_v7, this distribution could important implications for the pathophysiology and management of GWI pain.

The increase in vascular nociceptor excitability (see figure 15 and Table 2) took the form of decreased threshold, and increased AP discharge following stepped current injection induced depolarization and paralleled persistent shifts in activity of $Na_v1.9$,. Because $Na_v1.9$ is a voltage dependent, non-desensitizing channel, it would especially contribute to step depolarizations that were used in excitability testing (figure 15).

Stepped depolarization occur naturally during exposure to numerous pro-inflammatory mediators. As these were vascular nociceptors, it is important to consider those specific mediators released

by vascular endothelial cells, blood stream constituents or vascular efferents (ACh, bradykinin, prostacyclin, thrombin, serotonin, noradrenaline, NO (nitric oxide), SP, CGRP). These agents are associated with, not only with vascular damage, but also with routine vasoactive mediators of dilation and constriction. As we show below (TASK 3), rats exposed to GWI chemicals exhibited chronic vasodilation fundamentally linked to anticholinesterase exposure (PB). CGRP (calcitonin gene related peptide) is a mediator of vasodilation. The vascular nociceptors we study express both CGRP and SP (substance P) and release these agents during burst discharge (Petruska et al., 2002; Rau et al., 2014; Iyengar et al., 2017). In addition to vasoactivity, SP acts on resident mast cells to produce a localized neurogenic inflammation (Geppetti et al., 2005). The muscarinic burst discharge (MDBD) we identified could be an important path to chronic vasodilation and neurogenic inflammation. However, only subset of vascular nociceptors exhibited MDBD. As yet, we have been unable to reliably isolate this important subpopulation. Alternately, relatively minor inflammatory activity around vessels could be amplified by maladapted $Na_v1.9$ into a chronic pain condition.

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

Timeline: months 1-5

We reported that DEET accelerated the development of pain behaviors (Flunker et al., 2017), and that is associated with changes in the physiology of $Na_v1.9$ and K_v7 ion channels in vascular and/or muscle nociceptors. These maladaptations might occur due to chronic interaction of pesticides or repellants with these ion channels during the exposure period. Accordingly, in Task 2.4, we examined the hypothesis that DEET accelerated the development and lengthened 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. We isolated membrane currents expressed in muscle and vascular nociceptor neurons that were harvested from young adult male rats. Using whole cell patch clamp methodology, we exposed these neurons to DEET (10-100 μM; 2 minutes).

There was no evidence that DEET modified the activity of K_v proteins (K_v7, K_{DR}; figure 17). Nor was there clear evidence that DEET altered activity of Na_v ion channels at physiological dosages ($Na_v1.8$; $Na_v1.9$; figures 18 and 19respectively). Although some DEET mediated shifts in the activity of $Na_v1.9$ were produced at a dose of 100 μM (figure 10), we did not consider such effects to be physiologically significant at that dose.

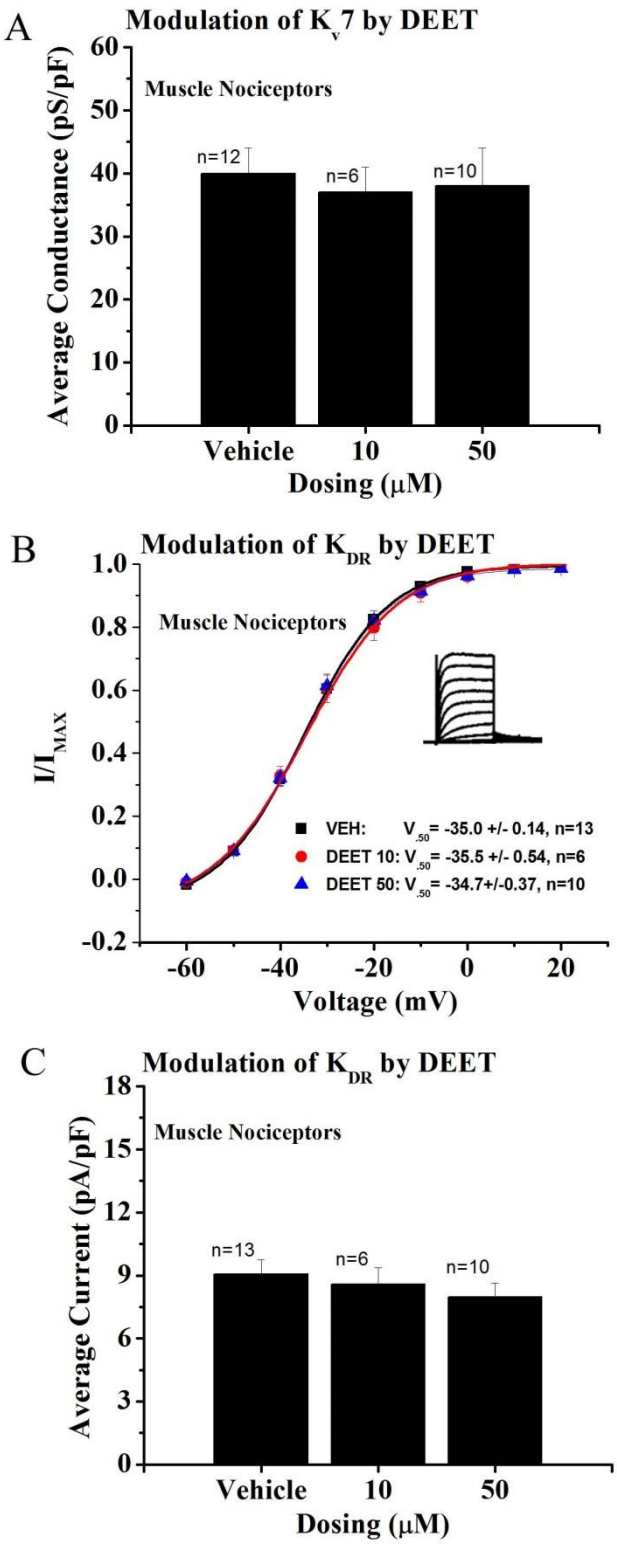

Figure 17. Voltage Activated K^+ Channels were Unaffected by Acute Exposure to DEET. A) The average conductance of muscle nociceptor K_v7 channels was not altered by DEET (10-50 μM); **B) and C)** Following a 12 minute exposure, there was no indication that either the voltage dependence or the average 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.

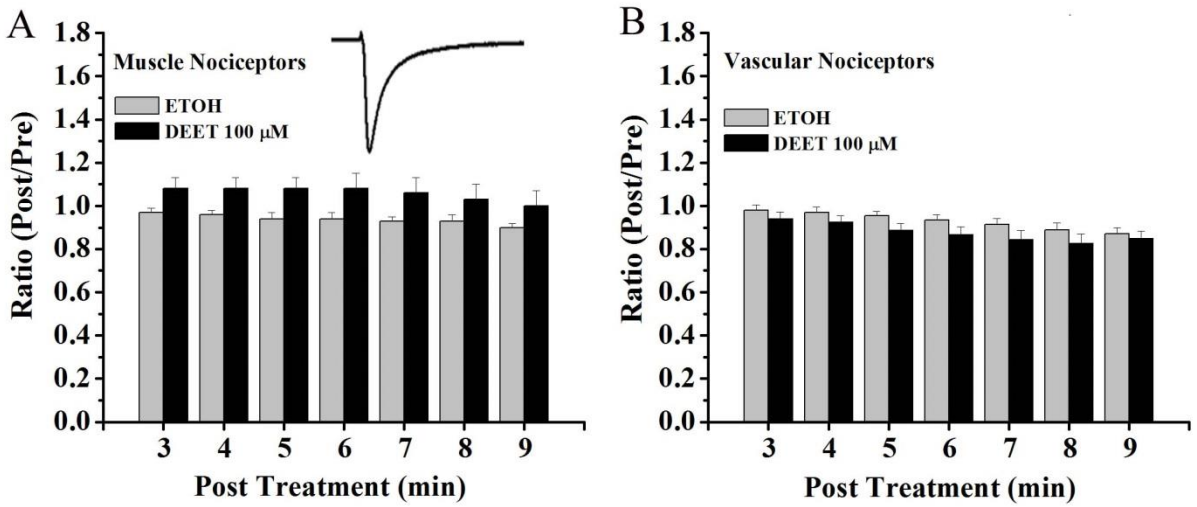

Figure 18. Time Dependent Modification of $Na_v1.8$ by DEET. A) The amplitude of muscle nociceptor $Na_v1.8$ was not changed by DEET (100 μM). A representative $Na_v1.8$ current is inserted. **B)** Vascular nociceptor $Na_v1.8$ amplitude was not altered by DEET (100 μM). Baseline records were taken prior 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.

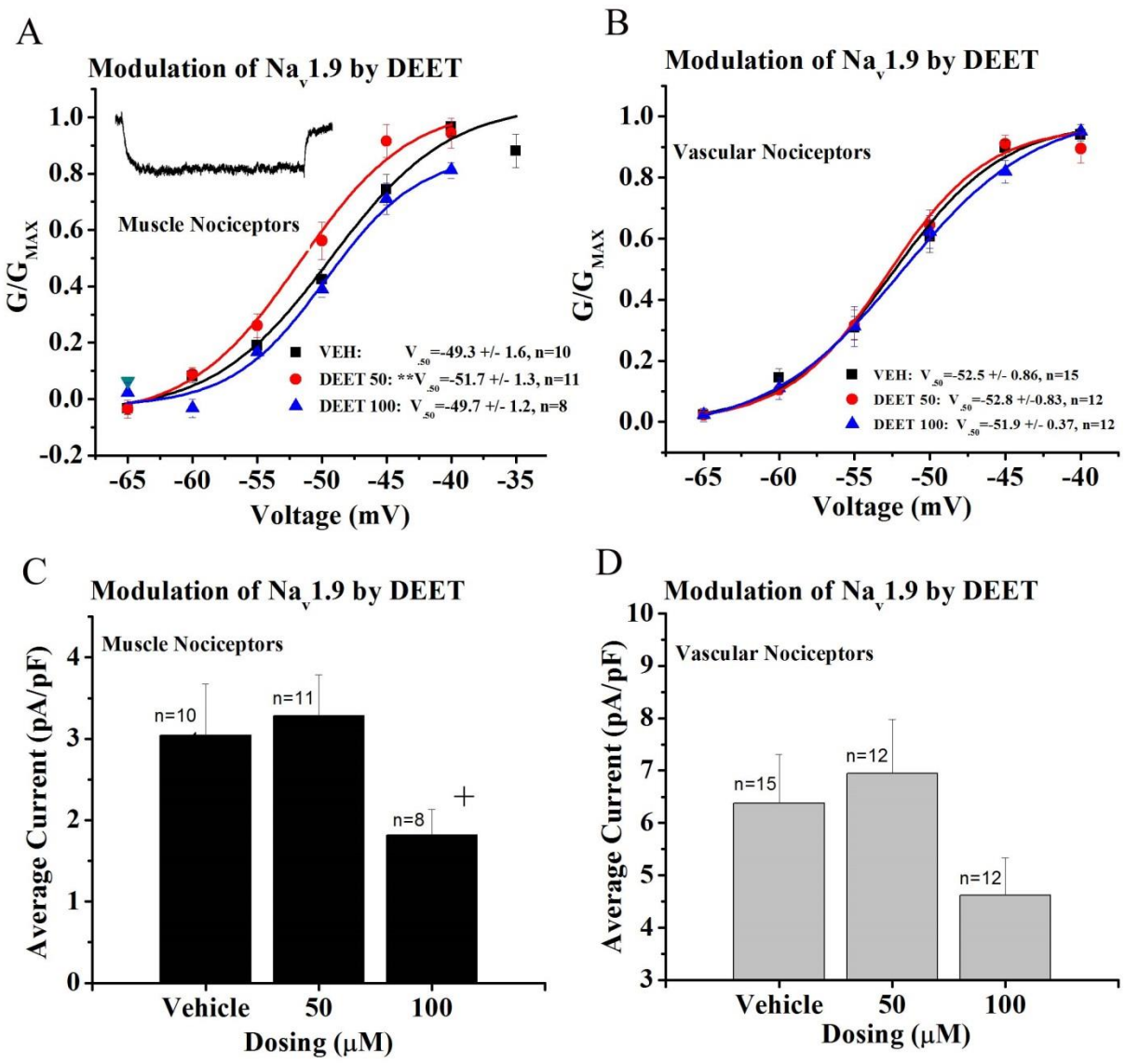

Figure 19. Voltage Activated, Na$_v$1.9, Channels were Weakly Modulated by Acute Exposure to DEET. A) The voltage dependent activation of muscle nociceptors was hyperpolarized at 50 but not at 100 μM DEET. A representative trace of a Na$_v$1.9 current (step to -50 mV; V_H=-120 mV) is included as an insert. **B)** The voltage dependent activation of vascular nociceptors was unaffected by DEET. **C)** Average currents of muscle nociceptors were unaffected by DEET ($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).

TASK 3.1: 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.

METHODS:

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. 84).

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 (vasodilation) in the hindpaw heels (figure 20). Blood flow was at control levels at 16WP. 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. 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 (figure 21). This suggests the increased vasodilation may have been due to overactive vascular afferents releasing vasodilator transmitter like CGRP, supporting our hypothesis.

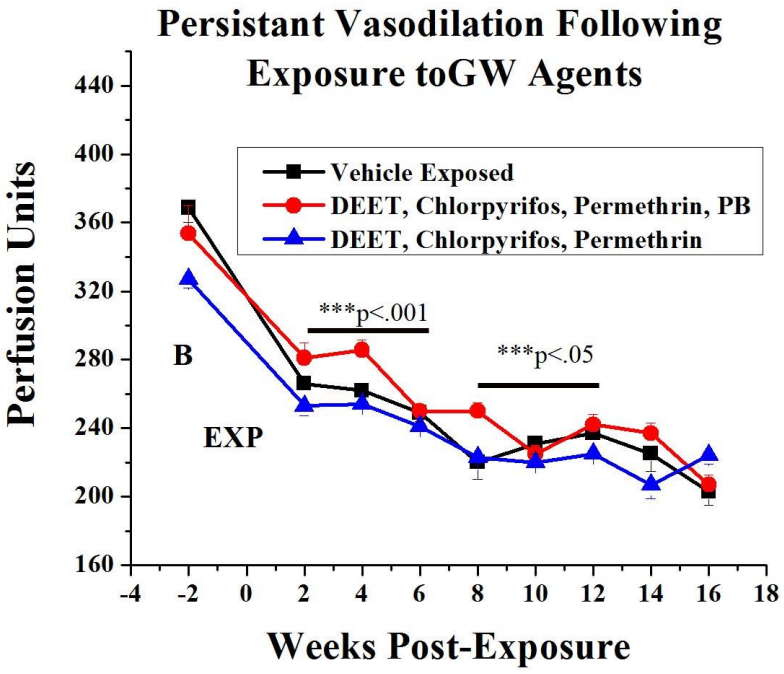

Figure 20. Exposure to GWI chemicals produces increased vasodilatation. A) Rats exposed to GW agents for 4 weeks develop persistent vasodilation (p<.001, weeks 2-6; p<.05 weeks 8-12). When PB is absent from the exposure, vasodilation is significantly reduced relative to 4 GW agent exposed rats (p<.001 at weeks 2-6 and 8-12). Laser speckle contrast imaging of hindpaw (n=6). B: baseline; EXP: 4 week exposure to PB and/or permethrin, chlorpyrifos, and DEET. No measures were taken during the 4 week exposure.

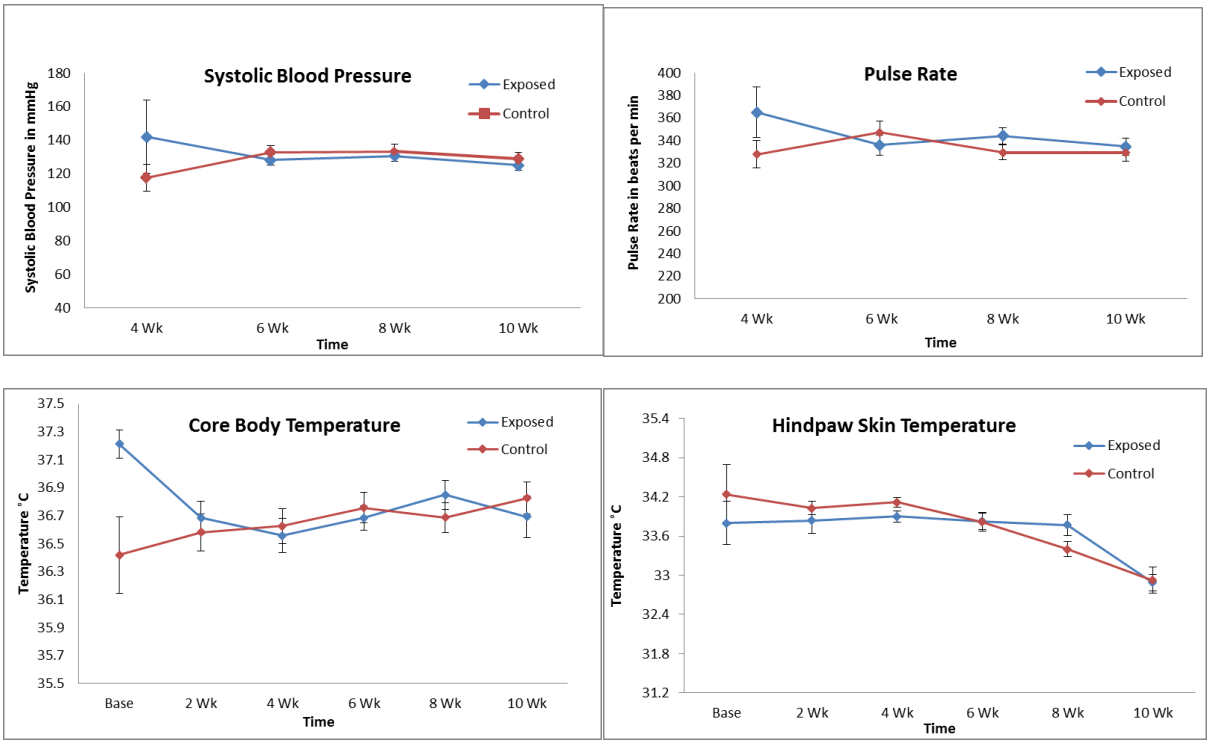

Fig. 21. Exposure to 4 GWI chemicals does not produce changes in autonomic vital signs parameters for up to 10W after end of exposure period. Mean ± SEM of systolic blood pressure, pulse rate, core body temperature, and hindpaw skin temperature measured before (baseline for temp data) and after the 4-week exposure period. There were no significant differences between exposed animals (Group X, n=14) compared to control animals (n=16).

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 (Figure 22). 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 (Figure 20). 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.

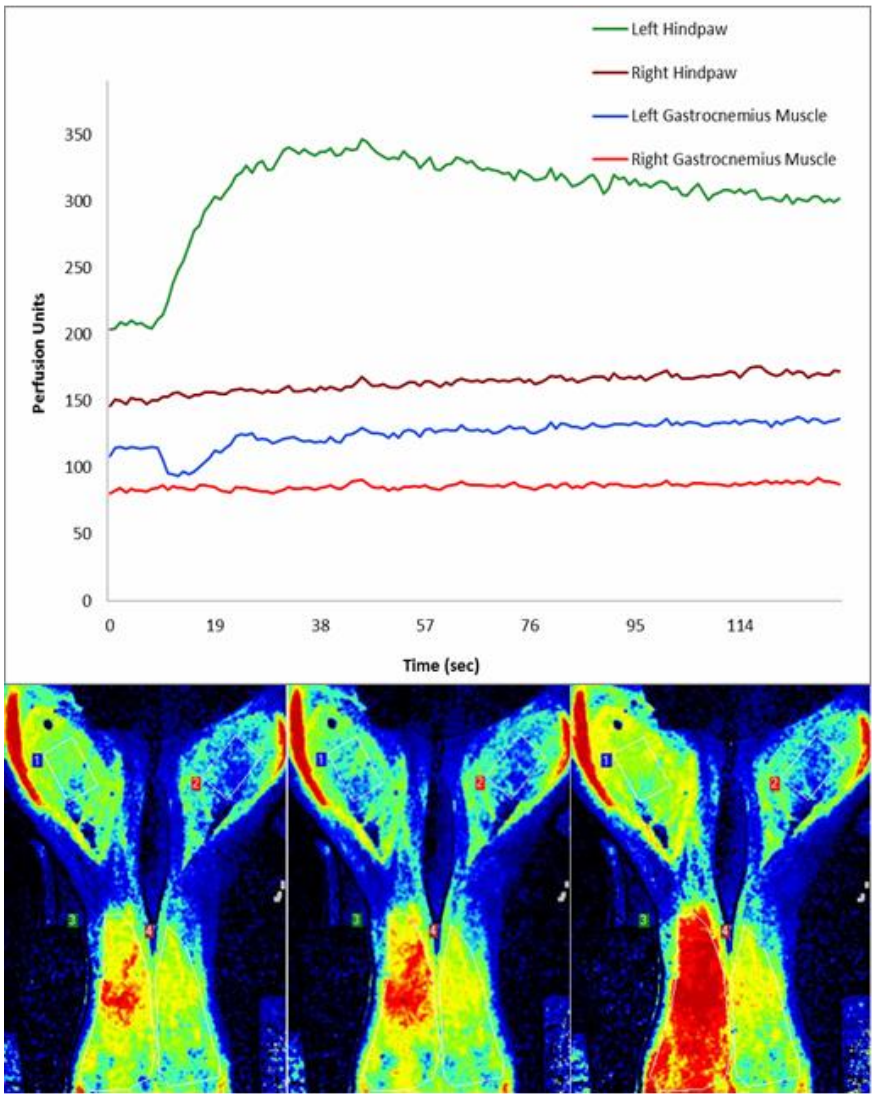

Figure 22. Left Decentralized Distal Sciatic Nerve Stimulation. Top panel: Blood flow measurements with the LSCI of the distal left/right gastrocnemius muscles and left/right hindpaws (lower images) expressed graphically with perfusion values (in perfusion units) plotted on the y-axis and elapsed time 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

This data is in progress

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

Task 3.3 was not executed as we were no longer able to obtain the CGRP blocker (BMS-927711)

as it was removed from clinical trials.

4. Impact

Impact on Principal Disciplines

Veterans suffering from GWI have struggled with a wide variety of sensory, motor and cognitive symptoms. While insecticide exposure has been long suspected as contributor to GWI, the pathophysiology of the multisymptom complex has remained obscure (Binns et al., 2008; RAC, 2014). Moreover, excessive emphasis has been unduly placed upon the delayed nature of the syndrome, when in fact, about 25% of warfighters developed symptoms of GWI while still in theater (Kroenke et al., 1998).

Through our rat model system, we have now demonstrated that: 1) the rate of development of pain-like symptoms can be manipulated by inclusion/exclusion of high concentrations of DEET in the exposure protocol (i.e.: DEET, permethrin, chlorpyrifos, PB); 2) the development of any pain-like symptoms are dependent upon inclusion of the anticholinesterases (PB or Chlorpyrifos); 3) PB exposure is critical to molecular and cellular maladaptations that accompany behavioral pain-like symptoms; 4) $Na_v1.9$ and TRPA1 are critical to the pathophysiology of GWI pain; 5) Exaggerated responses to muscarinic agonists dominate any specific maladaptation to TRPA1; and 6) Chronic autonomic nervous system malfunction, in the form of vasodilation, develops in parallel to behavioral deficits.

The presentation of the symptoms of GWI are likely to arise from molecular maladaptations or tissue damage consequent to these exposures. The molecular targets of these exposures have been clarified. We were able to demonstrate, that GW chemical induced shifts in the molecular activity of K_v7 did not fully parallel behavioral assessments of ambulation deficits that appeared over the course of testing. The divergence of K_v7 from the behavioral outcomes, in the presence/absence

of PB and also at the 16 WP tests, indicated that the voltage dependence of K_v7 was not definitively linked to the pathophysiology of chronic pain in our rat model. It is alternately possible that it is the muscarinic coupling to K_v7 that is actually the important factor. We have shown, in published reports, changes in functional muscarinic activity via the mAChR (muscarinic form of the cholinergic receptor) persist following exposure to GW agents (Cooper and Nutter, 2016). Because mAChR are diversely distributed throughout the nervous system (i.e.: M1, M2, M3, M4), differential alterations in their individual expression and function could be mediated through K_v7 as well as other ion channels that couple to this G-protein receptor. Clarification of the differential role of mACh receptors could lead to alternative treatment strategies for GW symptoms.

In contrast, our examination of $Na_v1.9$ activity in relation to behavioral effects clearly indicated that the activity of this ion channel covaried with the presence and absence of PB exposure and behavioral signs. The importance of this ion channel in the pathophysiology of the condition was amplified by these findings. We have reason to believe that $Na_v1.9$ is also sensitive to mAChR activation (preliminary data) and may also contribute to pain behaviors via muscarinic maladaptions brought about by GW chemicals.

Despite our inability to verify a direct link between K_v7 function and the pathophysiology of GWI, we were able to demonstrate that Retigabine, a specific K_v7 opener, could reverse pain-like symptoms that had developed 9-12 weeks following exposure. Reversal of these signs were incomplete and transient. Ambulation deficits associated with exposure to GW chemicals reappeared following withdrawal of treatment. Nevertheless, this data predicts that Retigabine and other K_v7 openers (e.g., Flutirpine) could be effective palliatives for GWI pain. As we only treated rats with Retigabine once per day, more extensive dosing might have produced a better result. In clinical applications, Retigabine is administered 3 times daily. Staff limitations and the concurrent

execution of other experiments prevented the implementation of multiple daily dosing regimens. Alternately, other human use approved K_v7 openers are available for testing, and many are currently undergoing development for chronic pain applications.

Finally, for the third time we have demonstrated the critical importance of PB to the development of delayed GWI-like pain. While we do not expect that PB alone is responsible for the symptoms of GWI, it clearly plays a fundamental role. We have now shown that cellular, molecular and behavioral maladaptations are closely tied to the presence or absence of PB in the exposure set. There also seems to be a growing focus on the role of muscarinic receptor maladaptations as a critical pathophysiological event; ultimately, nociceptor discharges arise from effectors of mAChR activation (TRPA1, $Na_v1.9$, K_{DR})

There was no impact on technology transfer-nothing to report

There was no impact on society-nothing to report

5. Changes/Problems

Although we originally proposed a treatment test series using a CGRP blocker (BMS-927711) we were unable to execute this portion of the SOW. When the project was submitted in 2014, the CGRP receptor blocker, BMS-927711, was in a stage II clinical trial for use as a migraine treatment. However, it was withdrawn from trials in 2016 due to side effects. We were unable to obtain the compound from Burroughs Welcome or any other vendor despite repeated attempts over a 4 month period. We could not identify another suitable CGRP blocker to use in these studies.

Some electrophysiological studies were planned to include experiments at 16 and 24 weeks post-exposure. We were unable to complete the excitability (and other) studies at 24 weeks post-exposure due to time constraints; in part, due to expansion of the Retigabine studies of TASK 1.2. It was decided that the Retigabine treatment studies showed promise and should be followed out for 2 additional months.

6. Products

Journal Publications

The following manuscripts published during the funded period are attached (Appendix, pp. 102, 146 and 205).

The data for this publication was collected during the previous grant from CDMRP, but was published during the present grant (p. 102):

Cooper, BY Johnson RD and Nutter TJ. Exposure to Gulf War Illness chemicals induces functional muscarinic receptor maladaptations in muscle nociceptors. Neurotoxicology 2016, 54: 99-110.

The data for this publication was collected and published during the present grant:

Flunker, L.K., Nutter, T.J., Johnson, R.D. and Cooper, B.Y. DEET Amplifies Anticholinesterase Dependent Chronic Pain Signs in a Rat Model of Gulf War Illness Pain. Toxicology and Applied Pharmacology, 2017, 316: 48-62.

The following manuscript was recently submitted for publication:

Cooper, B.Y., Flunker, L.D., Johnson, R.D. and Nutter, T.J. Behavioral, Cellular and Molecular Maladaptations Covary with Exposure to Pyridostigmine Bromide in a Rat Model of Gulf War Illness Pain, submitted to Toxicology and Applied Pharmacology.

Abstracts and Presentations

Contributions of DEET to a Rat Model of Gulf War Illness Pain

Cooper, B.Y., Nutter, T.J., Johnson, R.D and Flunker, L.

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 $Na_v1.9$.

Molecular maladaptations to vascular nociceptor $Na_v1.9$ covaries with exposure to pyridostigmine bromide in a rat model of Gulf War Illness pain

Cooper, B.Y., Nutter, T.J.., Flunker, L.K. and Johnson, R.D

Introduction. Many veterans of Operation Desert Storm (ODS) still struggle with symptoms of GWI. Symptoms are manifested as diverse cognitive, motoric and sensory abnormalities that include chronic pain. In order to understand the pathophysiology of GWI pain, our laboratory has developed rat models of this multisymptom disorder.

Method. We examined the influence of 4 GW agents on the ambulatory and resting behaviors of rats. Young adult male rats were exposed to either 3 or 4 GW chemicals for a period of 4 weeks. One group was exposed to DEET (400 mg/kg; topical, 50%), permethrin (2.6 mg/kg; topical), chlorpyrifos (120 mg/kg; s.c.) and pyridostigmine bromide (PB, 13 mg/kg; oral; GRP A, n=56). A second group received the same exposure but PB was excluded from the protocol (GRP PB, n=10). A third group served as a vehicle control (Grp C, n=28; ethanol topical, corn oil s.c., and water gavage). Ambulation and resting scores were measured weekly by an automated infrared detection system. Nine weeks after exposure, thirty rats received treatments intended to ameliorate deep tissue pain (Riluzole, 3 mg/kg; Retigabine, 7 mg/kg; 14 days). Sixteen weeks after chemical exposures ended, an additional 34 rats were euthanized, and their dorsal root ganglia prepared for

whole cell patch studies. ANOVA was used to assess changes in rat behaviors due to exposures and treatments. Student t tests were used to assess molecular data.

Results. Rats exposed to 4 GW chemicals (GRP A) developed pain-like deficits in ambulation and resting that persisted 13-16 weeks post-exposure (16 WP; $p<.001$ and $p<.001$, respectively). Rats exposed to only 3 GW agents (PB excluded) did not exhibit pain-like signs in weeks 13-16. Compared to vehicle exposed rats (GRP C), the amplitude of vascular, but not muscle nociceptor $Na_v1.9$ was elevated in GRP A (16WP; $p<.02$); but K_v7 activity was unchanged. When PB was excluded from the exposure (GRP PB) vascular nociceptor $Na_v1.9$ amplitude was similar to controls and significantly reduced relative to neurons harvested from GRP A rats (16 WP; $p<.005$); Treatment with $Na_v1.9$ inhibitor, Riluzole, did not improve behavior scores at 9-10WP; but K_v7 opener Retigabine did produce positive trends ($p<.07$).

Conclusion. Exposure to PB was critical for the emergence of persistent pain signs in a rat model of GWI. Maladaptations of vascular, but not muscle nociceptor, $Na_v1.9$, covaried with the manifestation of pain-like behaviors. Treatments targeting $Na_v1.9$ were not effective at 10WP.

Long-term increases in hindlimb vasodilatation following exposure to Gulf War Illness (GWI) chemical prophylactic agents is independent of cardiovascular parameters and suggests involvement of CGRP release from vascular nociceptor endings in a rat model of GWI pain

Tournade, CM, Nguyen, HD, Cooper, BY, Johnson, RD

Gulf War veterans experienced high levels of exposure to insecticides/repellants and nerve gas chemoprophylactic agents, which resulted in a series of chronic clinical symptoms (i.e. Gulf War Illness). These symptoms include unusual complexes of headache, joint, muscle and abdominal pain. We previously developed a rat model of GWI pain produced by a 4-week exposure to 3 or 4 GWI chemical agents, measurements of ambulatory/resting behaviors during the exposure and the 16W post exposure period, and determination of electrophysiological profiles of single vascular nociceptors. In the present study, we hypothesized that GWI chemical-induced maladaptations in vascular afferents (shown by our previous data) would produce increased peripheral release of their predominant constitutive vasodilatory neuropeptide, CGRP, resulting in subsequent vasodilatation and increased detectable blood flow. This study used a noninvasive laser scanning contrast imaging (LSCI) to measure hindpaw blood flow. Young adult male rats were exposed to either (i) DEET (400 mg/kg; topical, 50%), permethrin (2.6 mg/kg; topical), chlorpyrifos (s.c.; 120 mg/kg) and pyridostigmine bromide (PB,13 mg/kg; oral; GRP A, n=16), (ii) all chemicals except for PB (GRP PB, n=14), or (iii) vehicle control (Grp C, n=16; ethanol topical, corn oil s.c., and water gavage). Before and every two weeks after chemical exposure, rats were briefly anesthetized with isoflurane (10-15min) and blood flow in the plantar hindpaws recorded for five minutes with a PeriCam LSCI laser probe. The analysis area was set by programmable software based on spatial landmarks and was used for all animals to validate inter-animal and within-animal comparisons. Cardiovascular parameters of tail cuff blood pressure (systolic and mean arterial pressures), heart rate, and temperature (body core and hindpaw) were also measured. There were highly significant increases in hindpaw blood flow (vasodilatation) at post exposure weeks 4-10 in GRP A rats compared to GRP C controls, corresponding to behavioral measures of pain, despite the lack of differences in cardiovascular measures of blood pressure,

heart rate, and temperature. Interestingly, the GRP PB rats that lacked exposure to PB, did not show increased blood flow during the post exposure period. We conclude that in animals exposed to the four GW chemicals, long-term increases in blood flow (vasodilatation) were produced for at least 8 weeks after the exposure period, and in the absence of changes in cardiovascular parameters, suggests that hyperactivity in vascular afferents leads to increased release of vasodilator neuropeptides (e.g. CGRP) from the terminal endings.

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, 1.2, 1.3, 2.1, 2.2, 2.3, 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, 3.2, 3.3)

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 1.2, 1.3, 2.2)

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, 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3; 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, 3.2, 3.3)

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

Change in the Support for the PI:

A no-cost-extension of the current project is in place until 12/30/2017

Partner Organizations:

Nothing to report

Changes/Problems**:**

Nothing to report

8. Special Reporting Requirements

none

9. Appendices

Supplementary Figures

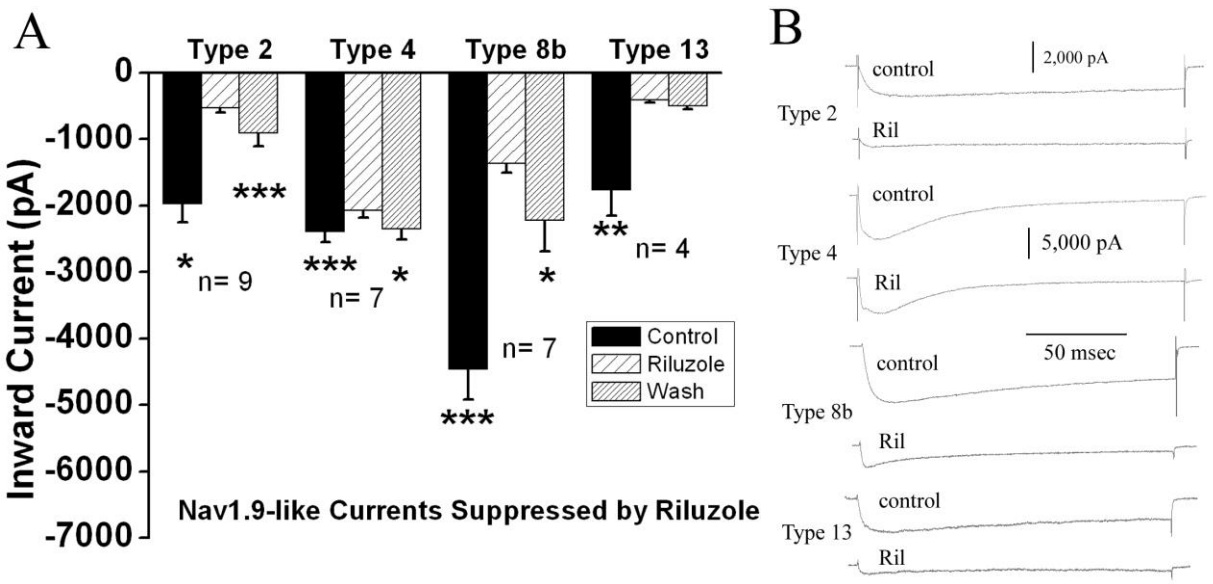

Figure A1. Vascular Nociceptor Nav1.9 Currents were Inhibited by Riluzole. A) Type 8 vascular nociceptors $Na_v1.9$ is significantly inhibited by Riluzole (10 μM; $p<.01$). The current recovers after a 2 minute wash out of Riluzole ($p<.05$). **B)** Representative $Na_v1.9$ current traces in superficial and deep tissue nociceptors (skin: types 2, 4 and 13; vascular nociceptor: type 8).

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 Floric
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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Exposure to Gulf War Illness Chemicals Induces Functional Muscarinic Receptor Maladaptations in Muscle Nociceptors

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Abstract

Chronic pain is a component of the multisymptom disease known as Gulf War Illness (GWI). There is evidence that pain symptoms could have been a consequence of prolonged and/or excessive exposure to anticholinesterases and other GW chemicals. We previously reported that rats exposed, for 8 weeks, to a mixture of anticholinesterases (pyridostigmine bromide, chlorpyrifos) and a Na_v (voltage activated Na^+ channel) deactivation-inhibiting pyrethroid, permethrin, exhibited a behavior pattern that was consistent with a delayed myalgia. This myalgia-like behavior was accompanied by persistent changes to K_v (voltage activated K^+) channel physiology in muscle nociceptors (K_v7, K_{DR}). In the present study, we examined how exposure to the above agents altered the reactivity of K_v channels to a muscarinic receptor (mAChR) agonist (oxotremorine-M). Comparisons between muscle nociceptors harvested from vehicle and GW chemical-exposed rats revealed that mAChR suppression of K_v7 activity was enhanced in exposed rats. Yet in these same muscle nociceptors, a Stromatoxin-insensitive component of the K_{DR} (voltage activated delayed rectifier K^+ channel) exhibited decreased sensitivity to activation of mAChR. We have previously shown that a unique mAChR-induced depolarization and burst discharge (MDBD) was exaggerated in muscle nociceptors of rats exposed to GW chemicals. We now provide evidence that both muscle and vascular nociceptors of naïve rats exhibit MDBD. Examination of the molecular basis of the MDBD in naïve animals revealed that while the mAChR depolarization was independent of K_v7, the action potential burst was modulated by K_v7 status. mAChR depolarizations were shown to be dependent, in part, on TRPA1. We argue that dysfunction of the MDBD could be a functional convergence point for maladapted ion channels and receptors consequent to exposure to GW chemicals.

1. Introduction

Following the 1991 Persian Gulf War, thousands of returning soldiers developed a syndrome comprised of a varying mixture of cognitive, motor, sensory and autonomic dysfunctions that came to be known as Gulf War Illness (GWI; Binns et al., 2008; Haley et al., 2013; White et al., 2016). A large portion of those suffering from GWI complained of chronic pain symptoms that were described as a mixture of headache, back pain, muscle, joint, and abdominal pains (Haley et al., 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006). While symptoms typically arose after warfighters had returned from their deployments, a significant portion of veterans (25%) reported symptoms of GWI while still in theater (Kreonke, et al., 1998). In succeeding years, the severity of GWI symptoms tended to remain constant or worsen (Hotopf et al., 2003).

Diverse risk factors, including exposure to Sarin gas, depleted uranium, oil fires, vaccination adjuvants, organophosphates and combat stress have been proposed as factors contributing to the development of GWI. However, no single factor has been able to account for the wide ranging symptoms of this complex multisymptom disease. The Research Advisory Committee on Gulf War Illness concluded that pesticides could have contributed to the development of the symptoms of GWI (Binns et al., 2008; RAC2014). While deployed to the Persian Gulf, soldiers were potentially exposed to 67 insecticides and repellants that contained 37 distinct 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. Coincident with exposure to these agents, warfighters were self-administering a nerve gas prophylactic, pyridostigmine bromide (PB), which shares the anticholinesterase properties of many insecticidal chemicals. Pesticides were to be used at specific concentrations and with specific application methods and procedures. These application procedures were not always carefully followed and warfighters often supplemented designated agents with others they acquired on their own (US, DoD Environmental Exposure Report: Pesticides, 2003). While the levels of exposure to any one of these pesticides may not have posed a significant detriment to the health of the troops, synergisms arising from multiple chemical exposures could have converged on one or more molecular targets to produce long lasting physiological maladaptations and/or physical damage to nervous system components.

Utilizing a variety of exposure protocols under laboratory conditions, it has been demonstrated that combinations of permethrin, chlorpyrifos, pyridostigmine bromide and other GW chemicals, produce a variety of motor and cognitive signs (Servatius et al., 1998; Servatius et al., 2000; Abou-Donia et al., 2001; Abdel-Rahman et al., 2004a; Abou-Donia et al., 2004; Parihar et al., 2013), induce inflammatory agents (Li et al., 2001; Singh & Jiang, 2003; Terry, 2012), cause microvascular injury (Ojo et al., 2014), degrade the blood brain barrier (Grauer et al., 2001; Abdel-Rahman et al 2002; Abdel-Rahman et al., 2004b), suppress enzyme activity (Abdel-Rahman et al., 2002; Casida and Quistad, 2005), and damage DNA (Falcioni et al., 2010). However, a laboratory model suitable for the study of GWI related chronic pain has been elusive (Scremin et al., 2003; see also Lotti and Moretto 2005).

Several years ago we began a series of studies that were designed to reproduce the myalgia and arthralgia of GWI in a rat model. Certain GW chemicals have properties that could directly (pyrethroids) or indirectly (anticholinesterases) interact with important membrane ion channel and receptor proteins expressed in peripheral nociceptors (e.g., voltage activated Na^+ channel 1.8 ($Na_v1.8$): Narahashi et al., 1998; Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006; Jiang et al., 2013; Muscarinic receptors: Abou-Donia et al., 2003; Abou-Donia et al., 2004; voltage activated K^+ channel 7 (K_v7): Marion 1997; Robbins, 2001; Brown and Passmore, 2009). Following a 60 day exposure to the anticholinesterases pyridostigmine bromide and chlorpyrifos and the pyrethroid insecticide permethrin, we discovered that the physiology of vascular and/or muscle nociceptor ion channel proteins $Na_v1.9$ and K_v7 were altered 8 to 12 weeks after exposures had ceased (Nutter et al., 2013; Nutter and Cooper, 2014). In contrast, the physiology of $Na_v1.8$ was unaffected in these nociceptor pools (Nutter et al., 2013). Despite a relatively consistent pattern of perturbed ion channel physiology, we could not demonstrate behavioral changes consistent with the development of a chronic pain syndrome.

Recently, we reported that a revised exposure protocol, modified so as to increase the frequency of anticholinesterase exposure, did have a substantial effect on post-exposure rat behavior patterns. Disturbances in movement and rest patterns that evolved over a 12 week observation period suggested the development of a delayed myalgia/arthralgia. Molecular studies tended to support this interpretation, as K_v7 and other K_{DR} (voltage activated delayed rectifier K^+ channel) currents were greatly diminished in muscle nociceptors during the manifestation of pain-like signs. Moreover, action potential bursts induced by muscarinic agonists were significantly increased during periods of heightened pain-like behaviors (Nutter et al., 2015). The emergence of pain signs following an increased frequency of exposure to chlorpyrifos and PB suggested that

anticholinesterases played a critical role of in the development of some GWI related pain. The changes we observed in the physiology of K_v7 proteins could reflect changes in expression of these channels or alteration in the pathways that regulate their activity**.**

Exposure to chlorpyrifos or PB can increase expression of muscarinic receptors in neocortex (Abou-Donia et al., 2003; Abou-Donia et al., 2004). Potentially, the expression of muscarinic receptors (mAChR) could have been altered in DRG (dorsal root ganglion) following exposure to chlorpyrifos and PB. In the experiments described below, we examined whether a 60 day exposure to permethrin, chlorpyrifos and PB altered nociceptor K^+ channel reactivity to a muscarinic agonist, and whether the pattern of alteration was consistent with the development of a GWI pain syndrome.

2. Methods

2.1. Behavioral Studies

2.1.1. Exposure Protocol

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. Juvenile male rats, initially weighing between 90 and 110 g, were used in all studies (chronic studies: n=35; acute studies: n=17; Sprague-Dawley; Harlan/Envigo). In chronic experiments, 15 rats 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), and pyridostigmine bromide (PB; 13 mg/kg), for 60 days. Permethrin, in ETOH, was applied every

day to a shaved area of the back between the forelimbs. Chlorpyifos was administered by a subcutaneous injection (corn oil) once every 7 days. PB was given daily by oral gavage (tap water). The latter represented a standard military dose of PB (assuming a 70 kg body weight). Twenty additional rats received only vehicle exposures using an identical administration schedule. Rats were sacrificed for electrophysiological studies 12 weeks after chemical exposures had ended. All rats underwent behavioral testing before, during and after chemical exposures (see below). There was little indication that chemical exposures affected body weight. The average body weights of vehicle and chemically exposed rats did not differ at the 8 week post-exposure period (442.4 +/- 5.0 g; n=20, Vehicle; and 430.8 +/- 8.6 g; n=15, Exposed; p<.28). Rats were weighed once per week and doses were adjusted according to body weight.

2.1.2 2.1.2. Assessment of Pain Behaviors

On arrival, rats were acclimated to the behavioral procedures for 2 weeks before dosing began. Testing continued throughout the entire dosing and post-dosing periods. Pressure pain withdrawal thresholds were measured using a computer monitored, hand held test device (PAM; Ugo Basile). Pressure was applied via a 5 mm diameter ball force transducer to the semitendinosus and biceps femoris muscles (left hind limb). During force application, the applied pressure was monitored and displayed to the experimenter on a video screen. Video feedback enabled the rate of force application to be regulated according to a visual standard. When the rat withdrew its limb, the force at withdrawal was automatically registered and stored. To complement pressure pain testing, activity levels (movement distance, movement rate, and rest times) were recorded automatically by infrared sensors in an activity box (15 min test period). Behavior tests were conducted on both chemical-exposed (permethrin, chlorpyrifos, PB) and vehicle treated (ETOH, corn oil, water) animals over an identical time course. From baseline testing (figure 1) until they were sacrificed,

the rats were tested once per week on the 4 behavioral tasks. In order to avoid potential experimenter test bias, all the manual PAM tests were conducted under 'blinded' conditions (with the exception of two days). Automated activity testing did not require experimenter blinding. None of the 'non-blinded' PAM data fell within the periods of statistical testing.

2.2. Electrophysiological Studies

2.2.1. Preparation of Cells

Dorsal root ganglion neurons (DRG) were harvested from GW chemical and vehicle exposed rats 12 weeks after termination of chemical exposures. 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 (4 mg/ml) and Dispase II (10 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 subsequently spun at 1000 RPM (30 sec). The supernatant was discarded. The remaining pellet was dispersed into 2 ml of Tyrode's, triturated and plated on 8-10, 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 on specific ion channels (voltage clamp tests on K_v7 and K_{DR}) were conducted at room temperature (20 °C) within 10 hours of plating. Studies on membrane excitability were conducted at 34 °C (current clamp testing). Only one cell was used per Petri dish (9 Petri dishes per daily

experiment). Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). Unless otherwise noted, 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. 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 Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 60-70% with Clampex compensation circuitry. Whole cell resistance and capacitance were determined by the Clampex 9.0 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), type 8 (vascular) nociceptors or type 19 (vascular) using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2000; 2002; Cooper et al., 2014; see also Xu et al., 2010; Ono et al., 2010). Anatomical targets of type 5, type 8 and type 19 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). Cells not fitting the classification criteria were discarded.

2.2.3. Current Clamp Experiments

Type 5 muscle, type 8 and type 19 vascular neurons were identified in voltage clamp. Studies were then conducted in current clamp mode (see also Nutter et al., 2015). The recorded cell was exposed to 10 μM oxotremorine-M for 30 seconds (OXO-M; Sigma-Aldrich). In some tests,

linopirdine (10 μM in ethanol; Sigma Aldrich) or HC-030031 (10 μM in DMSO; Sigma Aldrich), were presented for 2-3 minutes prior to the application of OXO-M. Linopirdine is a specific antagonist of K_v7 channels. It was applied when it was necessary to remove resting and evoked K_v7 channel activity (aka M-current; Brown and Passmore, 2009) from a given recording. OXO-M is a broad activator of mAChRs that is used to inhibit resting and evoked K_v7 activity that follows muscarinic receptor activation in many neurons. K_v7 inhibition can produce a depolarization due to the presence of a resting or voltage activated outward K^+ current (Brown and Passmore, 2009). HC-030031 is a specific antagonist of TRPA1 (McNamara et al., 2007). It was used to determine whether TRPA1 contributed to a depolarization evoked by OXO-M. When an antagonist (linopirdine or HC-030031) preceded OXO-M presentation, the antagonist (or its vehicle) was included in the OXO-M application.

To ensure that multiple channels involved in burst discharge operated with physiological kinetics, the current clamp tests were conducted at 34 ºC. The superfusion temperature was controlled by a heated probe positioned ~1 mm from the target cell (Cell Microsystems; HPRE probe). Net depolarization and total number of action potentials discharged were scored off-line. Depolarization was determined as the difference between the resting membrane potential 10 sec prior to the application of OXO-M and the peak depolarization that occurred 30 sec following application.

2.2.4. Isolation and Characterization of K_v Currents

K^+ currents (K_{DR}, K_v7) were characterized in a 'Kiso' (potassium current isolation) solution containing (in mM): 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 10 glucose, 10 HEPES, adjusted to pH 7.4 with HCL. On the day of the experiment, 1mM $CsCl_2$ and 2 mM 4-amino pyridine were added to block H-currents (I_H) and A-currents (I_a), respectively. 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. The osmolarity was approximately 290 mOsm.

For the purpose of this study, the K_{DR} current was defined as the residual total K^+ current following removal of the linopirdine sensitive K_v7 component. Attempts to remove other components using specific K_v1 inhibitors Maurotoxin ($K_v1.1$ and Kv1.2; Visan et al., 2004), Margatoxin ($K_v1.3$; Alomone Labs) or Dendrotoxin-K ($K_v1.1$ $K_v1.2$; Alomone Labs) were unsuccessful.

Stromatoxin (StTX) is a highly specific K_v toxin that targets K_{DR} channel proteins $K_v2.1$ and $K_v2.2$ (Escoubas et al., 2002). Following a 3 minute application of the Kiso solution, a series of strong depolarizations were used to evoke the K_{DR}, and to examine whether it contained Stromatoxin-sensitive components. From a holding potential of -60 mV, a 2,000 msec conditioning pulse (-100 mV) was followed by a 500 msec step to +20 mV. A pre-toxin baseline was established over the course of 6 to 9 test pulses (until the evoked current stabilized). Approximately 25 seconds separated each test. Subsequently, a solution containing Dendrotoxin (20 nM), Margatoxin (20 nM), Maurotoxin (100 nM) or Stromatoxin (100 nM) was applied for 5 minutes. Bovine serum albumin (BSA, 0.1%) was added to all solutions as a carrier for the toxins and for vehicle test contrasts against toxin testing. Toxins were applied continuously by close superfusion (~1 mm). Following toxin presentation, the Kiso solution (BSA and linopirdine added) was applied for 3

minutes as a washout test. There was little indication that the effect of a toxin could be washed out in 3 minutes.

Stromatoxin consistently inhibited substantial portions of the K_{DR} current in type 5, muscle, and type 8, vascular, nociceptors. The Stromatoxin-sensitive current was determined as the amount of current that decreased after 5 minutes in the presence of the toxin. Current amplitudes were measured off line. The amplitude of the evoked current was determined from a point 2 sec prior to the depolarizing step to a point 50 msec prior to the termination of the step. The baseline current (base) was determined as the average of the last 3 tests currents recorded prior to presentation of the toxin. The effect of a given toxin (tox) on the current was assessed as the difference of the baseline current from the average of the last 3 test pulses of the 5 minute toxin application series (base-tox). The measured values were normalized to cell capacitance. The Stromatoxin-insensitive current was determined from the currents remaining after 5 minutes in the presence of the toxin. The baseline current (base) was the average of the last 3 tests currents recorded prior to presentation of toxins. The toxin insensitive current (I_{tox}) was the average of the last 3 tests evoked after 5 minutes in the presence of the toxin. The percentage of toxin insensitive current in the K_{DR} was then determined as a ratio: I_{tox}/base.

2.2.5. Muscarinic Influences on K_{DR}

K_{DR} currents, expressed by type 5 and type 8 nociceptors harvested from GW chemical or vehicle treated rats, were exposed to a muscarinic agonist (OXO-M). The K^+ isolation (Kiso) solution was used to isolate K_{DR} current as described above. Ten micromolar (10 μM) linopirdine was added to block K_v7 components of the current. Following a 3 minute application of the Kiso solution, a series of strong depolarization were used to evoke the K_{DR}. From a holding potential of -60 mV,

a 2,000 msec conditioning pulse (-100 mV) was followed by a 500 msec step to +20 mV. The evoked current was leak corrected using the P/N procedure utility of pClamp 9.0. A pre-OXO-M baseline was established over the course of 6 to 9 test pulses (until the evoked current stabilized). Approximately 25 seconds separated each test. Subsequently, a solution containing 10 μM oxotremorine-M (OXO-M; Sigma Aldrich) was applied for 4 minutes by close superfusion.

The OXO-M sensitive current was determined as the percentage of current that increased after 2 minutes of application. Current amplitudes were measured off line. The amplitude of the evoked current was determined from a point 2 sec prior to the depolarizing step to a point 50 msec prior to the termination of the 500 msec step to +20 mV. The baseline current (base) was measured as the average of the last 3 tests currents recorded prior to presentation of OXO-M. The effect of oxotremorine-M (OXO) was the average of the first 4 test evocations following the initial application of OXO-M. The percentage of OXO-M sensitive current in the K_{DR} was then determined as a ratio: (OXO/base).

Statistics. The influence of chemical exposure on dependent measures of muscle pain threshold (grams), movement distance (cm/15 min), movement rate (cm/sec) and rest time (sec/15 min) were assessed by ANOVA for repeated measures. Follow-up tests were not conducted on significant main effects or interactions. Separate analyses of pain behaviors were performed on periods in which rats were sacrificed for molecular studies (5-8 weeks and 9-12 weeks post chemical exposure). We did not perform analyses on other time periods either during or after chemical exposures. Student's t-test was used to compare amplitude measures of K_{DR} and K_v7 activity in GW exposed and oxotremorine-M exposed nociceptors with vehicle treated cell groups. The alpha level was set at .05. Multiple tests within the same experiment were corrected for Type 1 error using the Bonferroni procedure.

3. Results

3.1. Behavioral Studies

Following an 8 week exposure to GW chemicals (PB, permethrin, chlorpyrifos), rats exhibited a delayed increase in resting behavior that was consistent with an emergent pain syndrome 9-12 weeks after exposures had ended (figure 1D). Relative to vehicle treated control rats, movement distance and movement rate measures were not modified by GW toxicants during the 9-12 week test period (figure 1B and C).

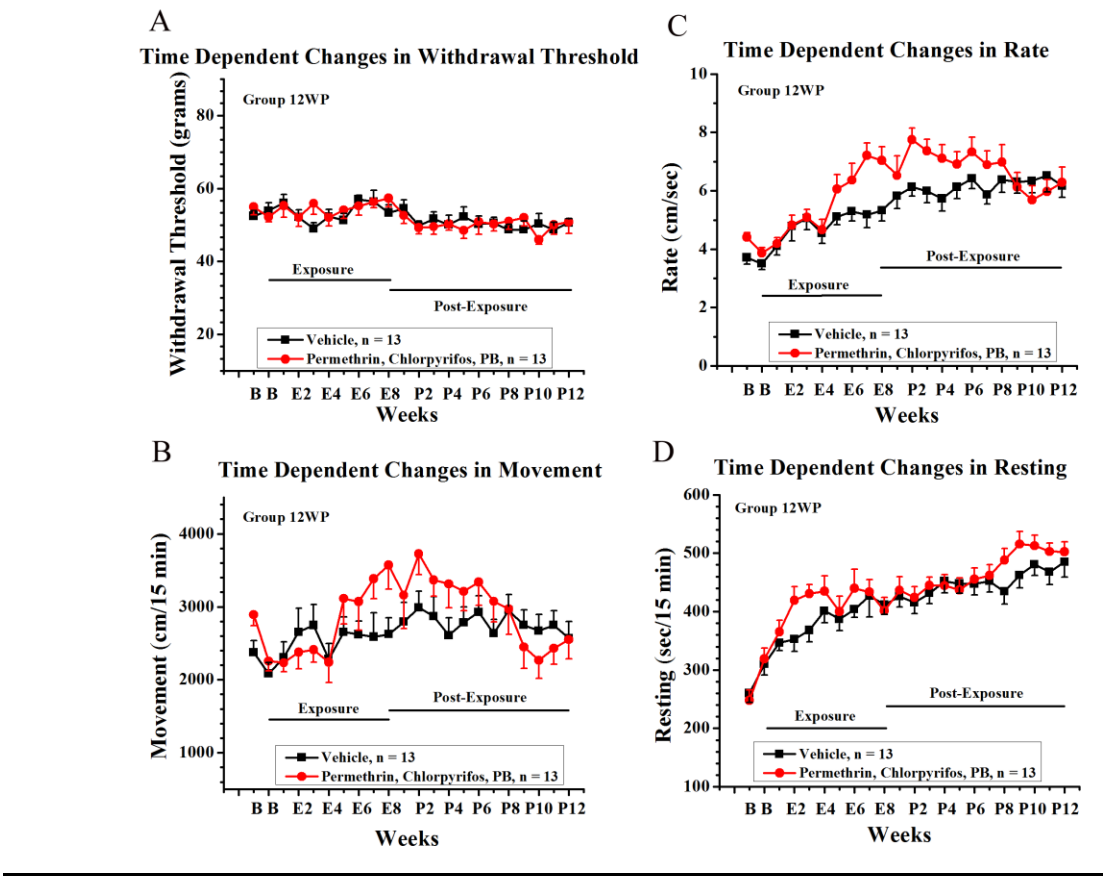

Figure 1. The Intensified Exposure Protocol Produced a Delayed Pain-Like Behavior Pattern in the 12 Week post Exposure Group. A)-B) Muscle pain pressure threshold, and movement distance were unchanged following the 60 day exposure to GW chemicals. **C)** Movement rate significantly increased during weeks 5-8 ($F=6.92$; $p<.01$) but returned to normal levels during weeks 9-12. **D)** Resting was significantly increased 9-12 weeks post-exposure ($F=5.82$; $p<.02$). Statistical tests were not conducted on any measure 1-4 weeks post exposure or during exposure to GW chemicals. B-baseline tests; E-exposure week; P-post-exposure week.

3.2. Molecular Studies

3.2.1. Subcomponents of the K_{DR} Current Modified by GW Chemicals

We had previously demonstrated that the average amplitude of K_{DR} currents declined following the 8 week GW chemical-exposure protocol (Nutter et al., 2015). Because certain chronic pain behaviors have been linked to the reduced expression of K_{DR} components, studies were conducted to identify the particular K_{DR} subunit(s) whose expression or function was altered by GW agents (e.g., $K_v1.2$, $K_v2.1$, $K_v2.2$; Zhao et al., 2013; Fan et al., 2014; Li et al., 2015; Tsantoulas et al., 2014a; Tsantoulas, 2014b), and whether these components exhibited reactivity to a muscarinic channel agonist.

Neurons were identified as muscle or vascular nociceptors as previously described (Petruska et al., 2002). Toxins that targeted $K_v1.1$, $K_v1.2$ and $K_v1.3$ (Dendrotoxin-K (20 nM); Maurotoxin (100 nM); Margatoxin (20 nM), respectively; n=10) were applied by close superfusion, but failed to produce significant reduction of the K_{DR} current. In order to isolate K_{DR} currents from other voltage activated K^+ currents (i.e., A-currents), 2mM 4AP was included in the K_{iso} solution (Castle et al., 1994; Grissmer et al., 1994; Russel et al., 1994). As 4-AP also blocks a portion of the K_{DR}, it was possible that most of the current attributable to $K_v1.1$, $K_v1.2$, and $K_v1.3$ were already blocked by 4AP. Stromatoxin (StTX) targets K_{DR} channel proteins $K_v2.1$ and $K_v2.2$. When presented by close superfusion, StTX (100 nM; 5 min) produced substantial reductions of K_v currents in both muscle and vascular nociceptors (figure 2). There was no indication that exposure to GW chemicals changed the total amount of $K_v2.1$ and $K_v2.2$, StTX sensitive current, expressed in either muscle or vascular nociceptors (figure 2).

After exposure to StTX, a substantial portion of the K_{DR} current remained. It was apparent that the $K_v2.1$ and $K_v2.2$ currents comprised about 25 and 35% of the 4AP and linopirdine insensitive K_{DR} currents present in muscle and vascular nociceptors, respectively (figure 3). As the precise composition of the residual current is unclear, we will refer to this as the StTX insensitive current. In contrast to the StTX sensitive current, the 8 week exposure to GW chemicals significantly decreased the StTX insensitive K_{DR} current in muscle nociceptors (figure 3A). Therefore, those specific muscle nociceptor K_{DR} component currents that were down regulated by exposure to GW chemicals were not $K_v2.1$ or $K_v2.2$, nor were they likely to be $K_v1.1$, $K_v1.2$ or $K_v1.3$.

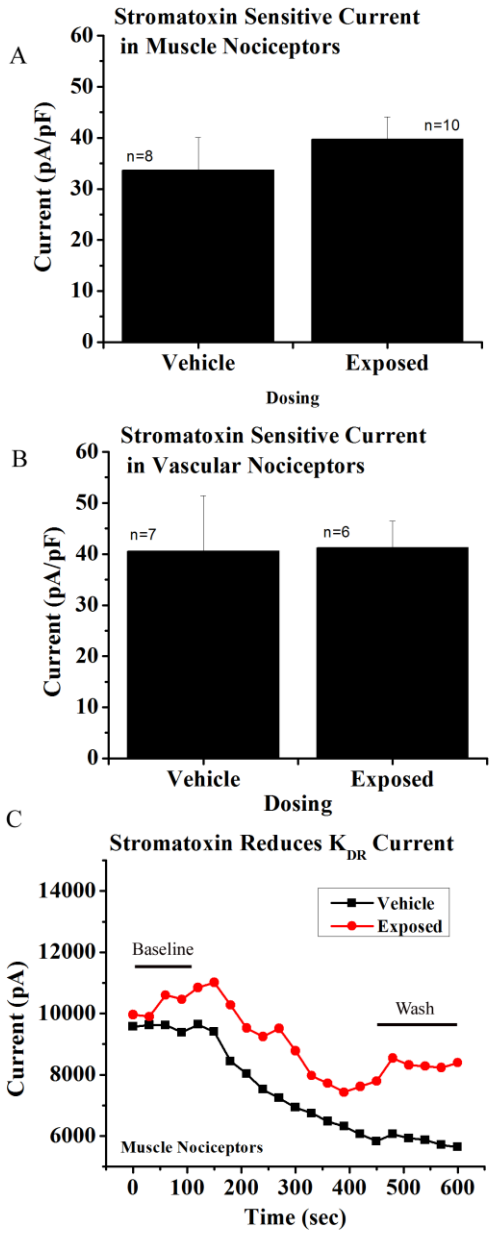

Figure 2. Stromatoxin-Sensitive Currents ($K_v2.1$, $K_v2.2$) in GW Exposed Nociceptors. The total $K_v2.1$ and $K_v2.2$ currents were not modified by chronic exposure to GW chemicals. **(A)** Muscle nociceptors **(B)** Vascular nociceptors. **C)** A representative case showing the time dependent change in peak K_{DR} current after exposure to StTX. Attempts to washout StTX and restore the K_{DR} currents to baseline levels generally failed. Cells were harvested 12 weeks after exposures had ceased. Experiments were conducted on cells collected from 13 rats.

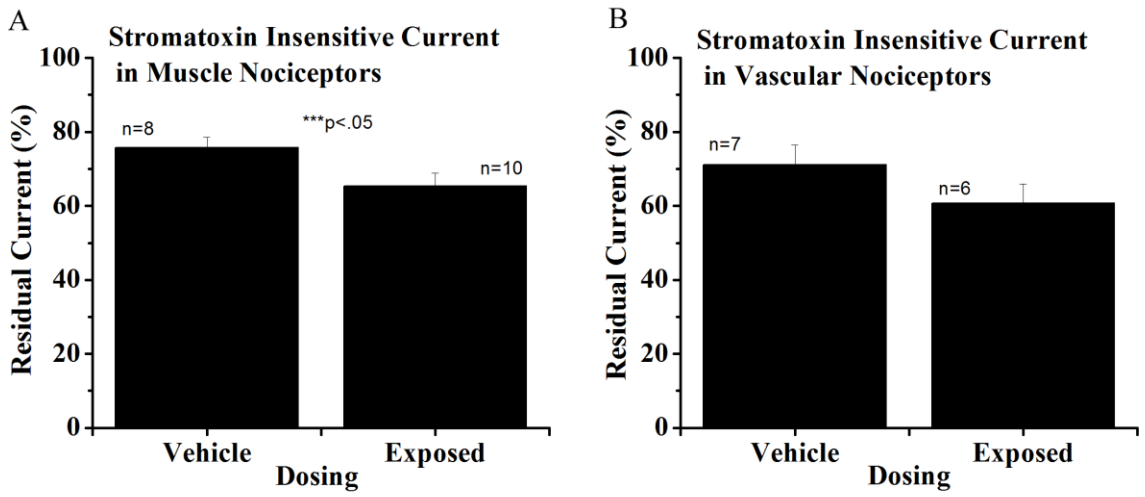

Figure 3. Stromatoxin-Insensitive Currents in GW Exposed Nociceptors. StTX sensitive currents ($K_v2.1$ and $K_v2.2$) normally comprised about 25 to 30% of the total K_{DR} current in muscle and vascular nociceptors, respectively (vehicle tested rats). The StTX insensitive current, the proportion of K_{DR} currents, in muscle nociceptors that remained after pre-treatment with linopirdine and StTX, were modified in muscle nociceptors by chronic exposure to GW chemicals. **(A)** Muscle nociceptors **(B)** Vascular nociceptors. Cells were harvested 12 weeks after exposures had ceased. Experiments were conducted on cells collected from 13 rats.

3.2.2. Modification of mAChR Reactivity after Exposure to GW Chemicals

We have shown that increasing the frequency of exposure to GW anticholinesterases (chlorpyrifos, PB) produced long term changes to rat activity levels (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al., 2015). These behavior patterns were consistent with the emergence of a delayed myalgia. Parallel studies indicated that there were corresponding perturbations to a number of ion channel proteins expressed in muscle nociceptors; we also reported that the AP (action potential) discharge, in response to a muscarinic agonist OXO-M, was significantly exaggerated in GW

chemical-exposed rat nociceptors (Nutter et al., 2015). Due to the apparent importance of anticholinesterases to development of pain-like behaviors, we examined whether presentation of a muscarinic agonist (OXO-M) would modify the activity of K_v7 and K_{DR} currents in toxicant and vehicle exposed neurons.

The functional status of the mAChR pathway was assessed in muscle and vascular nociceptors. In order to block contributions from K_v7 ion channels, studies were conducted in a K-iso solution that contained 10 μM linopirdine. The baseline K_v reactivity was determined using a series of strongly activating voltage steps (2 sec pre-pulse to -100 mV, followed by a step to +20 mV; 6-9 tests). OXO-M was then applied for 4 minutes by close superfusion. The total K_{DR} current was significantly increased within 60 seconds of OXO-M exposure. A ~20% increase in current amplitude was maintained over the duration of OXO-M application (4 minutes) and showed little tendency to reverse during a 3 minute wash (not shown). The influence of OXO-M was significantly reduced in muscle nociceptors harvested from rats exposed to GW chemicals (figure 4A). Vascular nociceptor K_{DR} from these same rats were not affected (figure 4B).

A portion of the K_{DR} in muscle nociceptors is composed of Stromatoxin-sensitive $K_v2.1$ and $K_v2.2$ (figure 4). We were able to show that the StTX sensitive, $K_v2.1$ and $K_v2.2$, current was not modified by GW chemicals. We now used StTX to determine whether the component of the muscarine sensitive K_{DR} was $K_V2.1$ and $K_v2.1$. K_{DR} currents were isolated as above. Following 5 minutes of StTX exposure, OXO-M was applied for 4 minutes to muscle nociceptors that were excised from rats exposed to GW chemicals. As shown in figure 4C, removal of the StTX sensitive current did not block the influence of OXO-M on the K_{DR}. Because the K_v2 toxin failed to block the effect of OXO-M on the StTX resistant current, we concluded that the OXO-M modulation occurred mainly against that portion of the K_{DR} current that was modified by chronic

exposure to GW chemicals. Because we have not yet been able to break this component down further, it is not clear whether the entire StTX resistant component was modified by GW chemicals or only some distinct portion of it. Therefore, we cannot be certain that the portion of the GW toxicant modified current was identical to the current that was enhanced by OXO-M.

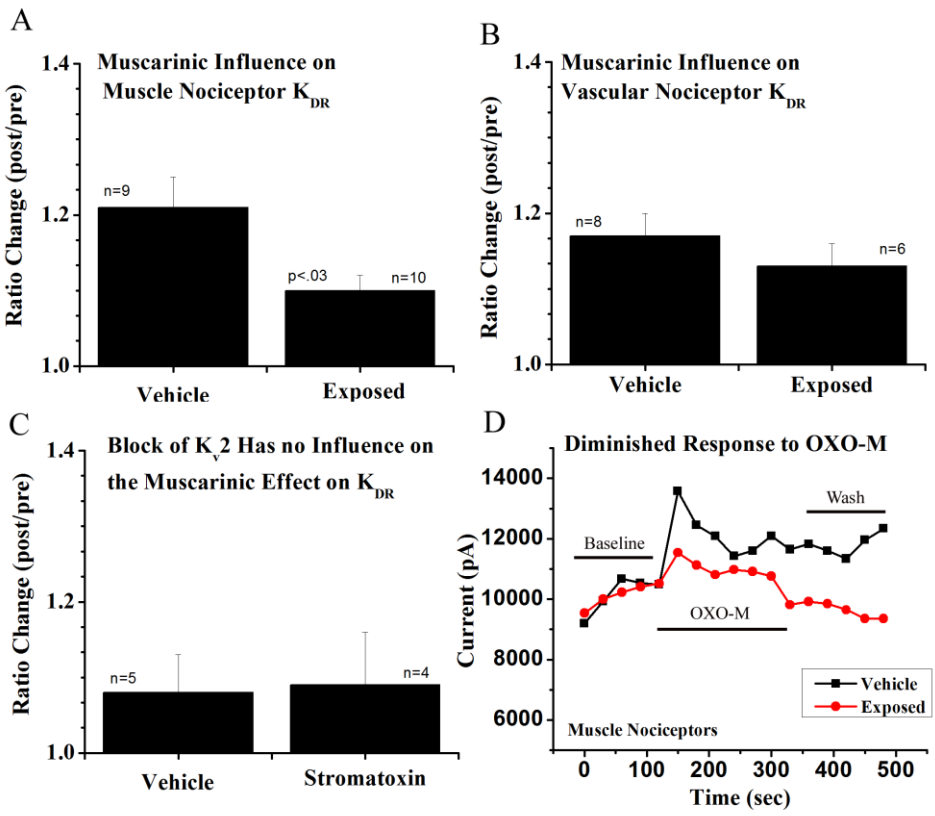

Figure 4. GW Chemicals Decreased the Muscarinic Sensitivity of K_{DR} Currents. A) A broadly active muscarinic agonist (OXO-M) increased K_{DR} currents of muscle nociceptors harvested from both vehicle exposed and GW chemical-exposed muscle nociceptors. Muscle nociceptors from exposed rats were significantly less reactive to the muscarinic agonist. **B)** Vascular nociceptor K_{DR} reactivity to OXO-M was not influenced by GW chemicals. **C)** Block of the $K_v2.1$ and $K_v2.2$ current by StTX did not prevent the increase in K_{DR} current by OXO-M in muscle nociceptors. **D)** A representative case showing the time dependent change in peak K_{DR} current after exposure to OXO-M. Values in (A) and (B) represent amplitudes measured 1 minute after the superfusion of OXO-M. Experiments were conducted on cells collected from 13 rats.

K_v7 is a member of the broader K_{DR} family. We have shown previously that the activity of K_v7 currents was modified by an 8 week exposure to GW toxicants (Nutter et al., 2013; Nutter et al., 2015). One level of modification could occur via activation of muscarinic receptors. Cholinergic modulation, via the muscarinic receptor activated pathway, is one of the ways by which inhibition of K_v7 potentiates neuronal activity (Brown and Passmore, 2009). We examined how exposure to

a broadly active muscarinic agonist, OXO-M, altered the resting currents in both muscle and vascular nociceptors harvested from rats exposed to GW agents (OXO-M, 10 μM; 30 sec).

Muscle and vascular nociceptors were identified as above. K^+ currents were isolated in a Kiso solution (see 'Methods'). Following exposure to OXO-M (close superfusion), the holding current shifted, in a depolarizing direction, within 20 seconds of OXO-M application (figure 5D). The depolarizing shift was consistent with the closure of K_v7 currents. We confirmed this by blocking the depolarizing shift by pretreatment with linopirdine (figure 5C). The depolarizing shift in the resting current was significantly greater in muscle nociceptors harvested from GW exposed rats 12 weeks after exposures had ceased (figure 5A). Vascular nociceptors did not exhibit similar effects (figure 5B).

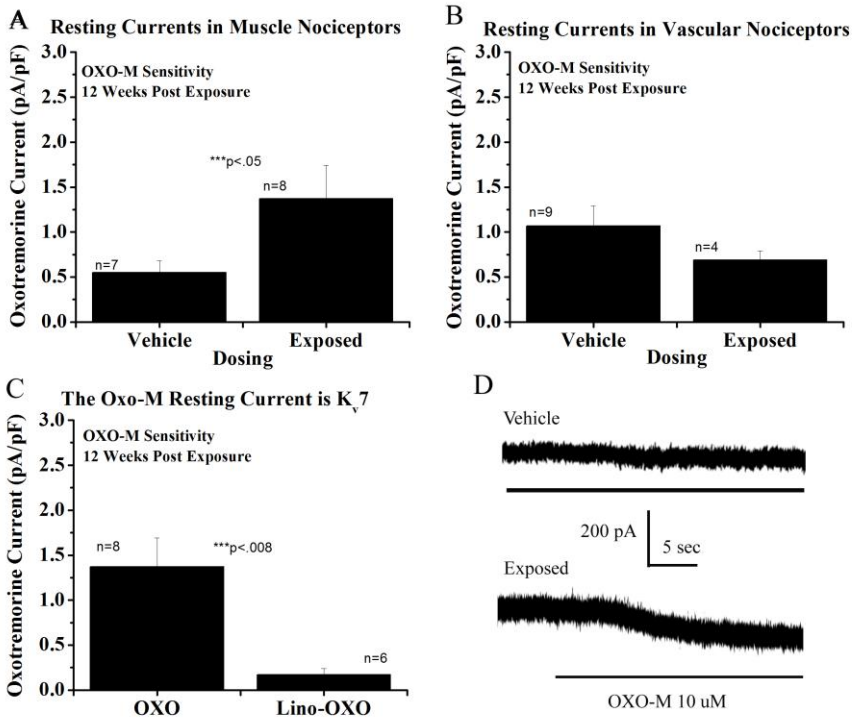

Figure 5. Muscle Nociceptors Exhibit Enhanced Sensitivity to a Muscarinic Agonist. A) Shifts in the holding current following a 30 second exposure to OXO-M (10 μM) suggested enhanced suppression of K_v7 currents in muscle nociceptors exposed to GW chemicals. **B)** Resting currents in vascular nociceptors were not affected by exposure to GW toxicants. **C)** OXO-M induced shifts in the holding current were prevented by pretreatment with linopirdine (10 μM; 3 minutes; muscle nociceptors of exposed rats). **D)** Representative cases of OXO-M current shifts in a vehicle (upper) and GW chemical-exposed (lower) muscle nociceptor. Studies were conducted in a Kiso solution (see 'Methods'). Experiments were conducted on cells collected from 9 rats.

3.2.3. Molecular Basis of the MDBD

In a recent publication, we demonstrated that a muscarinic agonist evoked a burst discharge (MDBD) in muscle nociceptors. This burst discharge was significantly potentiated in Type 5 muscle nociceptors 12 weeks following exposure to GWI chemicals (Nutter et al., 2015**).** As this was the first demonstration of such a mechanism in DRG, and as it could have important

implications for the role of anticholinesterase-induced maladaptations in GWI, we further developed this line of investigation.

The MDBD is a mAChR instigated discharge that is similar to a phenomena described in hippocampus (Klink and Alonso, 1997; Chiang et al., 2010; Yamada-Hanff and Bean, 2013). Studies of the molecular basis of the hippocampal MDBD suggested muscarinic receptor activation coupling to a persistent TTXs Na^+ current and a non-selective cation current (Yamada-Hanff and Bean, 2013). We examined whether the molecular basis of the MDBD in DRG was similar to that described in hippocampus.

Experiments were conducted on young adult male 'naïve' rats (n=13) in current clamp mode (Tyrode's bath solution). Muscle and vascular afferents were identified in the usual manner (see 'Methods'). After entry into current clamp mode, the cell was exposed to one or more, conditioning, pharmacological agents or their vehicle (2 or 3 min). Subsequently, OXO-M (10 μM) was applied for 30 sec. The OXO-M solution contained the same 'blocking' agent(s) that was present during the conditioning period. The superfused solutions were heated to 34 ºC by a servo-controlled probe during the conditioning phase and thereafter (see 'Methods').

Under vehicle test conditions, both type 5 muscle and type 8 vascular nociceptors manifested MDBD. When challenged with OXO-M, 14 of 15 nociceptors tested exhibited rapid and substantial depolarization (figure 6). One third of these cells also emitted burst discharges (133.0 +/- 44.6 APs, n=3 of 10 and 18.5 +/- 8.5 APs, n=2 of 5 cases; muscle and vascular nociceptors respectively). Additional tests with OXO-M were conducted on, capsaicin insensitive, type 19 afferents that were recently identified in tracing studies to be part of the vascular afferent pool (Cooper et al., 2014; Henao et al., 2015). Type 19, non-nociceptive vascular afferents did not

exhibit significant depolarization or any AP discharge during tests with OXO-M (0.91 +/- 0.99 mV; n=4; not shown).

MDBD could be the result of inhibition of K_v7 currents that are active at the resting membrane potential. Activation of mAChR is the classic pathway to K_v7 channel inhibition (Linely et al., 2008; Brown and Passmore, 2009). To test this hypothesis, we applied, by close superfusion, the specific K_v7 inhibitor, linopirdine (10 μM), to muscle and vascular nociceptors for 3 minutes prior to the presentation of OXO-M (10 μM). Despite pretreatment with linopirdine, muscle and vascular nociceptors were strongly depolarized by OXO-M (figure 6A, 6B). Seven of 14 cases also emitted AP bursts (73.0 +/- 17.9 APs, n=6 of 8; 115 APs, n=1 of 6, muscle and vascular nociceptors, respectively). Although depolarization amplitude was essentially unchanged in those cases pretreated with linopirdine, the duration of the AP burst discharges was significantly increased ($p<.04$; 6.73 +/- 1.98 vs 17.83 +/- 4.01 sec, OXO-M, n=6, and linopirdine treated, n=7, respectively; not shown). Therefore, pre-treatment with the K_v7 blocker, linopirdine, did not block the mAChR dependent depolarizations, but did modify the AP bursts that often accompanied depolarization.

In hippocampus, and other sites, mAChR mediated depolarizations are strongly dependent upon an unidentified, Ca^{++} activated, non-selective cation current referred to as the I_{can} (see 'Discussion'). The identity of this current is unknown, but the I_{can} has also been linked to a muscarinic dependent phenomenon called ADP (afterdepolarization potential). ADP related depolarizations are sensitive to block by, the non-selective I_{can} antagonist, flufenamic acid (Hoffman and Frazier, 2010; Tsuruyama et al., 2013; Alvares et al., 2014). One hypothesis contends that the I_{can} supporting ADP is mediated by TRPC (Yan et al., 2009; Tai et al., 2011;

Dasari et al., 2013). We examined whether we could identify a non-specific cation channel in DRG that contributed to depolarizations produced by OXO-M.

TRPA1 is a Ca^{++} activated non-selective cation channel that is expressed in about half of the capsaicin sensitive nociceptor population (Julius, 2013). We examined whether TRPA1 was an I_{can} mediating MDBD in capsaicin sensitive muscle and vascular nociceptors (Petruska et al., 2002; Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). Using the methods described above, nociceptors were pretreated with linopirdine and the specific TRPA1 antagonist HC-030031 (10 μM) for 3 minutes prior to presentation of OXO-M.

Pre-treatment with a TRPA1 antagonist significantly reduced depolarizations associated with MDBD (figure 6A and B). Significant reductions were demonstrated both with respect to vehicle treated cases (muscle and vascular nociceptors; 6A and B) and 'linopirdine only' pre-treated cases (pooled nociceptors; 6F). Despite the block of TRPA1, about half of the mAChR induced depolarization remained intact. Therefore, an I_{can} supporting MDBD in muscle and vascular nociceptors is mediated, in part, by TRPA1.

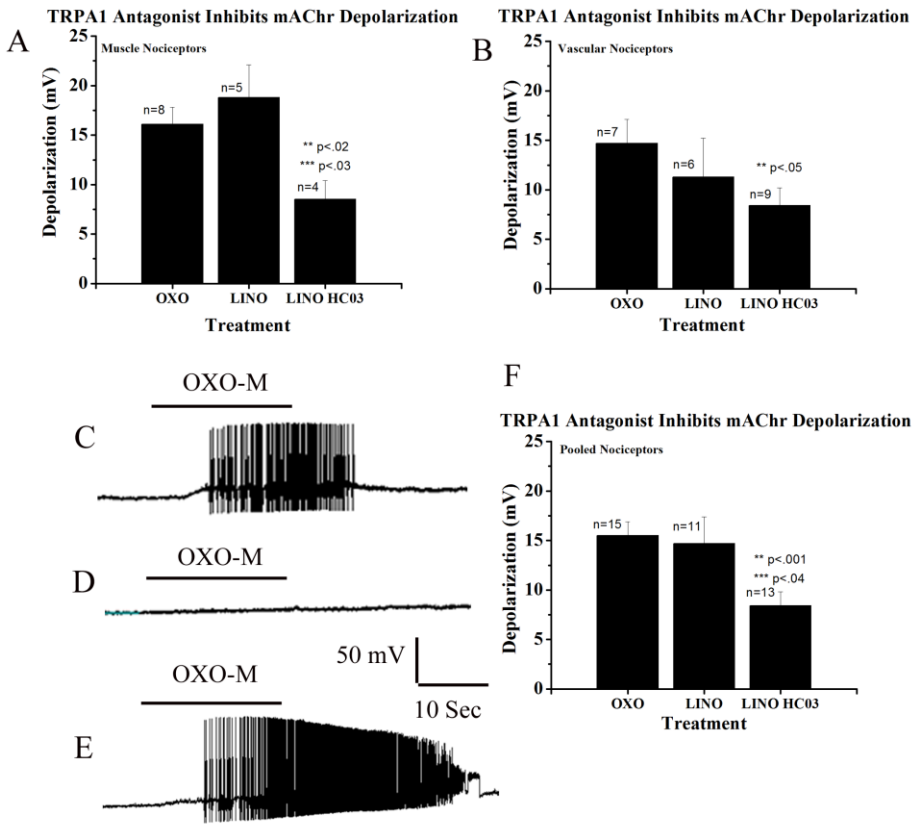

Figure 6. Block of TRPA1 Reduces mAChR Depolarization. A) The OXO-M induced depolarization in muscle nociceptors is reduced by TRPA1 antagonist HC-03003 (10 μM; HC03). Linopirdine pre-treatment has no influence on depolarization produced by OXO-M (LINO; 10 μM). **B)** The OXO-M induced depolarization in vascular nociceptors is also reduced by the TRPA1 antagonist HC-03003 (10 μM; HC03). Vascular nociceptor depolarization is unaffected by pre-treatment with the K_v7 antagonist, linopirdine (LINO; 10 μM). **C)** Representative burst discharge in a muscle nociceptor following OXO-M. **D)** Block of muscle nociceptor depolarization by OXO-M following pretreatment with HC-03003 and linopirdine (3 minute application). **E)** Following a 5 minute wash out of HC-030031 and LINO, OXO-M application produced a powerful depolarization and burst discharge (same cell as 'D'). **F)** The OXO-M induced depolarization in pooled muscle and vascular nociceptors is reduced by TRPA1 antagonist HC-03003 and linopirdine (10 μM; HC03). Linopirdine pre-treatment has no influence on depolarization produced by OXO-M (LINO; 10 μM). Experiments were conducted on cells collected from 17 rats. All tests were conducted at 34 ºC. ** significant vs OXO-M; *** significant vs LINO

4. Discussion

In the above study, rats were exposed to 3 GW chemicals (permethrin, PB, chlorpyrifos) for a period of 8 weeks. This procedure has been shown, previously, to produce a delayed myalgia-like pattern of behavior (increased resting and decreased movement) that was accompanied by molecular maladaptations to certain K_v ion channels. Those maladaptations persisted at least 12 weeks after chemical exposures were terminated (Nutter et al., 2015). The delayed development of pain-like behaviors mimicked the emergence of GWI pain syndromes that appeared after soldiers returned from their Persian Gulf deployments (Kroenke et al., 1998). Following the first Gulf War, soldiers complained of a variety of deep tissue pains that included myalgia, arthralgia, back pain, abdominal pain and headache (Haley et al., 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). Because molecular maladaptations occurred specifically in muscle nociceptors, outcomes generally supported the interpretation that the behavioral manifestations were an expression of a myalgia. Still, the possibility that other types of pains were present cannot be excluded. In the present study, we partially replicated behavioral findings of Nutter and colleagues (2015) and examined distinct physiological consequences to K_v regulation that resulted from the exposure protocol.

4.1. Behavioral Studies

Despite some strong similarities between the behavioral changes recorded in this report and Nutter et al., (2015), the emergence of pain-like signs appeared to be far less robust in this group of rats. While resting time was modestly increased in these GW exposed rats, it was much more increased

previously and there were also significant decreases in movement distance that were consistent with an attribution to pain (Nutter et al., 2015). Despite this shortcoming, some strong similarities remained. During the exposure to GW toxicants, movement distance and rate exhibited periods of rapid increase. Subsequently, in the post–exposure period, those paradoxical rapid movements returned to, or below, normal levels. It is possible that several more weeks of testing would have produced an improved outcome. Regardless, it is clear that increasing the exposure rate to GW anticholinesterases is producing lasting changes to behavior.

Two of our previous attempts to develop a rat model of GWI pain syndromes failed to produce pain-like behaviors (Nutter et al., 2013; Nutter and Cooper, 2014). Those studies used a different protocol in which the rate of exposure to anticholinesterases was limited to a 50% and 7% duty cycle (PB and chlorpyrifos respectively). Doubling the duty cycle of anticholinesterase exposure induced changes in rat behavior that persisted 5-12 weeks after treatment (Nutter et al., 2015). Prolonged exposure to chlopyrifos and/or PB can produce lasting changes in cholinesterase activity (Abou-donia et al., 1996; Padilla et al. 2005; Abou-donia et al., 2006; Lopez-Crespo et al. 2007) that could impact cellular and molecular physiology. Because it appeared that an increase in the frequency of exposure to GW anticholinesterases (PB and chlorpyrifos) were critical to the development of delayed pain-like behaviors, we focused this report on physiological changes related to cholinergic signaling in nociceptors. We have shown that muscle and vascular nociceptors express nicotinic receptors and (Rau et al., 2005; Rau et al., 2014) and exhibit discharges to muscarinic agonists (Nutter et al., 2015). Because the binding of ACh at its muscarinic receptor is frequently coupled to down-regulation of K_v7 activity, we examined

whether muscarinic influences on nociceptor K_v7 and other K_{DR} channels were altered following GW chemical exposure.

4.2. Molecular Studies

Shifts of K_v7 channel activity can have profound effects on membrane excitability. The expression of K_v channels is controlled by a variety of neurotrophins and transcription factors whose activation and interactions with other extracellular, cytoplasmic and nuclear influences can be complex (Groth and Mermelstein, 2003; Im and Rao, 2004; Groth et al., 2007; Kim et al., 2014). K_v7 channel activity is regulated acutely by pathways leading to K_v7 protein phosphorylation/dephosphorylation, PIP_2 expression/cleavage and redox reactions (Gamper and Ooi, 2015). The former modulations typically result from activation of G-protein coupled receptors that are set into motion by extracellular derived pro-inflammatory agents (e.g., ACh, bradykinin, serotonin, serine proteinases, ATP, ROS; Brown and Passmore, 2009; Gamper and Ooi, 2015).

For our purposes, the influence of ACh at mAChR's is particularly relevant. Although the specific details of mAChR receptor distribution in nociceptive afferents is unknown, it is established that muscarinic M2 and M4 receptors are expressed on DRG neurons (Bernardini et al. 1999; Tata et al., 2000; Cai et al., 2009). The M2 subtype is widely expressed (Hayashida et al., 2006), while the M4 receptor is mainly present in small and medium sized DRG (Tata et al., 2000). We have identified type 5 muscle nociceptor phenotypes in both the small and medium sized pool of DRG neurons. The specific muscarinic receptor subtype(s) associated with modulation of K_v7, K_{DR} and MDBD in these nociceptors have yet to be determined.

Exposure to chlorpyrifos or PB alters the expression of muscarinic receptors in the CNS (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). We have now demonstrated that chronic exposure to GW agents disturbed the normal relationship between ACh muscarinic receptors and certain K_v proteins in the PNS. Twelve weeks after exposure to GW toxicants, the muscarinic up-regulation of an inhibitory StTX insensitive K_{DR} in muscle nociceptors was diminished; simultaneously the muscarinic down regulation of inhibitory K_v7 channel proteins in muscle nociceptors was enhanced. This is a combination that is highly suggestive of increased nociceptor excitability that could contribute to a chronic myalgia.

4.2.1. Role of MDBD in GW Pain

The depolarization and activation of certain DRG neurons by muscarinic agonists is similar to a burst discharge phenomenon that has been reported in hippocampus (Klink and Alonso, 1997; Chiang et al., 2010; Yamada-Hanff and Bean, 2013; see also Krnjevic et al., 1971). Mechanistically, it also closely resembles the phenomenon of ADP or sADP, in which a relatively brief induction of discharge, by current injection, in the presence of a muscarinic agonist, is followed by a long lasting depolarization (Schwindt et al., 1988; Andrade, 1991; Constanti and Bagetta, 1991). The depolarization is often accompanied by a burst discharge (Klink and Alonso, 1997; Egorov et al., 2002; Zhang et al., 2011). ADP has been described in a number of CNS regions, including neocortex, hippocampus, septal nucleus and olfactory bulb (Cole and Nicoll, 1983; Schwindt et al., 1988; Hasuo and Gallagher, 1990; Pressler et al., 2007). The molecular basis of ADP is unresolved and may be specific to functional cell types (Lawrence et al., 2006).

Following mAChR activation, ADP has been shown to depend upon rapid development of a persistent, TTX sensitive Na^+ current (Azouz et al., 1996; Yue et al., 2005; Chiang, et al., 2010; Yamada-Hanff and Bean, 2013; but see Hofman and Frazier, 2010) and/or activation of a non-selective cation current (I_{can}; Haj-Dahmane and Andrade, 1998; Hofman and Frazier, 2010; Zhang et al., 2011; Dasari et al., 2013). ADP is modulated by K_v7 activity (Yue and Yaari, 2004, 2006; Brown and Randall, 2009); and may require activation of LVA Ca^{++} currents, the phenotype of which may differ in distinct functional cell classes (Dreyfus et al., 2010; Geier et al., 2011; Park and Spruston, 2012).

In our studies, we found little evidence that mAChR induced depolarization in DRG muscle and vascular nociceptors was dependent on the inhibition of K_v7. Nevertheless, K_v7 played a significant role in MDBD by modulating the duration of AP discharges when such activity occurred. ADP has been shown to depend upon a flufenamic acid sensitive I_{can}, the identity of which remains unknown (Hoffman and Frazier, 2010; Tsuruyama et al., 2013; Alvares et al., 2014). We were able to demonstrate that inhibition of TRPA1, an I_{can} widely expressed in DRG nociceptors (Julius, 2013), reduced, by half, the mAChR evoked depolarization. The remaining current could represent activity in a distinct channel and/or residual activity in TRPA1.

It remains unclear whether these powerful mAChR dependent burst discharges in PNS nociceptors are mechanistically related to CNS ADP, or whether TRPA1 contributes to ADP in the CNS. Nor is it clear that ADP is a mechanistically uniform phenomenon throughout the nervous system. The prevalence of ADP in hippocampus, at times including muscarinic bursting discharges similar to those that we observed in DRG (Klink and Alonso, 1997; Egorov et al., 2002; Zhang et al., 2011), could have broad implications for the pathophysiology of GWI. In the CNS, ADP is believed to contribute to learning and memory formation (Rahman and Berger, 2011). We have shown that

MDBD is potentiated by chronic exposure to GW chemicals (Nutter et al., 2015). Those affected DRG ion channels and receptors are also expressed in hippocampus ($Na_v1.9$, K_v7, mAChR; see Nutter and Cooper, 2014; Nutter et al., 2015). If such GWI chemical maladaptations were also manifested in hippocampus, it could be a compelling functional link to cognitive deficits associated with this disorder (Haley et al., 1997).

In the periphery, GWI chemical-induced changes in K_v7, $Na_v1.9$, K_{DR} and muscarinic coupling to these channel proteins could converge to modify a functional burst discharge mechanism, such as MDBD. This would have important implications for the pathophysiology of GWI pain syndromes. In the PNS, postsynaptic spinal cord neuroplasticity (i.e., central sensitization) is associated with the development of some chronic pain syndromes (Willis, 2001; Latremoliere and Woolf, 2009). Type 5 muscle and type 8 vascular nociceptors co-express and release vasoactive neuropeptides SP and CGRP (Petruska et al., 2002; Nutter et al., 2013; Nutter and Cooper, 2014). These paracrine messengers also have powerful influences in the spinal and medullary dorsal horn. In the spinal cord, central sensitization can be initiated by high frequency discharge dependent release of SP from primary afferent nociceptors onto spinal cord relay neurons (Holz et al., 1988). Afferent derived SP produces prolonged depolarizations that relieve Mg^{++} blockade of the NMDA receptor, initiating a series of downstream amplifications that affect both presynaptic and postsynaptic aspects of sensory processing (Dougherty et al., 1993; Willis, 2001). Central release of the co-expressed neuropeptide, CGRP, activates CNS microglia that synthesize and/or release of a number of pro-inflammatory cytokines (IL-1β, IL-6) chemokines (CCL2), nitric oxide and other agents that contribute to central neuroplasticity (Li et al., 2008; De Corato et al., 2011; Malon et al., 2011).

5. Conclusion

We have now shown that increasing exposure to GW anticholinesterases (chlorpyrifos, pyridostigmine bromide) played an important role in the development of a delayed myalgia-like pain behavior. One consequence of heightened exposure to anticholinesterases was modification of mAChR coupling to ion channels (K_v7) that contribute to a unique DRG nociceptor burst discharge mechanism (MDBD) that can be linked, through the release of SP and CGRP, to known pathways to chronic pain.

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

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Abstract

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

Introduction

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

During their relatively brief deployment, the soldiers of ODS (Operation Desert Storm) were potentially exposed to 64 insecticides and repellants containing 37 distinct active ingredients (DoD Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008; RAC, 2014). Our laboratory developed a rat model of Gulf War Illness pain as part of an effort to explore cellular and molecular maladaptations associated with prolonged exposure to insecticides that were employed in ODS. Our early investigations focused on the role of three particular agents that possessed a unique potential to interact with membrane proteins expressed by deep tissue nociceptors. These agents included: 1) permethrin--- a type I pyrethroid that was supposed to be applied by soldiers to their uniforms every 4-5 days. Permethrin is a powerful Na_v (voltage

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

Exposing rats to various concentrations and durations of these three GW agents failed to produce a pattern of behavior changes consistent with chronic pain (Jiang et al., 2013; Nutter et al., 2013; Nutter and Cooper, 2014). We recently found that an intensified exposure to the AChE inhibitors (chlorpyrifos, PB) could produce pain-like behaviors that appeared and/or persisted up to 12 weeks following termination of exposure (Nutter et al., 2015). Although an 8 week protocol, utilizing an intermittent exposure pattern, and consisting of daily permethrin, chlorpyrifos (twice per month; 7% duty cycle), and PB (14 days per month; 50% duty cycle) could not produce any lasting changes in rat activity levels or superficial pain measurements (Nutter et al., 2013; Nutter and Cooper, 2014), a doubling of the duty cycle of the anticholinesterases (chlorpyrifos to 14%; PB to 100%), did induce a delayed pain-like syndrome that emerged 9-12 weeks after exposure (Nutter et al., 2015). Using this anticholinesterase intensified protocol, pain-like behaviors were

manifested as an increase in resting times and a decrease in free ranging ambulation. Patch clamp studies conducted on dorsal root ganglion neurons harvested from these same rats revealed the development of a variety of cellular and molecular maladaptations to muscarinic receptor (mAChR) signaling pathways and effectors in muscle nociceptors that were consistent with a chronic myalgia (Nutter et al., 2015; Cooper et al., 2016).

DEET (N,N-Diethyl-meta-toluamide) is an insect 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 weak anticholinesterase (Corbel et al., 2009; Wille et al., 2011; Swale et al., 2014). A recent publication demonstrated that high concentrations of DEET could inhibit both Na_v and K_v ion channel current amplitudes in rat cortical neurons (Swale et al., 2014; see also Corbel et al., 2009). Our laboratory has associated enhanced $Na_v1.9$ and decreased K_v7 activity with the appearance of pain-like signs in rats exposed to GW chemicals (Nutter and Cooper, 2014; Nutter et al., 2015; Cooper et al., 2016). In the experiments described below, we examined the impact of a DEET augmented exposure protocol on the development of pain signs in our rat model. Additional studies clarified the interaction of DEET with nociceptor ion channels implicated in the development of GWI pain.

Methods

Behavioral Studies

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

Chronic Exposure Protocol. Over a period of 4 weeks, rats (n=50) were exposed to permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), DEET (200 or 400 mg/kg; Sigma Aldrich) and pyridostigmine bromide (PB; 13 mg/kg; Sigma Aldrich). Permethrin, in ETOH, was applied every day to a shaved area of the back (~1square inch) between the forelimbs. 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). The latter group served as a positive control for DEET potentiation (Haley and Kurt, 1997). Two groups received only 3 agents: Group PB (PB excluded) and Group CP (chlorpyrifos excluded). Group C served as a vehicle control group. Agents were always administered in the same order. On the one day per week that rats were dosed with chlorpyrifos, the order was PB, chlorpyrifos, permethrin and then DEET. On the days in which chlorpyrifos was not administered, the order of administration was PB, permethrin and DEET. 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.

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 borosilicate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). In experiments on K_v channels, the pipette solution contained (in mM): 120 KCl, 5 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). 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).

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 (-30 to -70 mV) to obtain a mean normalized conductance. The peak conductance was determined by inspection.

Evocation and Characterization of K_{DR} Currents. For the purpose of this study, the K_{DR} current was defined as the total 4-AP insensitive K^+ current following removal of the K_v7 component with linopirdine. The voltage dependent activation of the total K_{DR} current, was assessed, as a tail current, after application of the K_v7 inhibitor linopirdine (10 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

A repeated measures ANOVA was used to assess influence of GW chemical treatments on the development of pain signs (post-exposure weeks 5-12). In order to assess the persistence of pain behaviors, an additional analysis was conducted on the 4 week span proceeding euthanasia (post-exposure weeks 17-20 and/or 21-24, Group A and C only). Dependent measures included: 1) muscle pain threshold (PAM; grams); 2) ambulation: movement distance (cm/15 min), average movement rate (cm/sec); and 3) rest duration (sec/15 min). The alpha level was set at .05. As noted above, 2 rats were euthanized for health related issues (one rat from Group A and one rat from Group HD). Both were terminated on the advice of the study veterinarian. To equalize the number of animals in each group, the corresponding rat (by date of entry), was excluded from each group. In order to adjust for the high variance, present in rat behavioral data, the means of movement and resting scores were trimmed (highest and lowest scores) prior to construction of plots and performance of analyses (Lix and Keselman, 1998; Wilcox et al., 2000; Mudholkar et al., 2013).

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

Results

Behavior

After a period of acclimation and baseline behavioral testing, rats were divided into 5 groups (n=50). One group (Group A) was treated with all four GWI chemicals for 4 weeks (permethrin 2.6 mg/kg, chlorpyrifos 120 mg/kg, PB 13 mg/kg, DEET, 400 mg/kg; 50% in ETOH). A positive control for the influence of DEET (Group HD) received all 4 agents, but DEET was administered at half the concentration (200 mg/kg; 25% in ETOH). Studies of GW veterans had indicated that skin application of high (75%), but not low concentrations (25%) of DEET, posed a risk factor for chronic pain (Haley and Kurt, 1997). Our preliminary studies indicated that a 50% level was sufficient to produce enduring pain-like signs. The remaining 2 groups received combinations of 3 agents where either chlorpyrifos (Group CP) or PB (Group PB) was not included in the dosing routine. A final group served as a vehicle treated control (Group C; corn oil, ethanol, water). Details of the dosing schedule are provided in 'Methods' and Table 1. Behavior assessment tests were conducted on all rats once per week for 26 weeks (Group PB, Group CP, Group HD) or 30 weeks (Group C, Group A). Tests included muscle pressure withdrawal threshold (PAM; left semitendinosus) and 3 open field activity measures: movement distance (cm), average movement rate (cm/sec), and rest time duration (sec). All PAM measures were carried out under blinded test conditions. Activity measures were assessed over a period of 15 minutes, in a 35 x 40 cm Perspex test chamber where movements were monitored and quantified by an automated infrared detection system (AccuScan).

After the 4 week exposure period had ended, we conducted a repeated measures ANOVA on 8 and 4 week blocks that extended from post-exposure week 5 through 24. We had previously

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

With the addition of DEET (50% in ETOH) to the same 4 week exposure protocol, a consistent pattern of pain-like signs emerged and persisted for up to 6 months (figure 1A, B, C). Both ambulation measures, movement distance and movement rate, were significantly reduced in the immediate weeks following exposure (weeks 5-12; $F=19.47$, $p<.001$ and $F=27.71$; $p<.001$, respectively). Although the depression of movement distance returned to normal levels by the final assessment period, (weeks 21-24), the slowing of movement rate, due to GWI agents, was still significant 21-24 weeks post-exposure (figure 1B).

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

As shown in figure 1, exposure to the 4 agents produced a pattern of activity suppression *during* the 4 week exposure period that was observed in nearly all experiments below (figures 2-

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

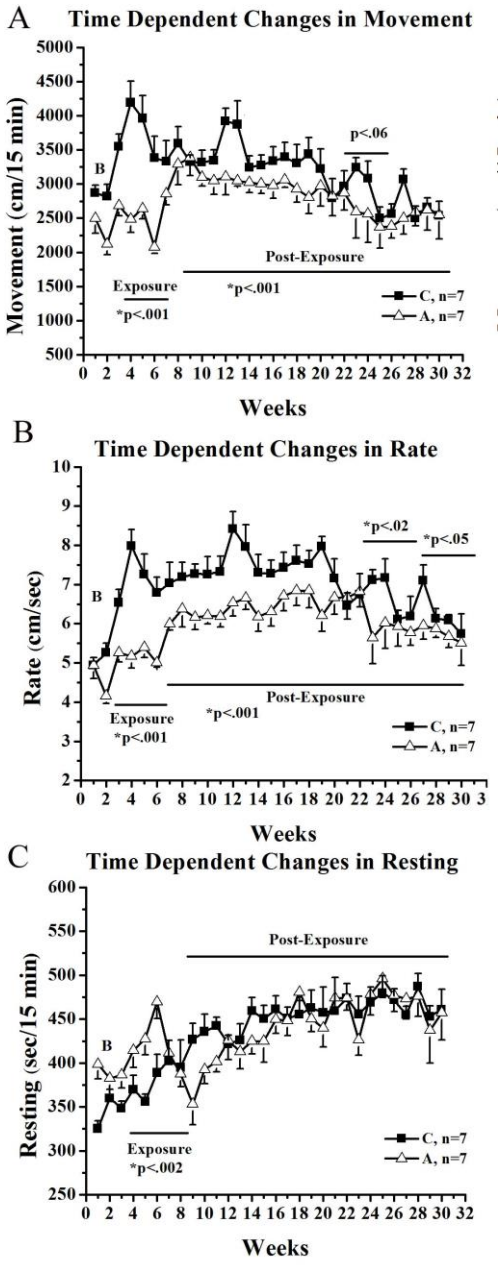

Figure 1. A DEET Augmented Exposure Protocol Produced Long Lasting Pain-Like Behaviors. A) Movement distance was significantly decreased at 5-12 weeks post exposure ($F=19.47$; $p<.001$). Movement distance pain signs approached significance 17-20 weeks post exposure ($F=3.72$; $p<.06$), but faded in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

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

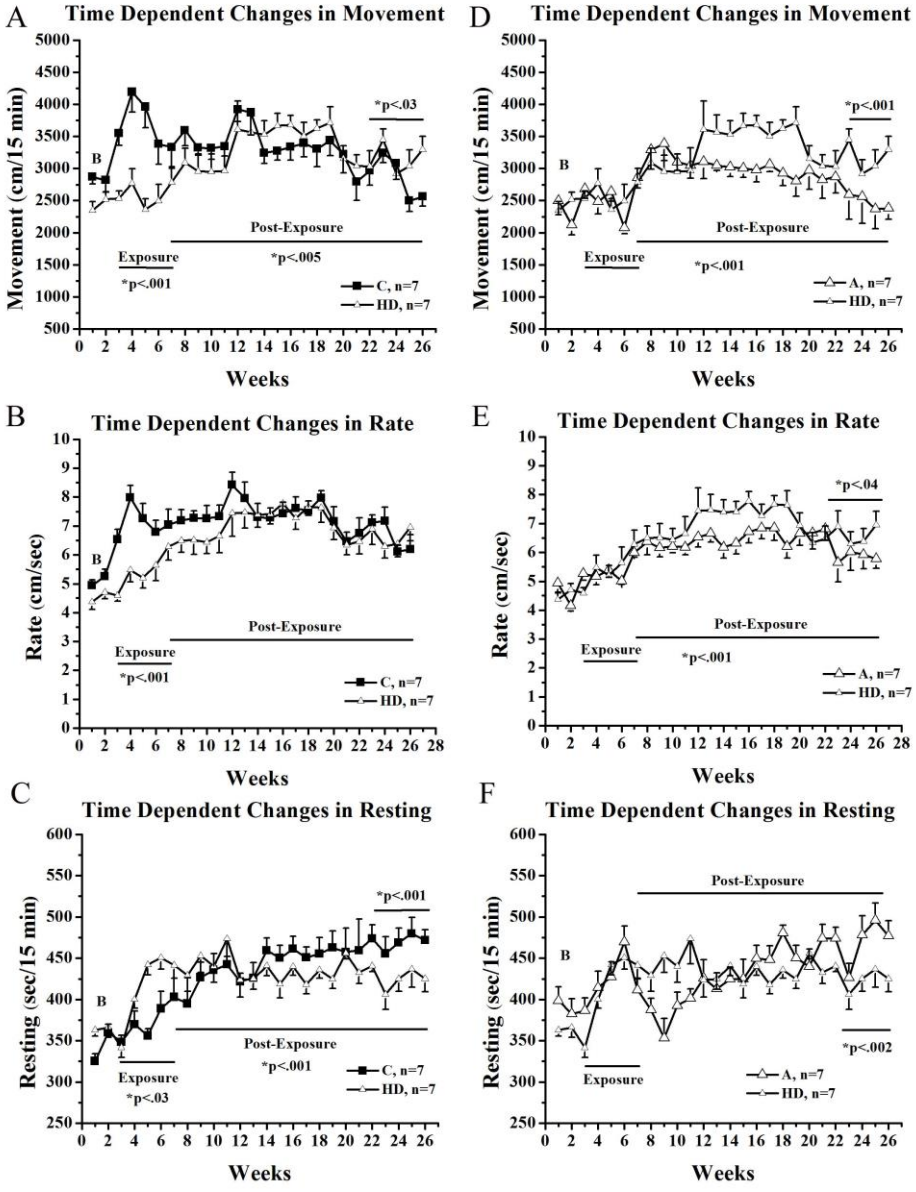

Figure 2. Pain-Like Behaviors Did Not Develop Nor Persist When DEET Concentration was Halved. A) Relative to control animals, movement distance was paradoxically increased in during post-exposure weeks 17-20 (F=5.50). **B)** Average movement rate was unchanged compared to vehicle exposed controls. **C)** Resting duration was paradoxically decreased relative to control rats in Group HD (weeks 17-20; F=19.51). There were no indications of pain signs during the early post-exposure period in any measure (weeks 5-12). **D and E)** When compared to Group A (50% DEET), the reduction of DEET concentration to 25% produced movement distance and rate scores that were significantly higher (more normal) during the last observation period (post-exposure weeks 17-20; F=12.4, p<.001 and F= 17.2, p<.001, distance and rate respectively). Pain behaviors appeared during the early testing phase in Group A (F=16.7 and F=16.1; p<.001, movement distance and rate, respectively). **F)** At week 17-20, resting was significantly reduced in the 25% DEET concentration group (F=11.24; p<.002), but there were no accompanying shifts during the early post-exposure phase (weeks 5-12). B: baseline testing; A: DEET (400 mg/kg; 50%), PB, chlorpyrifos, permethrin; C/Vehicle: (ethanol, corn oil, ethanol, water); HD: DEET (200 mg/kg; 25%), chlorpyrifos, PB, permethrin; *significantly different by ANOVA.

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.

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).

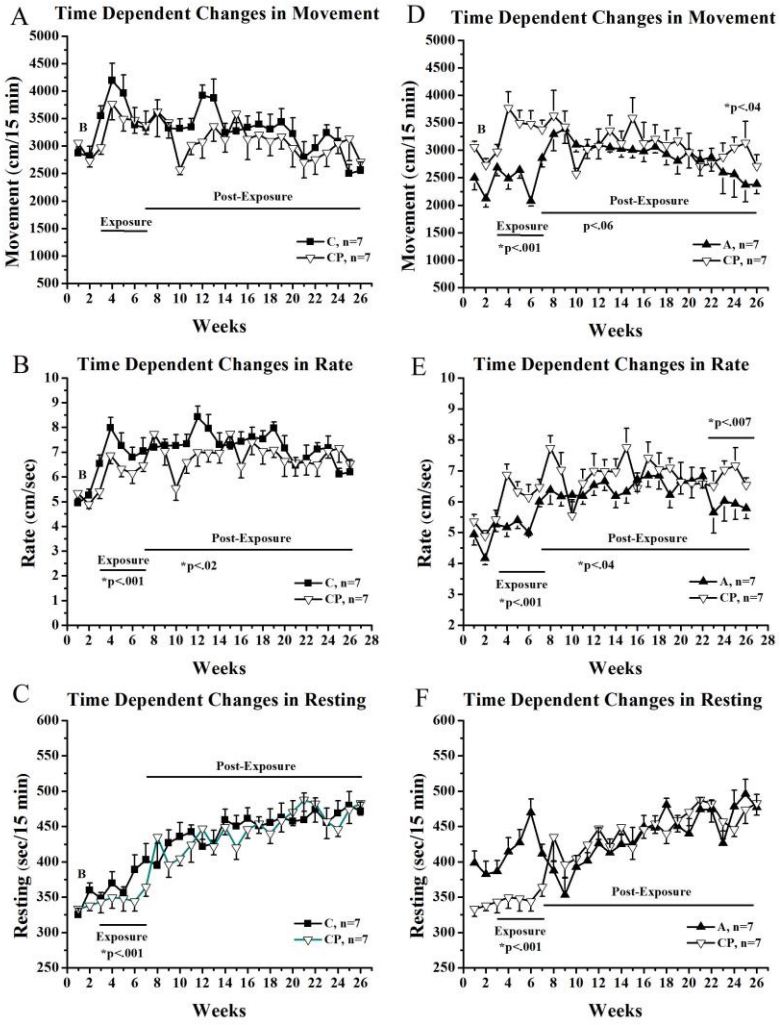

Figure 3. Excluding the AChE Inhibitor, Chlorpyrifos, from the Exposure Protocol Prevented Development of Persistent Pain Behaviors. A) The omission of CP prevented the suppression of movement (distance) by GW chemicals. **B)** Average movement rate was still significantly reduced in the early post-exposure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

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.

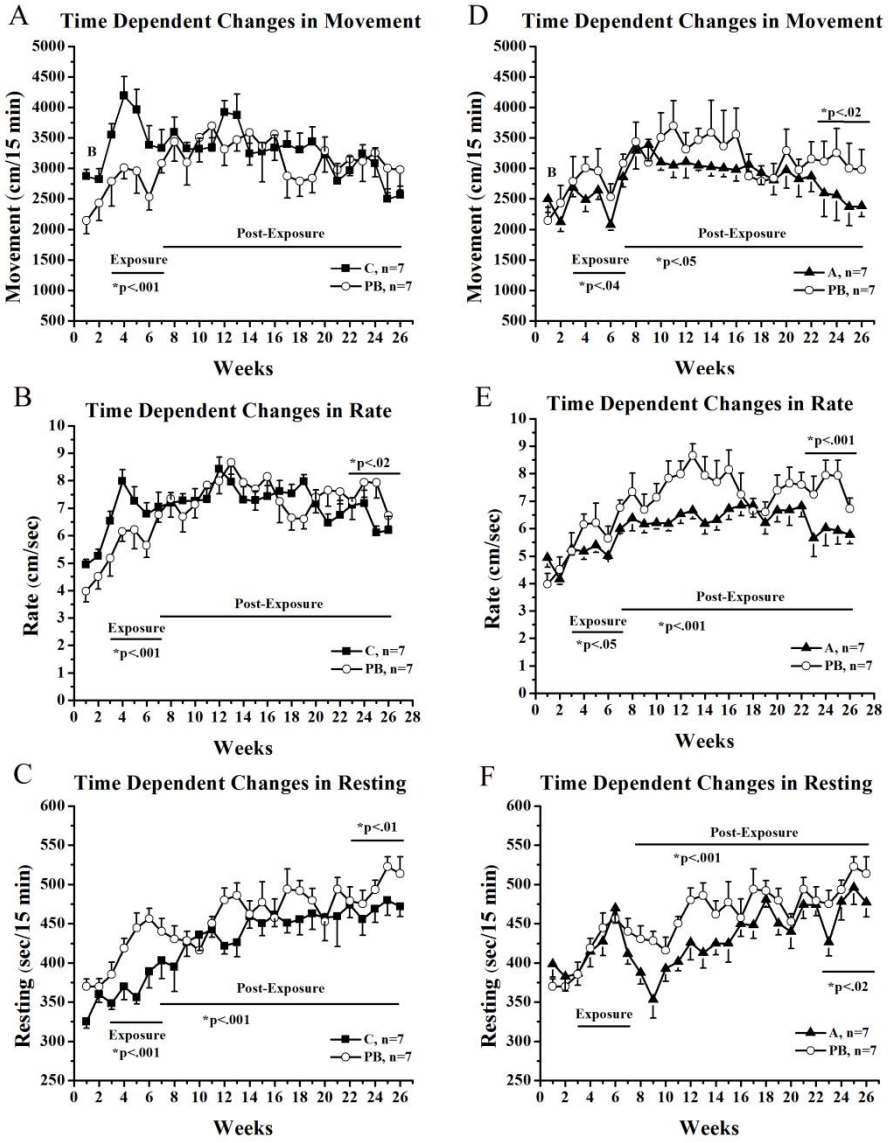

Figure 4. Excluding Pyridostigmine Bromide from the Exposure Protocol Differentially Contributed to the Development and Persistence of Pain Behaviors. A) Movement distance was unaffected in the absence of PB. **B)** Except for a paradoxical increase in weeks 17-20 (F=5.05), the average movement rate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

The Influence of DEET on K_v and Na_v Ion Channel Physiology

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

As the inclusion of DEET in the exposure protocol intensified and shifted the pattern of behavioral outcomes, we hypothesized that DEET might also influence the activity of K_v (and other ion channels) channels that have been associated with the development of pain-signs in rats (K_{DR}). It was recently shown that DEET (~100 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).

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.

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).

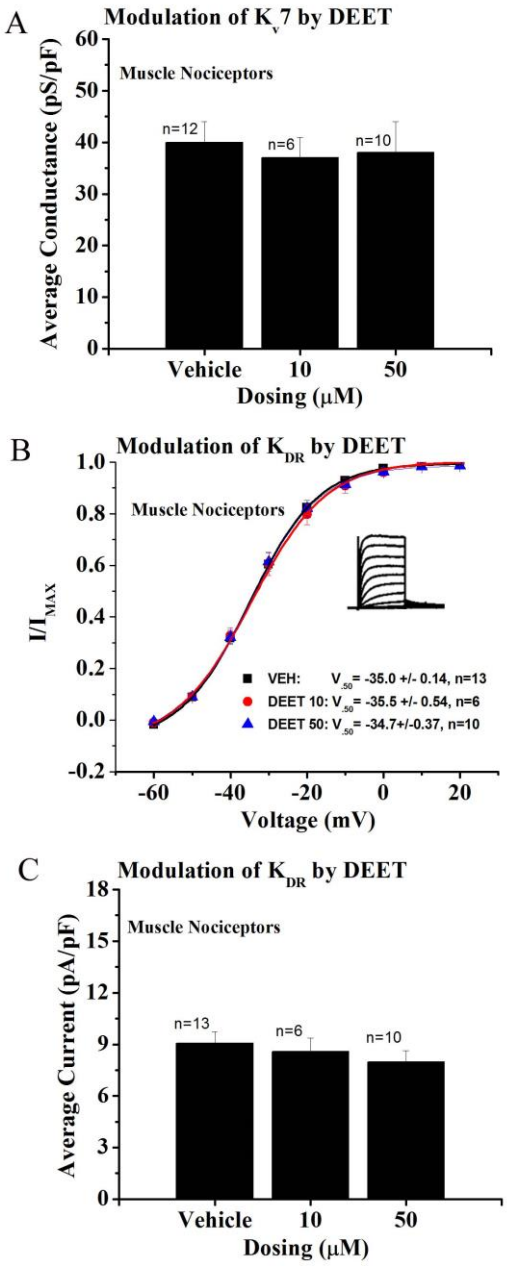

Figure 5. Voltage Activated K^+ Channels were Unaffected by Acute Exposure to DEET. A) The average conductance of muscle nociceptor K_v7 channels was not altered by DEET (10-50 μM); **B) and C)** Following a 12 minute exposure, there was no indication that either the voltage dependence or the average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

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).

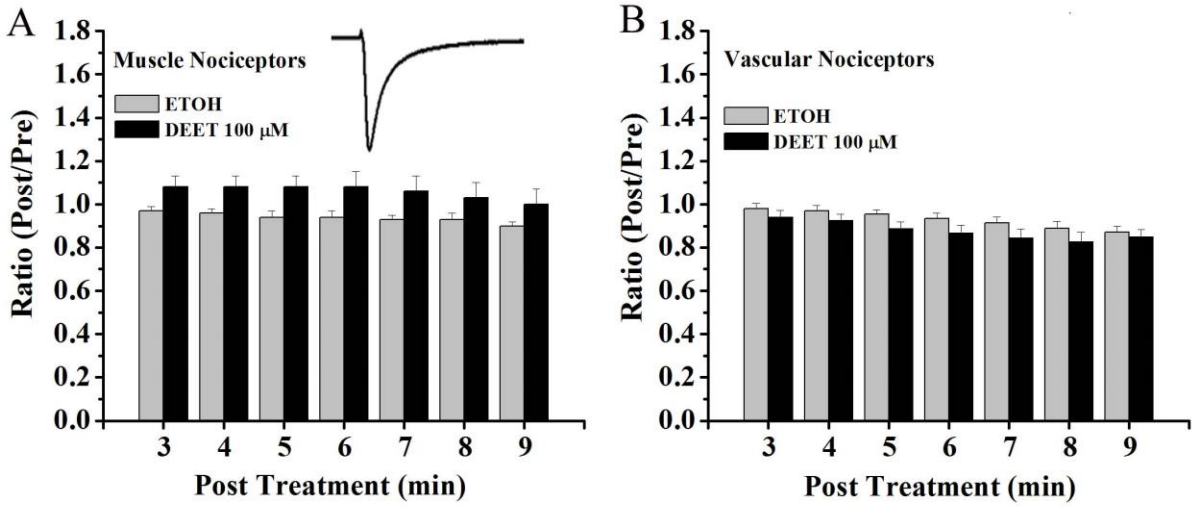

Figure 6. Time Dependent Modification of $Na_v1.8$ by DEET. A) The amplitude of muscle nociceptor $Na_v1.8$ was not changed by DEET (100 μM). A representative Nav1.8 current is inserted. **B)** Vascular nociceptor $Na_v1.8$ amplitude was not altered by DEET (100 μM). Baseline records were taken prior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

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.

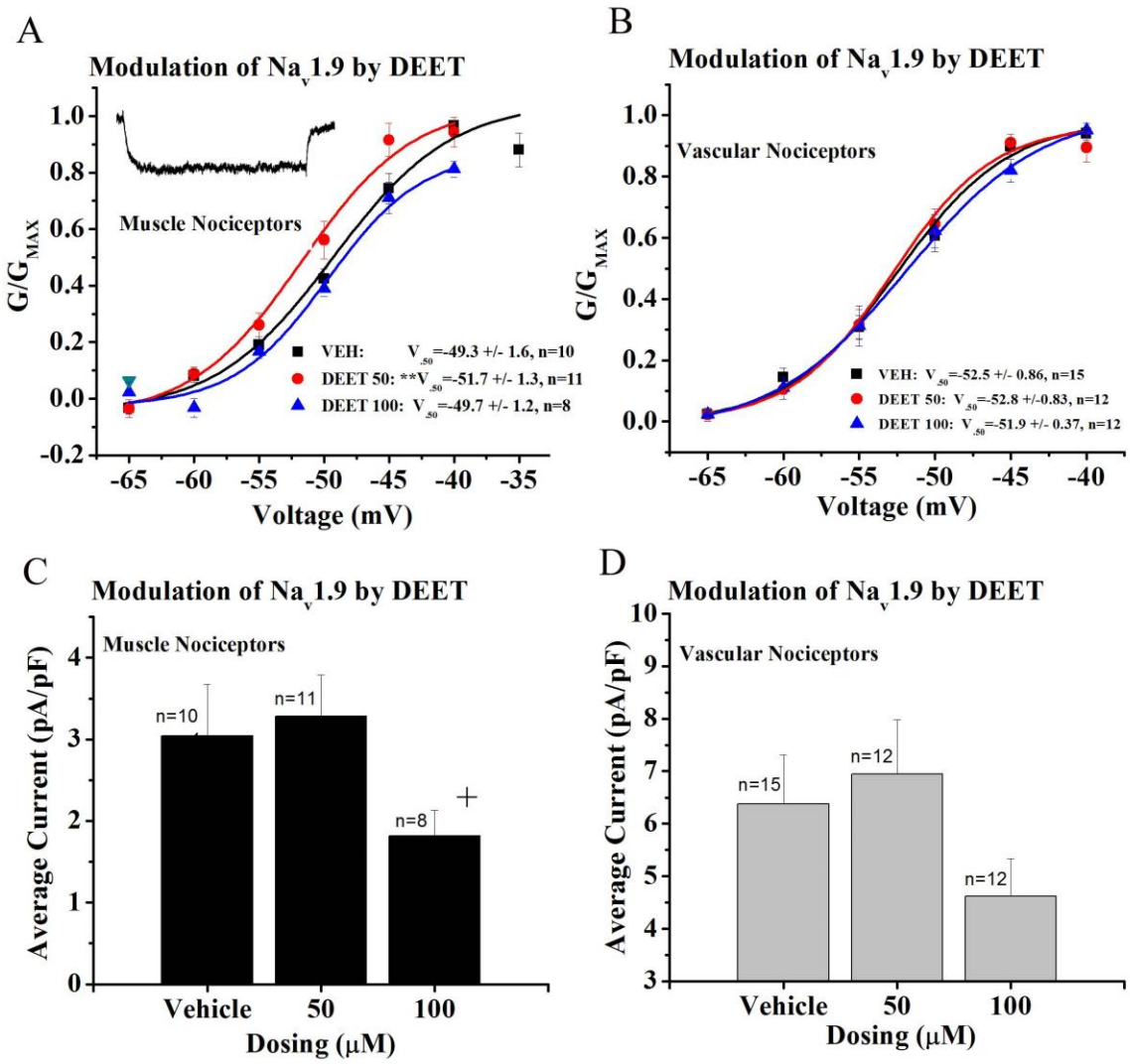

Figure 7. Voltage Activated, Na$_v$1.9, Channels were Weakly Modulated by Acute Exposure to DEET. A) The voltage dependent activation of muscle nociceptors was hyperpolarized at 50 but not at 100 μM DEET. A representative trace of a Na$_v$1.9 current (step to -50 mV; V_H=-120 mV) is included as an insert. **B)** The voltage dependent activation of vascular nociceptors was unaffected by DEET. **C)** Average currents of muscle nociceptors were unaffected by DEET ($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.

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

We examined whether the, DEET augmented, 4 week exposure protocol altered muscle nociceptor physiology. Experiments were conducted on Group A, Group PB and Group C rats. Twelve weeks after exposures ended, DRGs were excised from rats for whole cell patch clamp studies. Recordings were obtained from muscle nociceptor (type 5) $Na_v1.9$ ion channels using solutions and protocols described in 'Methods'. Following preliminary characterization procedures, muscle nociceptor, $Na_v1.9$ currents were isolated from other voltage dependent currents. From a holding potential of -120 mV, currents were evoked in 5 mV steps (-80 to -20 mV). A voltage of half activation ($V_{.50}$) was determined following fit of the evoked currents to a Boltzmann equation.

Regardless of the presence or absence of PB, there was no indication that DEET augmented protocols altered the $V_{.50}$ of activation (figure 8A). In contrast, the average evoked current of muscle nociceptor $Na_v1.9$ was increased in both Group A and Group PB relative to vehicle exposed controls. Although distinct exposure protocols led to distinct behavioral outcomes (figures 1 and 4), there were no differences in $Na_v1.9$ amplitude in neurons harvested from Group A versus those from Group PB (figure 8B).

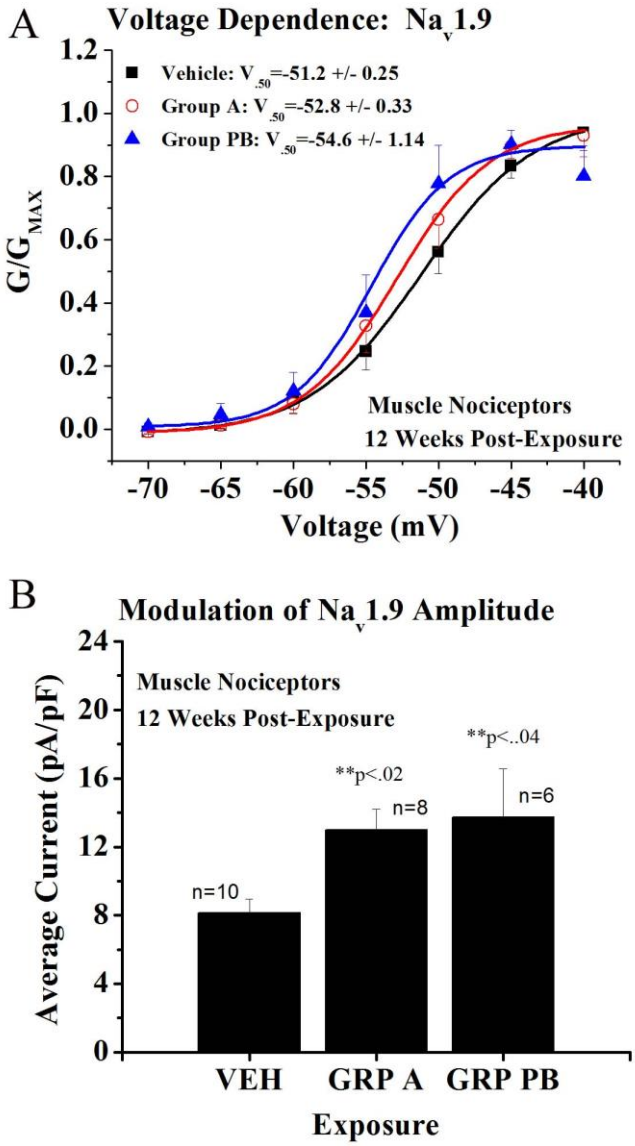

Figure 8. The Average Current of Muscle Nociceptor $Na_v1.9$ was Increased 12 Weeks Post-Exposure. A) The voltage dependent activation of $Na_v1.9$ was unchanged. The number of cells contributing to each curve are given in panel 'B'. **B)** Consistent with increased membrane excitability, the average current (-65 to -40 mV) was significantly increased in both Group A and Group PB muscle nociceptors. GRP A: PB, permethrin, chlorpyrifos and DEET; GRP PB: permethrin, chlorpyrifos and DEET; VEH: water, ETOH, corn oil, ETOH. To account for cell size variations, the average currents were normalized with respect to cell capacitance (pF). Thirteen rats contributed to these experiments. ** significantly different from vehicle exposed cases.

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. Relative to either vehicle or positive control groups, rat ambulatory behaviors were suppressed as early as 5-12 weeks post-exposure and remained suppressed out to the 17-24 week test periods. Consistent with the presence of a myalgia, these behavior shifts were accompanied by increased activity in muscle nociceptor $Na_v1.9$. Despite the powerful influence of DEET, the development and persistence of pain signs were shown to be mainly dependent, albeit in a complex fashion, on the anticholinesterase components of the exposure protocol.

Experiments in which chlorpyrifos or PB were eliminated from the exposure set gave clear indications of the importance of these anticholinesterases for the development and maintenance of pain behaviors. In the absence of CP, ambulation deficits were substantially reduced or prevented. 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.

DEET Potentiates the Development of a Chronic Pain Condition

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

We had reported previously that exposure to chlorpyrifos, permethrin and PB, at a low duty cycle, was insufficient to produce ambulatory and resting pain signs, but increased the activity of muscle nociceptor $Na_v1.9$ eight weeks following exposures (Nutter and Cooper, 2014). In the DEET augmented protocol, we now report a similar enhancement of $Na_v1.9$ activity that was manifested at the 12 week post-exposure interval. The production of 'plateau potentials' by $Na_v1.9$

could promote burst discharges from muscle nociceptor pools that would contribute to maintenance of a widespread myalgia (Copel et al., 2009, Herzog et al., 2001; Maingret et al., 2008; Nutter et al., 2015). It is possible that DEET intensified what had been a subclinical influence on $Na_v1.9$ to a level at which behavioral deficits were manifested. However, there is no specific evidence for that interpretation. It is simple to argue for a contribution of $Na_v1.9$ to the development and maintenance of pain signs, but it is difficult to see how dysfunctional $Na_v1.9$ currents could account, completely, for the observed patterns of ambulatory and/or resting pain.

The removal of PB from the exposure protocol shifted the pattern of behavioral signs, but there was no indication that this was reflected, in any way, by muscle nociceptor $Na_v1.9$. When PB was removed from the protocol, ambulation signs were substantially reduced and resting signs substantially increased. The amplitude of $Na_v1.9$ currents were not influenced by the presence or absence of PB in the exposure protocol. Finally, GW agents increased $Na_v1.9$ whether or not any pain signs were detected (Nutter and Cooper, 2014). We have reported that the physiology of other ion channels (K_{DR}, K_V7) can be altered by chronic exposure to GW agents. An examination of their properties might clarify the physiological influence of DEET and/or PB on pain system neurons (Nutter et al., 2015; Cooper et al., 2016).

While DEET clearly potentiated behavioral maladaptations, and may have potentiated shifts in muscle nociceptor physiology, we could not demonstrate any acute influences of DEET on $Na_v1.9$ activity. Nor did we observe acute influences, by DEET, on other ion channels that have been implicated in GWI agent-induced maladaptations (i.e., $Na_v1.8$, K_v7 and K_{DR}; Nutter and Cooper, 2014; Nutter et al., 2015; Cooper et al., 2016). The absence of effects of DEET on known and suspected GW agent maladapted ion channel proteins does not preclude a direct molecular

level interaction of DEET with deep tissue nociceptors through other pathways; nor does it rule out an indirect interaction through secondary pathways yet to be identified.

Chlorpyrifos is an AChE inhibitor, but one of its hepatic metabolites, chlorpyrifos-oxon is 1,000 fold more potent in that role (Huff and Corcoran, 1994). In a direct assessment of the hepatic interaction of these two GW agents, Usmani and colleagues reported that DEET increased the hepatic conversion of chlorpyrifos to its oxon form by a factor of 2.4 (Usmani et al., 2002). Potentially the amplification of pain signs, by DEET, occurs through its capacity to increase the peak levels of the more potent oxon form of CP (Abou-Donia et al., 1996; Abu-Qare and Abuo-Donia, 2008). Moreover, when PB was co-administered with DEET, it slowed hepatic DEET metabolism, and as a result could further enhance the DEET potentiated conversion of chlorpyrifos to chlorpyrifos-oxon (Abu-Qare and Abu-Donia, 2008; see also Chaney et al., 2000). Therefore, the amplification of pain signs by addition of DEET, in an otherwise ineffective protocol, could simply be due to an increase in the effective peak concentration of chlorpyrifos-oxon. Just as increasing the duty cycle of the anticholinesterases potentiated pain signs with an 8 week exposure (Nutter et al., 2015), increasing the functional concentration of chlorpyrifos-oxon, during a 4 week exposure, could have potentiated and prolonged ambulatory deficits.

It is well known that single high dose exposures to certain organophosphate anticholinesterases have been linked to the development of a progressive motor and sensory neurodegenerative disorder known as OPIDN/OPIDP (organosphophate induced delayed neuropathy/polyneuropathy; Johnson, 1975; Vale and Lotti, 2015). One to three weeks after a single exposure, subjects develop a variety of symptoms that include progressive ataxia, paresthesias, and muscle pain. These are accompanied by a peripheral axonopathy characterized by slowed action potential conduction and evidence of Wallerian degeneration (Lotti and Moretto,

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

Complex Influences of Chlorpyrifos and PB on Pain Signs

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

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

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

While PB has a demonstrated capacity to oppose the anticholinesterase effects of chlorpyrifos-oxon (Henderson et al., 2012), it has no capacity to oppose or prevent the extra-anticholinesterase effects of chlorpyrifos-oxon. The latter are considerable and well documented. Independent of any inhibition of AChE, 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). Repeated exposure to chlorpyrifos ultimately alters the expression of muscarinic receptors in the CNS (Nostrandt et al., 1997; Liu et al., 1999; Huff et al., 2001; Abou-Donia et al., 2003; Zhang et al., 2002; Padilla et al., 2005; Pung et al., 2006; Proskocil et al., 2010) and modifies the functional consequences of mAChR activation in PNS nociceptors (Nutter and Cooper, 2015; Cooper et al., 2016).

How these multiple cholinesterase and non-cholinesterase actions contribute to the development of specific pain signs cannot be readily resolved in this report. Our examination of muscle nociceptor $Na_v1.9$ did not reveal any differential influences attributable to the presence or absence of PB in the exposure protocol (see above). Soldiers suffering from GWI pain describe highly diverse symptom patterns (i.e., muscle pain, back pain, joint pain, abdominal pain, headache) that suggest pathology in multiple, functionally distinct, nociceptor pools that innervate these diverse tissues (Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). In the absence of PB, additional nociceptive pools, under the full influence of both the anticholinesterase and extra-cholinesterase actions of CP, could suffer significant maladaptations that are manifested as distinct forms of pain.

Summary and Conclusions

DEET substantially potentiated and prolonged the pain signs that developed after a 4 week exposure to GW agents. The pattern of pain signs associated with a DEET augmented exposure set diverged from those observed after an 8 week exposure to the same GW agents in the absence

of DEET (Nutter et al., 2015; Cooper et al., 2016). While we were unable to identify a specific linkage between nociceptor ion channel physiology and acute exposure to DEET, such interactions may yet be found. At present, it is more likely that DEET indirectly amplifies the physiological impact of chlorpyrifos on a variety of molecular targets.

Soldiers deployed to the Persian Gulf were potentially exposed to a large variety of insecticides, repellants, nerve agents, adjuvants, depleted uranium, and other toxins (Binns et al., 2008; RAC, 2014). A high percentage developed symptoms while still in theater (~25%; Kroenke et al., 1998), but most developed a wide variety of pain symptoms that were delayed in onset and worsened over time (Hotopf et al., 2003). The variations of the symptoms, as well as the timing of their onset, could represent different exposure patterns (and degrees of exposures) and how they ultimately interacted with the genetic makeup of each individual. Acknowledging that, it is likely that there were common risk factors that set into motion a definable set of maladaptations that resulted in the symptoms of GWI. Most of our research points to the fundamental role of the anticholinesterases, CP and PB, as primary risk factors for pain. Although it is not clear that it was specifically their anticholinesterase activity that posed the greatest risk, the manifestations of pain-like behaviors appeared after doubling the exposure duty cycle of PB and CP (Nutter et al., 2015; Cooper et al., 2016). Adding DEET to the exposure set accelerated the development, altered the pattern and prolonged the persistence of pain-like behaviors that were ultimately dependent upon the presence of chlorpyrifos and/or pyridostigmine bromide. The impact of DEET augmentation on muscle nociceptor $Na_v1.9$ physiology might account for some of the resultant behavioral signs, but $Na_v1.9$ status could not account for the differential influences of PB on these signs.

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

Acknowledgements

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Behavioral, Cellular and Molecular Maladaptations Covary with Exposure to Pyridostigmine Bromide in a Rat Model of Gulf War Illness Pain

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Abstract

Many veterans of Operation Desert Storm (ODS) struggle with the chronic pain of Gulf War Illness (GWI). Exposure to insecticides and pyridostigmine bromide (PB) are implicated in the etiology of this multisympton disease. We examined the influence of 3 (DEET (N,N-diethyl-meta-toluamide), permethrin, chlorpyrifos) or 4 GW agents (DEET, permethrin, chlorpyrifos, pyridostigmine bromide (PB)) on the post-exposure ambulatory and resting behaviors of rats. In three independent studies, rats that were exposed to all 4 agents consistently developed both immediate and delayed ambulatory deficits that persisted at least 16 weeks after exposures had ceased. Rats exposed to a 3 agent protocol (PB excluded) did not develop any ambulatory deficits. Cellular and molecular studies on nociceptors harvested from 16WP (weeks post-exposure) rats indicated that vascular nociceptor $Na_v1.9$ mediated currents were chronically potentiated following the 4 agent protocol but not following the 3 agent protocol. Muscarinic linkages to muscle nociceptor TRPA1 were also potentiated in the 4 agent but not the 3 agent, PB excluded, protocol. Although K_v7 activity changes diverged from the behavioral data, a K_v7 opener, Retigabine, transiently reversed ambulation deficits. We concluded that PB played a critical role in the development of pain-like signs in a GWI rat model and that shifts in $Na_v1.9$ and TRPA1 activity were critical to the expression of these pain behaviors.

Introduction

Gulf war illness (GWI) is a multi-symptom disease affecting ~60% of veterans of the 1991 Persian Gulf War. Although popularly associated with cognitive symptoms, GWI actually clusters into 3 major syndromes that include chronic pain, confusion-ataxia as well as cognitive deficits (Haley and Kurt, 1997). Those that suffer from chronic pain manifest an unusual variety of symptoms that defy simple explanation. These include combinations of muscle, back, joint, abdominal and headache pains (Haley and Kurt, 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013; White et al., 2016). While considering the obvious complexity of such a diverse constellation of symptoms, it is also important to recognize that although most soldiers developed delayed symptoms that were manifested only upon their return from deployment, about 25% developed symptoms while they were still in theater (Kroenke et al., 1997). After more than two decades, an effective treatment for GWI has not been identified.

There is growing evidence that the symptoms of GWI derived from excessive exposure to insecticides and other neurotoxicants that were present in the Gulf theater. During their relatively brief deployment, the soldiers of ODS 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). Many of these agents (organophosphates, pyrethroids) had demonstrated capacity to interact with the human nervous system (Narahashi et al., 1998; Soderlund et al., 2002; Jiang et al., 2013). Most insecticides were provided by the military, along with specific guidelines for their safe use. There is no record as to whether these guidelines were followed in the field. Others insecticides and repellants were obtained independently by soldiers to supplement military issued chemicals that were insufficiently effective. In addition to these

neurotoxicants, warfighters were also directed to ingest the anticholinesterase pyridostigmine bromide (PB) three times daily (Binns et al., 2008). In combination with atropine or other anti-muscarinics, PB had some capacity to reduce the lethality of certain nerve agents (Soman, 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).

Our laboratory has generated evidence that synergistic action of anticholinesterases (PB, chlorpyrifos), pyrethroids (permethrin) and the repellant DEET (N,N-diethyl-meta-toluamide) could produce, in various combinations, both delayed (Nutter et al., 2015) or relatively acute pain-like signs (Flunker et al., 2017) in rats that persisted for up to 24 weeks following 4-8 week exposures (Flunker et al., 2017). Moreover, the exclusion of certain agents from the exposure set could either prevent the development of symptoms (chlorpyrifos) or change the pattern of pain-like signs that persisted (PB; Flunker et al., 2017).

If our model accurately represents the development of delayed and acute pain symptoms of GWI, identification of the molecular underpinnings of signs that differentially emerge from distinct exposure sets could isolate the key molecular adaptations that are critical to the maintenance of chronic pain, and then direct strategies to ameliorate or permanently reverse the associated suffering.

Exposure to 3 GW agents for 8 weeks (permethrin, chlorpyrifos, PB) produces shifts in K_v7 and $Na_v1.9$ physiology that were identified variously in muscle ($Na_v1.9$; K_v7; Nutter and Cooper, 2014; Nutter et al. 2015) or vascular sourced nociceptors ($Na_v1.9$; Nutter and Cooper, 2014), 8 to 12 weeks following exposure. We also identified heightened muscarinic sensitivity in K_v7 and other K_{DR} ion channels that are expressed in deep tissue nociceptors (12 weeks post-

exposure; Cooper et al., 2016). When DEET was added to the exposure set (DEET, permethrin chlorpyrifos and PB), the development and persistence of pain signs were substantially magnified, and the necessary exposure time was reduced from 8 to 4 weeks (Flunker et al., 2017). K_v7 activity was again depressed; however, exclusion of PB from the exposure set had no influence on the suppression of K_v7 at 12WP (Flunker et al., 2017). Divergence of K_v7 activity from behavioral impact of GW agents indicated that this deep tissue nociceptor ion channels was not associated with the pathophysiology of GWI pain symptoms.

In the experiments described below, we examined the influence of the 4 week, 4 agent DEET augmented protocol on the activity of $Na_v1.9$ and K_v7, and whether molecular maladaptations associated with these ion channels at 16 weeks post-exposure covaried with the presence and absence of PB in the exposure protocol. Additional studies were conducted to determine the net effect of these exposures on nociceptor excitability in the presence and absence of PB.

Methods

Behavioral Studies

Subjects. Young adult male rats (n=101) were purchased from Envigo (Sprague-Dawley; 109.2 +/- 0.81 grams. 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). No animals exhibited signs of acute pesticide toxicity, but one vehicle exposed/ vehicle treated animal was found dead it its cage during the treatment phase and another vehicle treated rat was lost during the post-treatment phase.

Chronic Exposure Protocol. Over a period of 4 weeks, rats were exposed to either 3 or 4 GW chemicals. GRP A rats were exposed to 4 GW chemicals: DEET (400 mg/kg; 50% ETOH), permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), and pyridostigmine bromide (PB; 13 mg/kg). GRP PB rats were exposed to 3 GW chemicals: DEET, permethrin, chlorpyrifos. GRP C, control, rats were exposed only to agent vehicles using the identical procedures and volumes (ETOH, corn oil, water). DEET and permethrin (ETOH), were applied, daily, to a 1 inch square shaved area of the upper back 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 agent over several days (Smith et al., 2009). PB, in tap water, was administered daily by oral gavage. The dose represented 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 by body weight. Twelve (12) or 16 weeks after chemical exposures had ended, rats were sacrificed for electrophysiological studies. Rats developed normally and showed none of the classic signs of organophosphate or pyrethroid toxicity. Rats treated with the 4 agent or 3 agent protocol weighted

significantly less at the termination of studies (GRP C: 490.6 +/- 6.5. , GRP A: 468.3 +/- 6.4, GRP PB: 462.7 +/- 6.2 grams; $p<.01$ and $p<.02$, GRP A vs GRP C and GRP PB vs GRP C, respectively).

Assessment of Pain-Like Behaviors. Open field testing was conducted before, during and after chemical exposures. Upon arrival, rats were acclimated to the behavioral procedures for 2 weeks. Thereafter, testing continued weekly throughout the entire exposure and post-exposure periods (22 weeks). These measures included movement distance (cm), average movement rate (cm/sec), and rest times (sec). Events were detected and recorded automatically by infrared sensors that lined a 35 by 40 cm Perspex test chamber (Fusion System, AccuScan/Omnitech). The chamber was modified to prevent rearing behaviors. After each 15 minute collection period, the test chamber was cleaned with 70% ETOH.

Electrophysiological Studies

Preparation of Cells. Twelve (12) or 16 weeks following the termination of exposures, rats were anesthetized (Isoflurane) and rapidly euthanized by decapitation (Harvard Instruments). The spinal column was removed, bisected and the DRG were dissected free from T3 to S1. Ganglia were trimmed, cut into strips and digested in Tyrode's solution containing collagenase A (2 mg/ml) 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 pellet was dispersed, 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 boroscilate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). For experiments on K_v7 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. For experiments on $Na_v1.9$, 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 9 software and a Digidata 1322A (Molecular Devices). Axopatch circuitry was used to compensate series resistance (R_s; 65-70% (K_v7) or 75-80% ($Na_v1.9$)). 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).

Recording and Characterization of Muscle and Vascular Nociceptors. 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. Using 3 voltage characterization protocols (CP1, CP2 and CP3), we classified medium sized neurons as type 5 muscle or type 8 vascular nociceptors. The physiological signature of type 5 nociceptors included small I_H (<1.5 pA/pF; CP1), a high threshold I_A (0 mV; CP2) that exhibited a prolonged settling time (>50 msec) and a Na^+ current that was broad at its base (> 4 msec at 0

mV; CP1) and achieved maximum values at a voltage step to 0 mV; CP3). The physiological signature of type 8 nociceptors included small I_H (<1.5 pA/pF; CP1), an I_A threshold of -20 mV with prolonged I_A settling time (>40 msec; CP2), and a Na^+ current that exhibited a broad base (>3.5 msec at 0 mV; CP1) and achieved maximum values at a voltage step to -10 mV; CP3). The main distinguishing feature between type 5 and type 8 cells was the 20 mV difference in the threshold of I_A. These physiological current signatures are very different from other medium sized neurons encountered in DRG recordings, and are visually obvious (Petruska et al., 2000; 2002). Cells not fitting the classification criteria of type 5 or 8 were discarded. Anatomical targets of type 5 and type 8 neurons were determined by a series of anatomic tracing experiments (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). Type 5 and type 8 nociceptors are capsaicin/heat sensitive and co-express vasoactive neuropeptides (substance P and CGRP; Petruska et al., 2000, 2002; Rau et al., 2007).

Isolation and Characterization of $Na_v1.9$ Channel Currents. Currents were isolated in an external solution (Na_{iso}) containing (in mM): 70 NaCl, 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. The pipette solution contained 140 CsF, 10 NaCl, 5 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH. Whole cell patch mode was achieved in Tyrode's solution. Following characterization and series resistance correction, the cell was exposed to the Na_{iso} solution for 2 minutes. The voltage dependence of activation was then determined. 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. Junction offsets were not corrected.

After completion of the studies, peak currents of non-desensitizing $Na_v1.9$ were measured 250 msec from the start of the voltage step. This precaution was taken to avoid contamination by $Na_v1.8$. If fast desensitizing peaks were observed during the voltage step, no data was collected from that trace. To characterized the 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 $V_{.5}$ for 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 $V_{.5}$ is the voltage at which G is half maximal, and K is a slope factor. Average currents were determined from the normalized peak currents observed that were recorded over the active range (-65 to -45 mV).

Isolation and Characterization of K_v7 Channel Currents. Following cell characterization in Tyrode's solution, K^+ currents were characterized in an external solution containing (in mM): 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 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. The osmolarity was approximately 290 mOsm.

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 1000 ms step command to -20 mV was followed by a series of repolarizing 10 mV steps from -20 to -90 mV (1000 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 first applied in the K^+ isolation solution containing .001% ETOH (pre-applied for 2 min). This was followed by application of the K^+ isolation 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). A mean G was computed over the range of functional deactivation steps (-40 to -60 mV) to obtain a mean normalized conductance. The peak conductance was determined by inspection.

Statistics. A repeated measures ANOVA (QIMacros) was used to assess the influence of chemical exposure and weeks on dependent measures of movement distance (cm/15 min), movement rate (cm/sec) and rest (sec/15 min). Follow-up tests were not conducted on significant main effects or interactions. Separate analyses of pain behaviors were performed on periods in which rats were sacrificed for molecular studies (9-12 and 13-16 weeks-post (WP) chemical exposure). We did not perform analyses on other time periods either during or after chemical exposures. Student's t tests were used to contrast amplitude of K_v7 activity expressed in exposed, vehicle treated animals. The alpha level was set at .05.

Results

Behavioral Studies

We examined the ambulatory and resting behaviors of rats that were exposed to 3 or 4 GW chemicals (4 weeks; n=38). One group was exposed to DEET, permethrin, chlorpyrifos and PB (Grp A). A second group was exposed to DEET, permethrin, and chlorpyrifos, but PB was excluded (Grp PB). A third group served as a vehicle control (Grp C; water gavage, ethanol topical, corn oil s.c., and ethanol topical).

Behavioral testing was conducted once per week, lasted 15 minutes, and was fully automated by means of an array of infrared beams (AccuScan/Omnitech Electronics). AccuScan software converted beam interruptions into measures of ambulation (total distance moved, average rate of movement) and the total time at rest. Consistent with our previous published work, a repeated measures ANOVA was used to assess ambulation and resting scores captured in 4 week blocks (9-12 weeks and 13-16 weeks post-exposure; 9-12WP and 13-16WP).

Rats exposed to all 4 agents (Grp A) developed enduring pain-like behaviors. Movement distance was significantly reduced at both the 9-12 and 13-16 week post-exposure periods (figure 1A; Grp C vs A). Movement rate was also depressed, but emerged only at the late 13-16WP assessment (figure 1B; Grp C vs A). Reduced ambulation was accompanied by a significant increase in rest duration at both test periods (figure 1C; Grp C vs A).

Exclusion of PB from the exposure set significantly altered the pattern of behavioral outcomes. In the absence of PB, ambulation deficits were ‘rescued’ at 9-12WP (movement distance) and 13-16WP (figure 1A; Grp C vs Grp PB). This was evidenced as significant increases

in movement distance and rate for GRP PB relative to GRP A. It is noteworthy, that, in most comparisons, movement distance and rate scores of Grp PB were not only increased relative to GRP A, but also paradoxically elevated over scores of vehicle exposed rats (figure 1A and 1B; Grp C vs Grp PB). The absence of PB from the exposure set failed to rescue resting deficits at the 9-12WP assessment period (figure 1C; Grp C vs PB); however, resting deficits were eventually rescued at 13-16WP (figure 1C). At no time were any of the resting scores paradoxically shifted relative to GRP C.

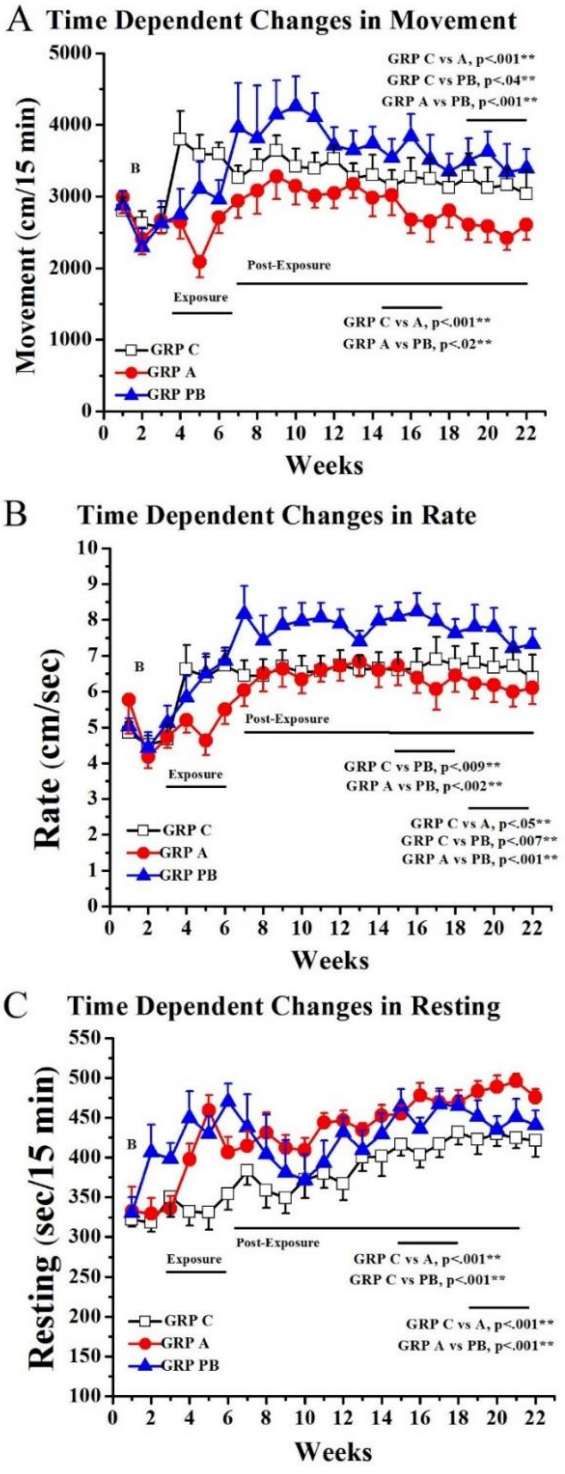

Figure 1. Exposure to 4 GW Chemicals Produces Persistent Pain-Like Behaviors that Required PB. A) Exposure to all 4 GW agents (GRP A) significantly decreased movement distance scores at both 9-12 and 13-16WP. When PB was excluded from the exposure set (GRP PB), movement distance was no longer suppressed. **B)** The 4 GW agents also significantly decreased movement rate scores, but declines in rate were delayed until 13-16WP. When PB was excluded from the exposure set, movement rate was significantly increased. **C)** Resting duration was significantly increased at both 9-12 and 13-16WP. In the absence of PB, resting scores were rescued only at 13-16WP. B: baseline testing; GRP A (n=10): DEET, chlorpyrifos, PB, permethrin; GRP C (n=10): (ethanol, corn oil, ethanol, water); GRP PB (n=10): DEET, chlorpyrifos, permethrin. **significantly different by ANOVA.

Retigabine Produces Transient Recovery of Some Pain-Like Behaviors

Our previous studies have linked diminished K_v7 ion channel, voltage dependent activity, to ambulatory deficits at 8 and 12WP exposures (Nutter et al., 2015; Flunker et al., 2017). As the voltage dependence of K_v7 functions as a brake on membrane excitability, a lessening of activity is fully consistent with a chronic pain syndrome (Brown and Passmore, 2009; Du et al., 2017). Conversely, the opening of K_v7 channels should decrease nociceptor excitability in those deep nociceptor populations that have been implicated in the pathophysiology of GWI. To examine this possibility, we prepared 3 additional groups of rats to test whether the K_v7 activator, Retigabine, could reduce pain-like behaviors of rats exposed to GW chemicals (Gunthorpe et al., 2012). As above, rats were prepared for behavioral studies (n=18). GRP A2 (n= 12) animals were exposed, for 4 weeks, to DEET, permethrin, chlorpyrifos and PB. GRP C2 (n=6) rats were exposed only to the corresponding vehicles. Ambulation and resting behaviors were assessed once per week.

Retigabine treatments were initiated at 9WP in half of the GRP A2 rats (GRP R). Rats were pre-assigned to GRP R when they first entered the experimental sequence (B: Baseline; see figure 2). Retigabine treatment continued daily for 4 weeks (9-12WP; 7 mg/kg in DMSO; oral gavage). GRP A2 and GRP C2 rats received DMSO during the treatment phase. Behavior testing occurred within 2-3 hours of Retigabine gavage. In order to determine the persistence of any significant Retigabine related recovery, testing continued for another month after the treatments ended (13-16WP).

Similar to the rats of figure 1, rats of GRP A2 developed pain-like behaviors manifested as a significant decrease in ambulation. The onset of effects were relatively delayed. Movement

distance and average movement rate were significantly decreased at 9-12WP following the 4 week exposure to GW chemicals (figure 2A and B; $p<.01$ and $p<.002$ respectively). In contrast to rats of figure 1 studies, rest times were unaffected by GW agent exposures (figure 2C).

Treatment with Retigabine (GRP R) produced mixed outcomes. On the one hand, Retigabine rescued the exposure-induced reduction in average movement rate. Average movement rate of GRP R rats was significantly increased relative to DMSO treated GRP A ($p<.006$) and was not depressed relative to vehicle treated GRP C2 animals (figure 2B). Movement distance score outcomes were less definitive. Although Retigabine treated animals 'improved' to the extent that their scores were not significantly decreased relative to GRP C2 ($p<.10$), trends were still strongly towards reduced movement distance. Moreover, Retigabine failed to significantly improve movement distance scores relative to GRP A2 rats (figure 2A). Although resting scores were not significantly affected by GW chemical exposure ($p<.12$), they were paradoxically increased by Retigabine treatment (GRP R vs GRP C2; figure 2C; $p<.02$).

The influence of Retigabine on the rate of ambulation was transitory. During the month that followed Retigabine treatment (13-16WP; Post-Treatment), GRP R rats did not retain their significant improvement in movement rate behaviors relative to GRP A2, but GRP R rats did come close to regaining significant rate suppression relative to GRP C2 ($p<.06$; figure 2B). GRP A2 continued to exhibit diminished ambulation rate relative to GRP C2 ($p<.002$; Post-Treatment; figure 2B). Movement distance scores, that had been partially rescued by Retigabine, slipped back to their diminished level once treatment ceased (GRP C2 vs GRP R; $p<.02$, 13-16WP). Therefore, Retigabine treatment produced a partial but transient rescue of pain-like ambulation behaviors associated with exposure to GW chemicals.

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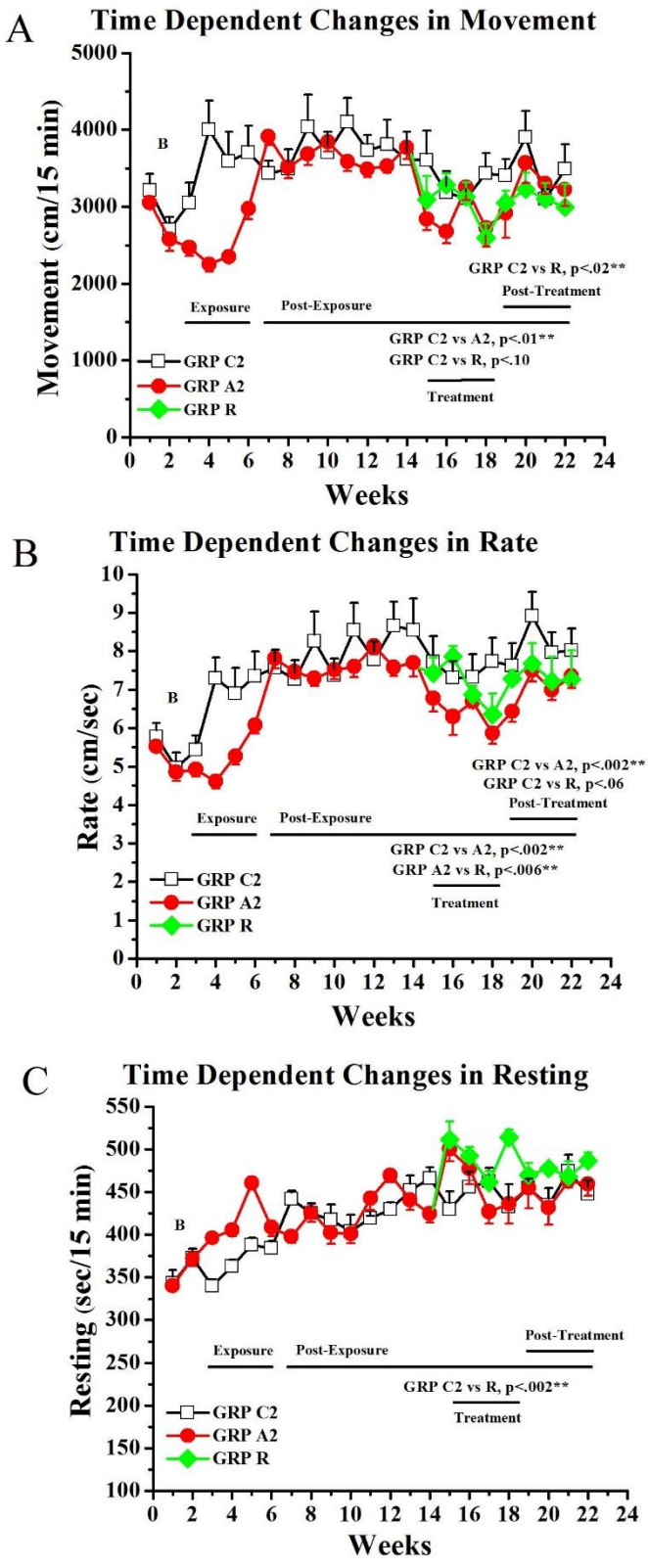

Figure 2. Treatment with Retigabine Produced a Transient Reduction of Pain-Like Behaviors. **A)** Exposure to all 4 GW agents (GRP A2) significantly decreased movement distance scores at both 9-12 and 13-16WP. Retigabine treated rats were not different from controls; but they were not significantly better than exposed animals. **B)** Exposure to GW chemicals also significantly decreased movement rate scores (9-12 and 13-16WP). Retigabine significantly improved rate scores relative to GRP A2 and rescued these scores relative to GRP C2 during treatment (9-12WP). In the post-treatment phase, movement rates were again significantly reduced (13-16WP). **C)** Resting duration was unchanged by exposure to GW agents at any observation period. Retigabine significantly increased resting scores at 9-12WP. B: baseline testing; GRP A2: DEET, chlorpyrifos, PB, permethrin; GRP C2 (n=6): (ethanol, corn oil, ethanol, water); GRP R (n=6): DEET, chlorpyrifos, PB, permethrin. Prior to treatment, GRP A2 was composed of 12 rats. During and after treatment GRP A2 was composed of 6 rats. **significantly different by ANOVA.

Molecular Physiology

Rats from GRPs C, A and PB (see figure 1) were euthanized 16 weeks post-exposure. Neurons were prepared for acute, whole cell patch experiments. Recordings were obtained from muscle and vascular nociceptors (see Method). Specialized solutions were used to facilitate isolation of $Na_v1.9$ or K_v7 currents (see Methods).

$Na_v1.9$ Activity in Muscle and Vascular Nociceptors. Following cell characterization and series resistance compensation, neurons were exposed to a Na_{iso} solution for 2 minutes. Voltage dependent activation was assessed by application of a series of voltage steps (-80 to -20 mV; 5 mV steps; V_H=-120 mV). Boltzmann functions were subsequently fit to the computed values of the peak conductance and plotted against the test voltage series. The voltage of half activation ($V_{.5}$) was determined for each individual cell recording. Cells that failed Boltzmann function fits were excluded from the study.

Shifts in the physiology of $Na_v1.9$ covaried with behavioral outcomes. The voltage dependent activation ($V_{.5}$) of vascular nociceptor $Na_v1.9$ was significantly hyperpolarized relative to vehicle exposed cases (GRP C vs GRP A; figure 3D, 16WP; $p<.04$). In contrast, we failed to find any significant effect on the half activation voltage ($V_{.5}$) of muscle nociceptor $Na_v1.9$ (GRP C vs GRP A; figure 3A and C). Moreover, when tests were conducted on nociceptors harvested from GRP PB rats, the $V_{.5}$ values of both muscle and vascular nociceptors were similar to Grp C, vehicle controls, but significantly depolarized relative to GRP A neurons (figure 3C and D).

Therefore, the removal of PB from the exposure protocol was associated with rescue of pain-like behaviors, and also rescue of the voltage shifts associated with vascular nociceptor excitability.

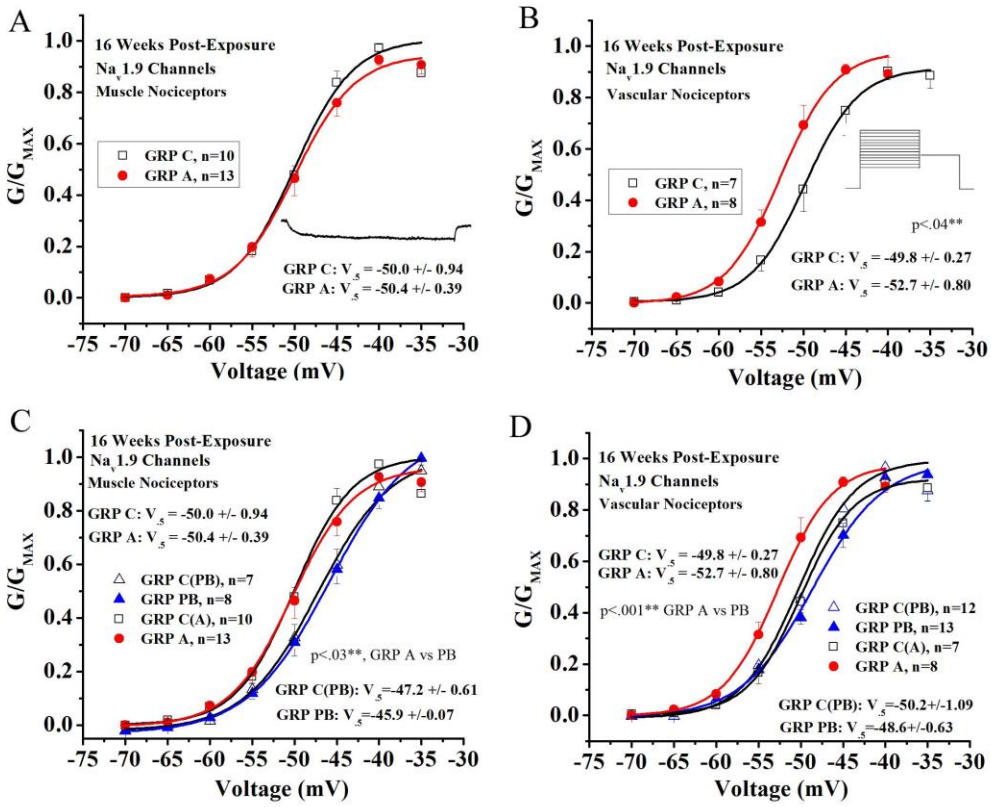

Figure 3. Voltage Dependent Activation of $Na_v1.9$ Shifts with Exposure to 4 GW Chemicals. A and B) Sixteen weeks after exposure, the $V_{.5}$ of activation was hyperpolarized in vascular but not muscle nociceptors (GRP C vs GRP A). An insert, in panel A, presents a representative trace that was evoked at -50 mV from a vascular nociceptor (300 msec duration; 1,997 pA amplitude). The voltage step protocol is presented as an insert in panel B. **C and D)** In the absence of PB in the exposure set (GRP PB), the activation $V_{.5}$ was depolarized in both muscle and vascular nociceptors relative to GRP A, but non-different from experiment specific controls (GRP PB vs GRP C(PB)). GRP A: DEET, permethrin, chlorpyrifos, PB; GRP PB: DEET, permethrin, chlorpyrifos; GRP C: ETOH, ETOH, corn oil, water; GRP C(A): control cases for GRP A; GRP C(PB): control cases for GRP PB.

Hyperpolarizing shifts in the voltage dependence suggest that more $Na_v1.9$ current will be evoked as neurons are depolarized. This could promote prolonged action potential bursting during depolarizing events (Copel *et al*., 2009; Herzog *et al*., 2001; Maingret *et al*., 2008; Nutter and Cooper, 2014). We examined the average evoked current in muscle and vascular nociceptors. The average evoked current was determined across the active range for each neuron (-65 to -45 mV) and then normalized to the cell dimension parameter capacitance (pF).

Statistical comparisons indicated that the average evoked nomalized current amplitudes were significantly increased in vascular nociceptors (GRP C vs GRP A, 16 WP; figure 4A). When PB was absent from the exposure protocol, no difference in average currents were detected (GRP C vs GRP PB, 16WP; figure 4B), but the average amplitude of GRP PB currents were significantly reduced relative to GRP A ($p < .001$). These molecular outcomes were consistent with the behavioral outcomes for GRPs A and PB at 16WP and tend to support a critical role of vascular nociceptor $Na_v1.9$ in the manifestation of pain-like behaviors that persisted following exposure to GW chemicals.

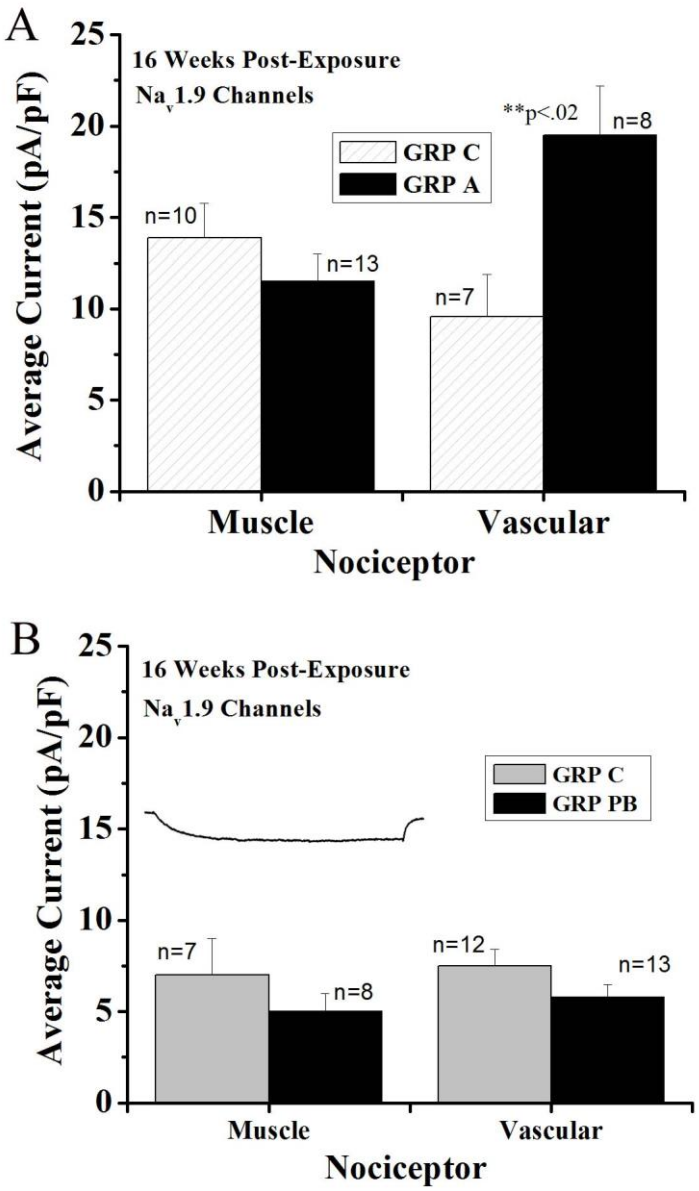

Figure 4. Exposure to GW Chemicals alter the Average Evoked Na$_v$1.9 Current. A) Sixteen weeks after exposure, the average current of vascular nociceptors was significantly higher (GRP C vs GRP A), but muscle nociceptors were unaffected. **B)** In the absence of PB, the average evoked currents were unchanged in either nociceptor class. An insert, in panel B, presents a representative trace that was evoked at -50 mV from a muscle nociceptor (300 msec duration; 961 pA). GRP A: DEET, permethrin, chlorpyrifos, PB; GRP PB: DEET, permethrin, chlorpyrifos; GRP C: ETOH, ETOH, corn oil, water.

K_v7 Activity in Muscle and Vascular nociceptors. We had previously shown that the DEET augmented protocol produced significant shifts in K_v7 conductance that were consistent with increased cellular excitability and ambulatory behavior changes that persisted at 12WP (Flunker et al., 2017). When PB was excluded during the exposure (GRP PB), ambulation was rescued and resting deficits emerged while K_v7 shifts were retained (12WP). Therefore, the decline in K_v7 activity diverged from ambulation deficits at 12WP; but might have retained some linkage to resting deficits. We now report that, at 16WP, changes in K_v7 conductance trended in a manner that was inconsistent with increased deep nociceptor excitability and the presence of ambulation and resting deficits (figure 5). Accordingly, it did not appear that shifts in K_v7 conductance were critical to either ambulation or resting status at 16 WP exposure.

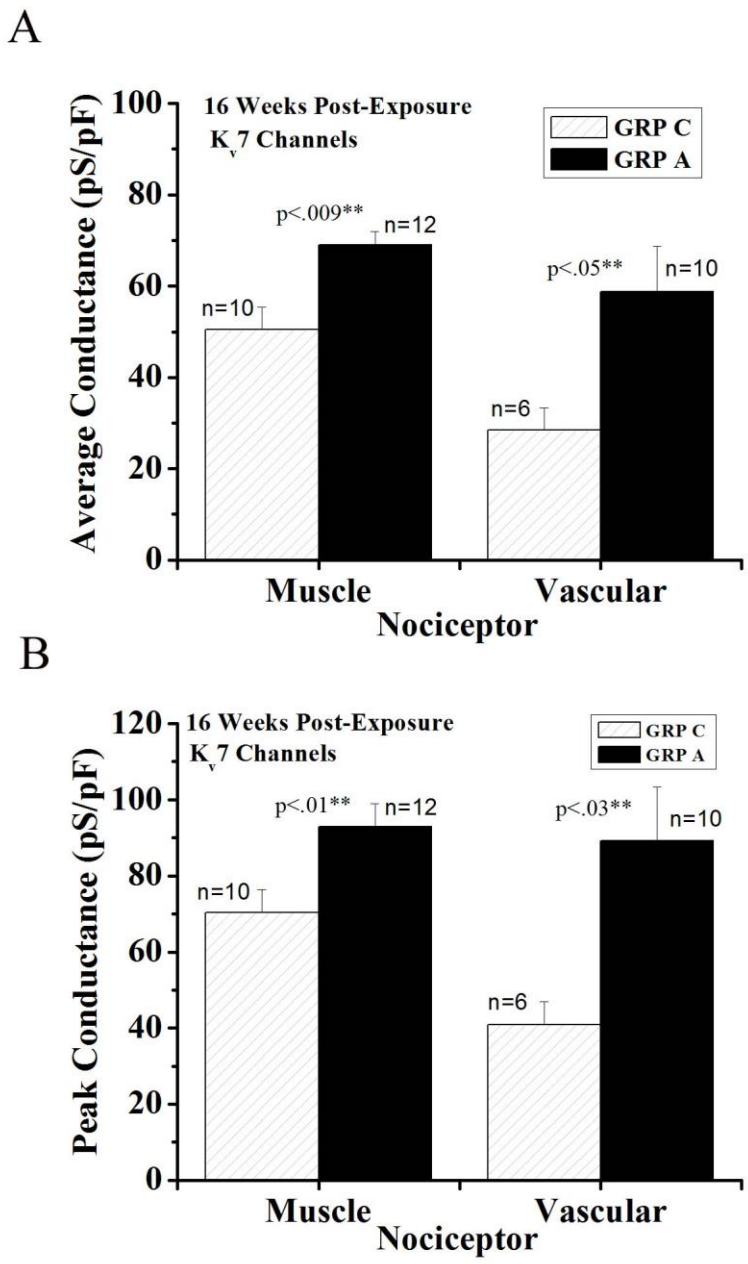

Figure 5. The Average and Peak Conductance of K_v7 ion Channels at 16 Weeks Post-Exposure. A) The average conductance was significantly increased in muscle nociceptors. Vascular nociceptor conductance was unchanged. **B)** The peak conductance was also significantly increased in muscle nociceptors at 16WP. Again, vascular nociceptor K_v7 peak conductance was unchanged. The average conductance was computed from averaged tail current amplitudes that were evoked following a series of stepped repolarizations (-30 to -90 mV, 10 mV steps) from -20 mV. GRP A: DEET, permethrin, chlorpyrifos, PB; GRP C: ETOH, ETOH, corn oil, water. Thirteen rats contributed to these experiments (n=7 GRP C; n=6 GRP A).

Cellular Studies

Maladapted physiology of $Na_v1.9$ and K_v7 ion channels predict changes in nociceptor excitability that could underlie GWI pain. Accordingly, we examined both the general membrane excitability and specific changes in excitability to neurotransmitter/paracrine messenger ACh in populations of vascular and muscle nociceptors. These studies were conducted in current clamp mode. As noted below, a portion of these studies were conducted at 35 °C.

Animals were prepared in the usual manner. Rats were exposed to either 4 GWI chemicals for 4 weeks (GRP A: DEET, permethrin, chlorpyrifos and PB) or 3 GWI chemicals for 4 weeks (GRP PB: DEET, permethrin, chlorpyrifos). Control rats received only vehicle (ethanol, ethanol, corn oil, water) exposures. Rats were euthanized 16 weeks following the end of the exposure period, and cells were harvested for electrophysiological experiments.

As presented in figure 6, the patterns of ambulation and resting deficits were similar, but not identical, to above studies (figures 1 and 2). Movement distance and average rate were substantially impaired at 16WP. Also similar to figure 1 and 2, the decline in rate was relatively delayed. When PB was not included in the exposure set, both ambulation measures were significantly improved relative to rats exposed to all 4 agents, and paradoxical increases in the rate of movement occurred in the PB groups. Rest time scores were elevated at 16WP, but not influenced by the absence of PB (see also figure 1). Some rescue of rest score were realized in figure 1.

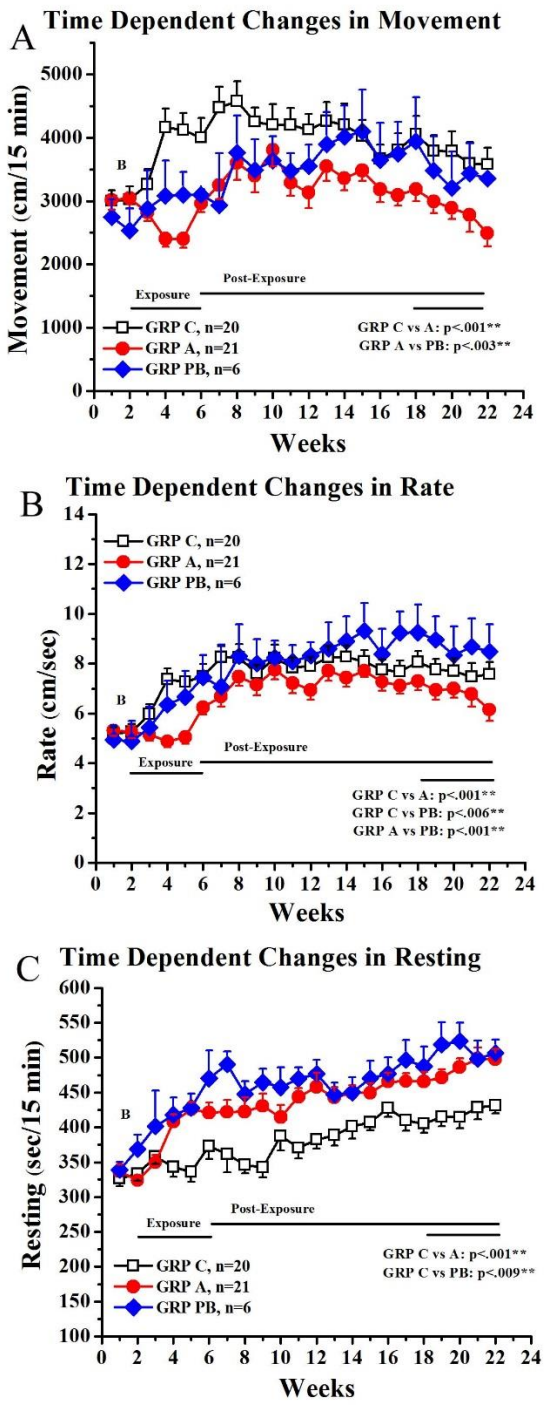

Figure 6. Exposure to 4 GW Chemicals Produced Persistent Pain-Like Behaviors at 16 Weeks Post-Exposure. **A)** Exposure to all 4 GW agents (GRP A) significantly decreased movement distance scores at 13-16WP. When PB was excluded, movement scores were significantly improved. **B)** Movement rate scores declined significantly at 13-16WP. In the absence of PB, rate scores improved significantly. **C)** Resting duration was significantly increased at 13-16WP. Despite the exclusion of PB, resting behavior remained elevated. B: baseline testing; GRP A (n=21): DEET, chlorpyrifos, PB, permethrin; GRP PB (n=6): DEET, chlorpyrifos, permethrin; GRP C (n=20): ethanol, corn oil, ethanol, water. Rats at 16WP were used in excitability experiments. **significantly different by ANOVA.

Following cell characterization and series resistance compensation, deep tissue muscle and vascular nociceptors were brought into current clamp mode in order to permit the assessment of action potentials. Following a two minute observation period in which cells were examined for spontaneous activity, current injection studies were initiated. Stepped, square wave injections ranged from 100 to 1200 pA (100 pA steps, ascending; 250 msec duration; 1 sec interstep interval). The threshold and total number of action potentials evoked were determined off-line. Cells whose resting membrane potential fell out of a range of -65 to -55 mV were excluded from current injection studies. Current injection tests were conducted at ambient temperature (20 °C). After a 1 minute recovery period, the temperature of the superfused solution was increased to 35 °C and maintained for 2 minutes. At this point, a 35 °C heated solution containing the highly specific muscarinic agonist, Oxotremorine-M, was applied to the cell by close superfusion (OXO; 10 μM; 1 minute).

Current inject studies revealed heightened membrane excitability in vascular nociceptors harvested from GRP A rats (figure 7A and B). This took the form of both decreased threshold and increased AP (action potential) discharge ($p < .01$ and .05, respectively; Table 1). In contrast, membrane excitability of muscle nociceptors was unchanged by exposure to GW chemicals. No spontaneous activity was observed in either class of neurons in either experimental group.

The application of a muscarinic agonist, OXO, depolarized muscle and vascular nociceptors. In muscle nociceptors, these depolarizations were often accompanied by powerful action potential bursts (figure 7C; Table 2). The pattern of depolarization was complex and appeared to be composed of multiple components. Muscle nociceptors exhibited large auto-

reversing depolarizations, with bursting AP discharges (6/7 exposed and 5/8 control cases). An additional 10 cases did not exhibit the auto-reversing current (7 exposed; 3 controls). All 25 muscle nociceptors exhibited a persistent depolarization that outlasted the OXO application by more than 1 minute (Table 2).

While all vascular nociceptors exhibited the persistent depolarization following OXO application (19/19 cases; figure 7D), only one of these nociceptors expressed an auto-reversing early depolarization with bursts of APs during the application (Table 2). Three additional cases also emitted burst discharges, but these cells did not survive the OXO challenge, and therefore the data could not be scored. In GRP C, the persistent OXO-induced depolarization of vascular nociceptors were significantly greater than those of muscle nociceptors (15.1 vs 9.8 mV; $p<.007$; Table 2). This difference was diminished following exposure to GW agents, as the persistent depolarization was significantly decreased at 16WP in the vascular group (Table 2).

Table 1

Membrane Excitability								
			RMP		**Threshold**		**Total APs**	
			mV		**pA**			
Vehicle Control Rats								
	Muscle		60.1 ± 1.0	16	387 ± 46	16	10.4 ± 1.1	16
	Vascular		60.0 ± 1.4	8	387 ± 55	8	10.3 ± 1.3	8
GWIC Exposed Rats								
	Muscle		59.8 ± 1.4	10	410 ± 66	10	14.2 ± 3.5	10
	Vascular		61.7 ± 0.8	14	285 ± 66*	14	16.4 ± 1.7*	14

RMP: Resting Membrane Potential
AP: Action Potential
GWIC: Gulf War Illness Chemicals
* significantly different from vehicle control

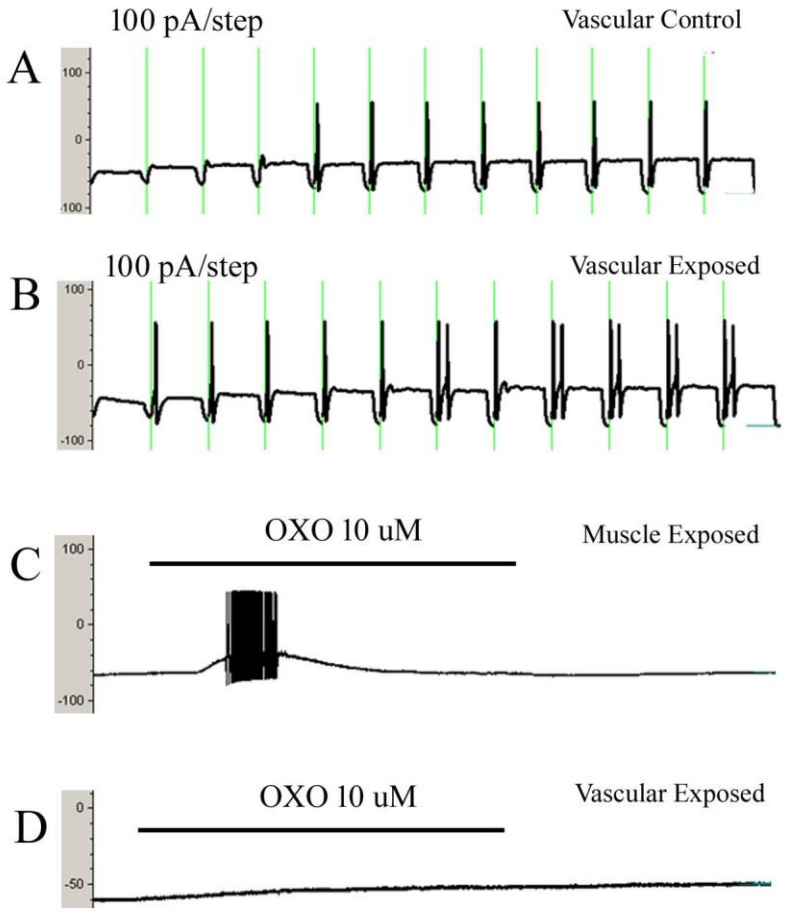

Figure 7. Deep Tissue Nociceptor Excitability Testing. A) Representative current-injection excitability testing of a vascular nociceptor from a control (GRP C) rat. **B)** Representative current-injection excitability testing of a vascular nociceptor from an exposed (GRP A) rat. **C)** Representative action potential burst from a muscle nociceptor from an exposed rat (GRP A). **D)** Representative slow depolarization of a vascular nociceptor from an exposed rat (GRP A). Some muscle nociceptors only exhibited this slow depolarization response to OXO application. Bold lines indicate the application of OXO-M (1 min).

Table 2

Muscarinic Reactivity																	
			RMP		**Depolarization**		**Net Reversed**		**Total APs**		**Burst Duration**		**Frequency**		**Persistent Depolarization**	
			mV		**mV**		mV				**sec**		**APs/sec**		**mV**	
Vehicle Control Rats																
	Muscle		60.9 ± 1.2[1]	11	20.8 ± 0.8	8	14.6 ± 1.0[2]	8	117.2 ± 26.3	5	12.3 ± 1.3	5	9.9 ± 2.5	5	9.8 ± 1.1[3]	11
	Vascular		62.0 ± 1.9[1]	5	9.4 ± 0.6	5	NA	5	0	5	NA	5	NA	5	15.1 ± 0.44[3]	5
GWIC Exposed Rats																
	Muscle		62.4 ± 1.0[1]	14	24.0 ± 0.9*	7	19 ± 1.2*	7	86.2 ± 28	6	17.8 ± 3.1	5	5.2 ± 1.6	5	11.3 ± 1.1[3]	14
	Vascular		62.7 ± 1.9[1]	8	7.6 0.9	7	NA	7	29	1	9.03	1	3.2 ± 12.6	1	12.3 ± 0.9[3]*	8
Muscarinic Reactivity Following HC-03001																
			RMP		**Depolarization**		**Net Reversed**		**Total APs**		**Burst Duration**		**Frequency**		**Persistent Depolarization**	
			mV		**mV**		mV				**sec**		**APs/sec**		**mV**	
Vehicle Control Rats																
HC Treated	**Muscle**		62.6 ± 2.9	7	19.5 ± 1.2	7	5.6 ± 3.2**	7	33.2 ± 8.5**	6	18.7 ± 2.3**	5	2.3 ± 0.5**	5	8.8 ± 1.1	7
GWIC Exposed Rats																
HC Treated	**Muscle**		60.6 ± 3.6	8	14.7 ± 1.0***Δ	8	2.9 ± 1.4***	8	81.3 ± 36.4	3	18.0 ± 4.7	3	5.0 ± 2.1	3	13.0 ± 1.9	8

1. Includes only cases with RMP between -55 and -65 mV
2. Peak depolarization minus minimum post-OXO RMP
3. Includes cases without auto-reversing depolarization

RMP: Resting Membrane Potential
AP: Action Potential
GWIC: Gulf War Illness Chemicals

* significantly different from vehicle control
** significantly different from vehicle control
*** significantly different from GWIC exposed
Δ significantly different from HC Vehicle control

Exposure to the 4 agent exposure protocol led to a significant increase in muscle nociceptor excitability to muscarinic agonists. Following application of the mAChR (muscarinic acetylcholine receptor) agonist OXO, the peak auto-reversing depolarization (that accompanied bursting; figure 8A), as well as the total reversed depolarization, were significantly greater in muscle nociceptors harvested from GRP A vs those from GRP C rats ($p<.03$ and $p<.02$; figure 8 and Table 3). Action potential bursting properties (number of evoked APs, burst duration ($p<.14$), average burst frequency) did not differ for muscle nociceptors (GRP A vs GRP C; Table 2). In contrast, GRP PB muscle nociceptors, harvested from rats exposed only to DEET, permethrin and chlorpyrifos, did not manifest greater peak and reversed depolarization; moreover, in the absence of PB, the peak and reversed depolarizations were significantly less than nociceptors from GRP A rats (figure 8A and 8B). Therefore, the mAChR instigated depolarization covaried with the presence and absence of PB in the exposure set, and appeared to be critically linked to the maintenance of pain behaviors in our rat model.

Because of the importance of the auto-reversing depolarization to mAChR-induced depolarization and nociceptor AP discharge, we examined the molecular basis of the auto-reversing depolarization in muscle nociceptors harvested from both exposed and control rats. We had previously shown, in non-exposed (naïve) rats, that the depolarization associated with OXO presentation was mediated, in part, by ion channel TRPA1 (Cooper et al., 2016). We have now shown that the OXO-induced depolarization was enhanced 16 weeks post-exposure to the four GWI chemicals. To determine whether this enhancement was due to a greater contribution of TRPA1 in exposed rats, we assessed the contribution of TRPA1 to the OXO-induced

depolarization in muscle nociceptors harvested from GRP A and GRP C rats. Studies were conducted 16 weeks after GW chemical exposures had ceased.

After preliminary cell characterizations, rat muscle nociceptors were exposed to the specific TRPA1 inhibitor (HC-030031; 3 min, 10 μM; 1 min at 20 °C and 2 minutes at 35 °C). Following pretreatment with HC-030031, we assessed depolarization and action potential discharge to OXO (1 min, close superfusion; 10 μM; 35 °C). As illustrated in figure 8, HC-030031 significantly decreased OXO-induced auto-reversing depolarization in muscle nociceptors from GWI chemical exposed rats ($p<.001$), but not in control rats (figure 8A; Table 2). Both the peak magnitude of the depolarization and the net reversing depolarization (peak depolarization minus minimum depolarization post-OXO) were significantly less in GRP A nociceptors treated with HC-030031 ($p<.01$). The net auto-reversed depolarization was nearly eliminated in exposed rat nociceptors ($p<.001$; figure 8B; Table 2).

In the presence of the TRPA1 inhibitor, only 3 of 8 exposed cases manifested AP burst discharges (vs 6/7 in GRP A, without HC-030031). These were too few cases to analyze; however, 6 of 7 control cases still exhibited AP bursting in the presence of the TRPA1 inhibitor. One GRP C case emitted a single AP and therefore did not have a frequency or burst duration score (Table 2). In these 6 cases, HC-030031 reduced OXO-initiated AP burst number and average burst frequency; burst duration was increased relative to cases not pre-treated with HC-030031 ($p<.01$, $p<.02$ and $p<.04$ respectively; Table 2). Therefore, a TRPA1 inhibitor reduced key AP burst discharge properties of muscle nociceptors, and differentially decreased the auto-reversing depolarization that produced these discharges in GWI exposed rats.

It is not clear whether the enhancement of muscle nociceptor depolarization was solely due to TRPA1 augmentation in GWI exposed rats, or whether the increase reflected greater coupling of TRPA1 to muscarinic receptors. Ultimately, both could be involved, and could have important implications for treatment of GWI pain. We used the TRPA1 specific agonist, cinnamaldehyde, to separate the contribution of the TRPA1 component from other factors contributing to muscarinic activation of the channel.

Muscle nociceptors were isolated from rats exposed to GWI chemicals or their respective vehicles (16 weeks post-exposure). Following preliminary characterizations, cells were exposed, by close superfusion, to a saturating dose of cinnamaldehyde (100 μM). Application continued until peak currents were achieved, as evidenced by desensitization, or a 4 minute maximal application period was reached. Studies were conducted at room temperature.

Cinnamaldehyde evoked powerful currents in muscle nociceptors (13 of 15 GRP C and 13 of 15 GRP A). There were no significant differences between the peak currents evoked in muscle nociceptors derived from exposed or control rats (figure 8D). This data indicated that, while TRPA1 was a critical effector in the production of mAChR-induced nociceptor discharge to OXO, it was the muscarinic linkage to TRPA1 that was fundamentally responsible for the enhanced TRPA1 depolarization.

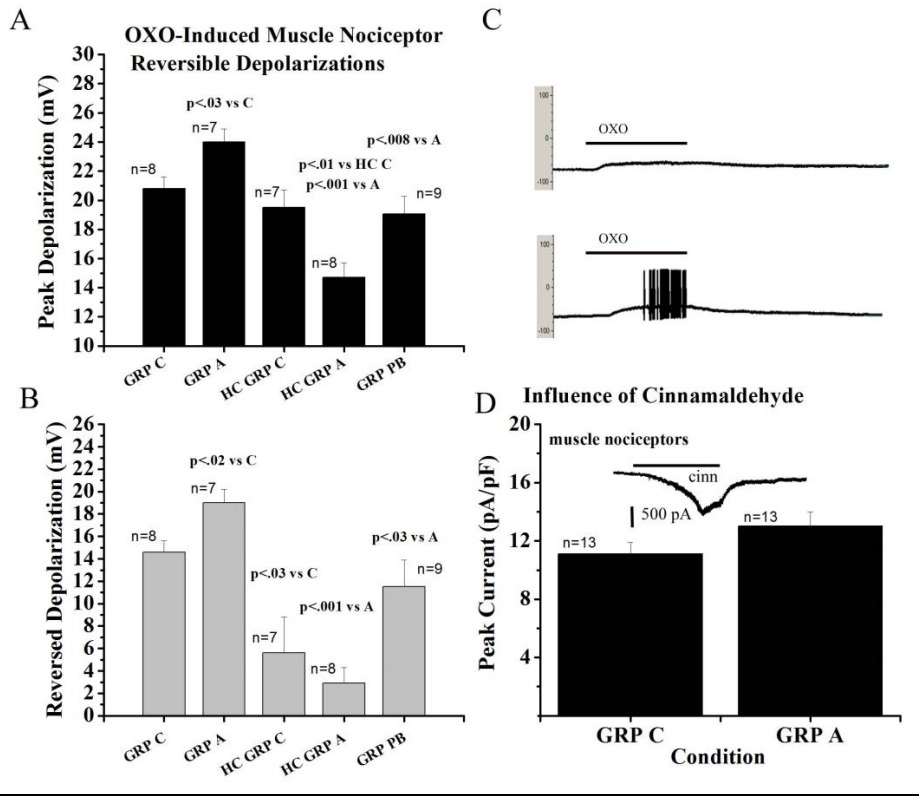

Figure 8. **TRPA1 Mediates Enhanced Reversible Depolarizations in Muscle Nociceptors. A)** The peak depolarization was greater in GW agent exposed nociceptors. The specific TRPA1 antagonist (HC-030031) inhibited the peak of the auto-reversing depolarization in GWI agent exposed muscle nociceptors, but not vehicle exposed. When PB was excluded, the effect of exposure was significantly less. **B)** The net auto-reversing depolarization was inhibited in both agent exposed and vehicle exposed neurons. When PB was excluded, the effect of exposure to GW agents was significantly less. **C)** Representative traces of HC-030031 pretreated OXO depolarization, with and without associated AP bursting. Note that reversing took place after OXO presentation ended, rather than during the OXO application (see figure 7). **D)** The TRPA1 agonist cinnamaldehyde (100 μM) evoked similar amplitude currents in GW exposed (GRP A) and vehicle exposed (GRP C) muscle nociceptors. Insert presents a representative response to cinnamaldehyde in a GRP A muscle nociceptor. HC: HC-030031; GRP A: permethrin, chlorpyrifos, PB, DEET; GRP PB: permethrin, chlorpyrifos, DEET GRP C: ethanol, corn oil, water, ethanol. OXO: Oxotremorine-M, 10 μM, 1 min at 35° C.; cinn: cinnamaldehyde 100 μM.

Discussion

Studies were undertaken to examine the impact of a DEET augmented, 4 agent, 4 week exposure protocol (DEET, permethrin, chlorpyrifos and PB) on ion channel maladaptations that were previously associated with an 8 week exposure to 3 GW agents ($Na_v1.9$: Nutter and Cooper, 2014; K_v7: Nutter et al., 2015). In the course of these investigations, we took advantage of the critical relationship between PB exposure and the development of pain-like behavioral deficits (Flunker et al., 2017) in order to test for potential disassociations between behavioral signs and deep tissue nociceptor physiology. We now report that maladaptations in $Na_v1.9$ physiology paralleled behavior shifts in the presence and absence of PB exposure. In contrast, K_v7 activity patterns diverged from both behavioral outcomes and the critical influence of PB on these measures at 16WP (see also Flunker et al., 2017). Although the importance of certain ion channels closely linked to muscarinic activity were diminished (K_v7), other downstream effectors of mAChR activation came more clearly into focus. Enhanced muscarinic coupling to TRPA1 was critical to a depolarization-induced burst discharge mechanism in muscle nociceptors (MDBD; Nutter et al., 2015; Cooper et al., 2016). Despite the absence of a critical relationship between K_v7 activity and the persistence of pain-like behaviors, Retigabine treatment produced transitory relief from established chronic pain signs. While it was confirmed that PB played a fundamental role in the development of ambulatory deficits, there was no evidence that chronic PB exposure offered any protection against rest time deficits (Flunker et al., 2017).

Pyridostigmine and Behavior

Our laboratory previously demonstrated, that a high (50%), but not a low (25%), concentration of DEET, in the presence of permethrin, chlorpyrifos and PB, greatly potentiated the onset and duration of pain-like behaviors in our rat model of GWI pain (Flunker et al., 2017). This finding mirrored patterns of pain symptom reports by veterans returning from the 1991 Gulf War. Specifically, there was a strong association between the development of pain symptoms and the use of 75% DEET formulations. In contrast, soldiers that applied 25% DEET formulations were significantly less likely to develop pain symptoms (GWI syndrome III; Haley and Kurt, 1997).

The present report confirms, in three independent experiments, that the DEET augmented exposure protocol produces relatively rapid development of, and substantial persistence of, pain-like signs in rats, and that the expression of these pain signs (ambulation) were critically dependent upon the presence of PB. In our original model, we chose the combination of PB, chlorpyrifos and permethrin, because they all had powerful influences on nociceptor function. Permethrin slows Na_v inactivation, and thereby permits more Ca^{++} to enter neurons during action potential discharge (Narahashi et al., 1998; Soderlund et al., 2002; Jiang et al., 2013). Chlorpyrifos and PB, non-competitive and competitive anticholinesterases respectively, increase and prolong the action of ACh at their nociceptive nicotinic and muscarinic receptors (K_v7.3; Nutter et al., 2013; Nutter and Cooper, 2014). It was hypothesized that prolonged influences of pyrethroids and organophosphates on affected surface ion channels (Na_v) and G-protein coupled receptors (mAChR) could lead to chronic functional effects on nociceptor channel activity and/or cellular excitability that would result in chronic pain. Although DEET substantially accelerated and

magnified the effects we recorded, we were unable to identify any direct actions of DEET on nociceptor $Na_v1.8$, $Na_v1.9$, K_{DR} or K_v7 that might contribute to persistent maladaptations to nociceptor ion channels (Flunker et al., 2017). There was substantial evidence in existing literature that DEET modifies hepatic catabolism of chlorpyrifos in a manner that greatly intensifies its anticholinesterase and extra-anticholinesterase activities (Abou-Donia et al., 1996; Chaney et al., 2000; Abu-Qare and Abuo-Donia, 2008). This pathway is the most likely avenue for DEET influences on pain-like behaviors (see Flunker et al., 2017).

Although DEET has a major impact on the timing and degree of pain-like signs, it is not a necessary component for the manifestation of pain-like behaviors. In the absence of exposure to the anticholinesterases, chlorpyrifos or PB, pain-like ambulation behaviors do not develop (Flunker et al., 2017). The relationship of PB to the development of pain behaviors was especially interesting due to its complex influence on, not only the appearance, but also the pattern of pain-like signs. The pattern of pain symptoms in GW veterans is highly diverse and includes constellations of joint, muscle, back, abdominal and headache pain components (Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). There is an increasing consensus that insecticides and PB played a role in the development of GWI, but the specific relationship between chemical exposure patterns and specific symptom manifestations is unknown (Binns et al., 2008; RAC 2014; White et al., 2016). In our model, exclusion of chlorpyrifos completely abrogated the development of pain-behaviors, but exclusion of PB abrogated only ambulation related deficits while aggravating resting deficits (Flunker et al., 2017). This finding was particularly interesting because the soldiers of ODS ingested PB daily as a prophylactic against nerve agent anticholinesterases. Our data suggested that PB did, in fact, protect rats from

some of the deleterious consequences of a powerful non-competitive anticholinesterase, like chlorpyrifos.

This report confirms that the DEET augmented protocol produces both rapidly developing and delayed ambulatory deficits (movement distance and rate respectively) and that these signs were critically dependent upon PB exposure. Absent PB, ambulation scores were 'normal' and significantly improved over full exposure cases (figures 1 and 6; see also Flunker et al., 2017). However, the relationship between PB and the development of resting time deficits was less clear than we observed previously. In our last report, we did not find evidence of resting deficits in rats exposed to the 4 agent protocol (Flunker et al., 2017), yet strong and persistent resting deficits *were* manifested in 2 of 3 independent behavioral studies above. When contrasted with groups in which PB was excluded during the exposure, it was clear that PB offered no 'protection' against resting deficits. In the absence of PB, resting scores remained elevated, relative to controls, in 3 of 4 test periods, but they never exceeded values in rats exposed to all 4 agents (figures 1 and 6). Therefore, we can confirm that there was a distinct relationship between PB exposure and the manifestation of ambulatory and resting deficits, but the influence of PB on resting behaviors was never 'protective'. It is best described as irrelevant. In the final analysis, there is significant variability in the resting measure that precludes simple explication of factors that modulated it from experiment to experiment. This study confirms that exposure to PB was critical to the expression of ambulatory pain-like signs, but its relationship to the resting behavior measure is unreliable and not a window into symptom diversity of GWI pain.

Pyridostigmine and Pathophysiology

In an animal model of chronic pain, changes in the physiology of certain ion channels could reflect the fundamental pathophysiological events that lead to the appearance and maintenance of chronic pain behaviors. This would clearly guide the development of effective treatments for such pain. Alternately, changes in molecular physiology could be instigated by a physical or chemical insult and yet not be critical to the expression of any measured behavior.

We previously identified, the voltage dependent channel, K_v7 as a channel whose function was altered by exposure to 3 or 4 GW chemicals (Nutter and Cooper, 2014; Flunker et al., 2017). Using the decisive contribution of PB to the development of ambulatory deficits, we subsequently disassociated voltage dependent K_v7 ion channel activity shifts from pain-like behaviors in our rat model of GWI. The anti-nociceptive ion channel, K_v7, was downregulated in parallel with the development of ambulation deficits at 12WP, but its measured activity remained downregulated when ambulation scores were rescued by exclusion of PB from the exposure protocol (Flunker et al., 2017). We have further documented this divergence, in the present study, by demonstrating that K_v7 function also diverged from ambulation deficits at 16WP.

Although K_v7 does not play a critical role in the pathophysiology of GWI pain, treatment of symptomatic rats with the K_v7 opener, Retigabine, resulted in significant improvements to pain-like behaviors. Given the role of K_v7 in nociceptor excitability, such an outcome might be expected in the absence of any specific dependency between GW chemical induced K_v7 channel maladaptations and the development of ambulation deficits. As a voltage sensitive ion channel that activates at hyperpolarized potentials, K_v7 serves as an active break against depolarizing Na^+, and Ca^{++} currents that promote action potential discharge (e.g., $Na_v1.8$, $Na_v1.9$, Ca_v3). Retigabine could lessen pain symptoms independently via its capacity to oppose depolarizing currents and

spontaneous activity (Marrion, 1997; Brown and Passmore, 2009; Gunthorpe et al., 2012; Du et al., 2017; Wu et al., 2017). In fact, certain depolarizing currents ($Na_v1.9$ and TRPA1) were shown to be potentiated in GW agent exposed rat nociceptors. Activation of K_v7 would be particularly effective against relatively slowly activating currents; including those mediated by $Na_v1.9$ or TRPA1. Although Retigabine produced only transient improvement in pain behavior outcomes in our rat model, these results predict that K_v7 openers have promise as palliatives in the treatment of GWI pain symptoms. Retigabine, and its close structural analog Flupirtine, have demonstrated clinical utility for relief of chronic inflammatory pain (Scheef, 1987; McMahon et al., 1987; Luben et al., 1994), myofascial and musculoskeletal pain (Worz , 1991; Worz et al., 1996; Ueberall et al., 2011; Uberall et al., 2013; Worz, 2014) small fiber neuropathy (Mishra et al., 2013) and headache (Podymova and Danilov, 2011; see also Devulder, 2010). We are unaware of any instances in which K_v7 activators have been tested on GW veterans.

While some molecular adaptations diverged from the behavioral influence of PB (K_v7), others tracked closely with its presence. The activity of a pro-nociceptive ion channel, $Na_v1.9$, was shown to be increased 8 weeks following an exposure to 3 GW chemicals (permethrin, chlorpyrifos and PB; Nutter and Cooper, 2014). We have now presented evidence that the DEET augmented protocol is also associated with increased activity of deep nociceptor $Na_v1.9$, and that this maladaptation persisted to 16WP (vascular nociceptors). Importantly, when PB was excluded from the exposure protocol, and ambulation scores were rescued, shifts in $Na_v1.9$ activity (voltage dependence and average current) were also significantly reversed.

$Na_v1.9$ is widely expressed in small and medium sized DRG neurons that comprise the classic A-delta and C-fiber pain system families (Dib-Hajj et al., 2002; Fang et al., 2002; Coste et

al., 2004; Jiang and Cooper, 2006). Nociceptor $Na_v1.9$ is upregulated in models of inflammatory pain (Baker, 2005; Priest et al., 2005; Maingret et al., 2008; Lolignier and Amsalem, 2011) and peripheral neuropathy (Huang et al., 2014; Leipold et al., 2015; Okuda et al., 2016; Han et al., 2017). Unlike other Na_v ion channels, $Na_v1.9$ does not participate in action potential formation; nevertheless, it makes significant contributions to nociceptor excitability during slow depolarizations. Unlike other Na_v, which rapidly inactivate, $Na_v1.9$ exhibits ultraslow inactivation kinetics (Dib-Hajj et al., 2002; Rogers et al., 2006; Rush et al., 2007; Liu and Wood, 2011). Due to the practical absence of inactivation, activation of $Na_v1.9$ results in a self-sustaining depolarization plateau that promotes burst discharges (Copel et al., 2009; Herzog et al., 2001; Maingret et al., 2008). In vascular nociceptors, where voltage and amplitude of $Na_v1.9$ underwent significant gain of function at 16WP, this enhanced bursting was evident during current step injection experiments that produced depolarizations sufficient to activate $Na_v1.9$. Inflammatory agents that are expressed and released by vascular and closely associated tissues act on nociceptor membrane ion channel receptors (e.g., ACh, serotonin, bradykinin, thrombin, ATP, prostacyclin, NO, ACh; Wessler, and. Kirkpatrick , 2008; Wessler, and. Kirkpatrick , 2012; Stitham et al., 2011; Westscott and Segal, 2013; Couture et al, 2014; Danese et al., 2014; Craige et al., 2015; Cirino et al., 2017). Any of these agents could serve as sources of *in vivo* step depolarization capable of recruiting an amplified $Na_v1.9$ to produce vascular nociceptor burst discharge and pain.

Maladaptations to nociceptor $Na_v1.9$ could contribute to a state of chronic oxidative stress and neurogenic inflammation that have been recently documented in animal models of GWI (Alhasson et al., 2017; Emmerich et al., 2017; Shetty et al., 2017; Zachirova et al., 2017). Both the type 5 (muscle) and type 8 (vascular) nociceptors, used in these experiments, express the vasoactive neuropeptides SP and CGRP (Petruska et al., 2002; Jiang et al., 2006; Rau et al., 2007;

Rau et al., 2014). These paracrine agents are released during burst discharges (Holzer, 1998; Iyengar et al., 2017). Subsequent to their release from nociceptor endings, they bind to receptors on vascular endothelial cells to induce the release of vasodilator and TRPA1 agonist, NO (nitric oxide; Westscott and Segal, 2013). If NO production becomes excessive (nitrosative stress), a number of NO reactive oxygen species (NO-ROS) can result (Badran et al., 2015; Craige et al., 2015). Lipid peroxidation products of NO-ROS include the powerful TRPA1 activator 9-nitro-oleic acid (9NOA; Taylor-Clark et al., 2009; see also Taylor-Clark et al., 2008). 9NOA is a highly reactive electrophile that produces neuronal depolarization, activation and pain via TRPA1 (Taylor-Clark et al., 2009). Nociceptor SP and CGRP also interact with resident mast cells to release a number of pro-inflammatory substances as part of the classic neurogenic inflammation cascade (Geppetti and Holzer, 1996; Geppetti et al., 2005). There are multiple reports of vascular dysregulation in GW veterans that could reflect these conditions (Haley et al., 2009; Liu et al., 2011; Li et al., 2011; Haley et al., 2013).

In 2015, we first reported that muscle nociceptors exhibited a unique ACh instigated burst discharge that was triggered by muscarinic receptor activation (Nutter et al., 2015). MDBD (muscarinic dependent burst discharge) was initiated by activation of mAChR, modulated by K_v7, and subsequently shown to have a TRPA1 component (Cooper et al., 2016). Presently, we examined whether MDBD and its components were modified in rat nociceptors following a 4 week exposure to the DEET augmented protocol. We were able to verify the muscarinic sensitivity of muscle nociceptor TRPA1, and demonstrated a significant enhancement of the TRPA1 mediated depolarization component in rats exposed to 4 GW agents. Importantly, these same outcomes (increased peak and net depolarization), were absent from muscle nociceptors harvested from rats whose exposure sets did not include PB and that did not develop ambulatory deficits. Despite the

clear and important contribution of TRPA1 to oxotremorine induced depolarization, subsequent experiments indicated that the mAChR links to TRPA1 were upregulated, rather than TRPA1 itself.

As the TRPA1 antagonist HC-030031 failed to fully block the muscarinic fast depolarization or diminish the late depolarization, it remains likely that other mAChR and non-mAChR sensitive currents were involved in MDBD. This could include K_v7, as a known mAChR linked channel, and more importantly $Na_v1.9$. There are no published reports documenting links between mAChR and $Na_v1.9$. Regardless, the large voltage shifts induced by oxotremorine (>20 mV) would recruit voltage sensitive $Na_v1.9$. The possible convergence of $Na_v1.9$ and TRPA1 in MDBD would have significant implications for the pathophysiology of GWI pain in general and muscle pain in particular. The subgroup of vascular nociceptors that also exhibited MDBD could provide a bridge between distinct nociceptor subtypes that would link a broad array of symptoms to a common mechanism founded upon muscarinic receptor dysfunction, oxidative stress and neurogenic inflammation.

Summary and Conclusions

DEET powerfully potentiated behavior changes that appeared and persisted following exposure to certain GW agents (permethrin, chlorpyrifos, PB). PB played a critical role in the emergence of pain-deficits, as the presence or absence of PB during the exposure period determined whether ambulatory signs of chronic pain would occur and persist. Certain molecular events ($Na_v1.9$ and TRPA1), and associated changes to nociceptor excitability, varied with

behavioral outcomes related to PB exposure. In some instances, molecular maladaptations were shown to be due to increased sensitivity to mAChR activation. While warfighters of ODS were potentially exposed to many neurotoxicants, exposure levels and patterns of exposure to anticholinesterases were generally unknown and variable. On the other hand, a large majority of veterans were taking PB 3 times daily, for weeks, as a prophylactic against nerve agent attack (Binns et al., 2008). In our model, it is clear that the impact of PB was extensive and pivotal to long term behavioral, cellular and molecular manifestations of GWI pain. The full effect of PB exposure is yet to be determined.

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