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14. ABSTRACT
Experiments were conducted in rats to determine the molecular basis of a widespread chronic pain that is frequently reported in veterans of the 1991 Gulf War. Building upon a previous behavioral model, we found that increasing the frequency of exposure to anticholinesterase components (pyridostigmine bromide, chlorpyrifos) could produce a pattern of behavior consistent with an emerging myalgia. Recordings from muscle and vascular pain sensing neurons, 12 weeks post-exposure to permethrin, pyridostigmine bromide and chlorpyrifos, revealed a diminished activity level in certain K_v ion channel proteins (K_v7, K_{DR}) that oppose neural excitability. Chronic exposure to Gulf war neurotoxicants also altered muscarinic receptor coupling to these K_{DR} and K_v7 channel proteins in a manner that reduced the capacity of neurons to resist excitation. Molecular maladaptations were prominent in muscle nociceptors.

15. SUBJECT TERMS
Pain, pesticides, pyridostigmine bromide, Kv channels, muscarinic, Gulf War Illness

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1. Introduction

Many veterans of the 1991 Persian Gulf War returned from that conflict with symptoms of chronic pain. Twice as many developed a widespread deep tissue pain following their return to the states (Kroenke et al., 1998). The type of symptoms that were reported were distinct from those associated with polytrauma, Complex Regional Pain Syndrome (CRPS) or Non-Battle Injury (NBI) pains that can develop from load bearing microinjuries. 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; RAC2014). Our laboratory initiated a series of investigations to determine whether a subset of the many pesticides and related GW chemicals that were used during the conflict could produce chronic pain behaviors in a rat model; and whether there were molecular correlates of this pain present in ion channel proteins expressed in families of deep tissue nociceptors. The identification of persistent molecular maladaptations could provide targets for treatment. In the progress report that follows, we present findings from examination of how pesticides and a nerve gas prophylactic affects the long term physiology of K_v channels in afferent neurons. K_v channels play a fundamental role in the control of neural excitability and axonal coding throughout the nervous system. Because the veterans report a variety of deep tissue pains, our studies focused on nociceptors innervating muscle and blood vessels.

2. **Keywords:** pain, pesticides, pyridostigmine bromide, K_v channels, Gulf War Illness

3. Overall Project Summary

Muscle and joint pain are common symptoms associated with GWI (Haley Syndrome 3; Haley et al., 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). 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; RAC2014). During the brief course of the Gulf War, veterans were potentially exposed to 67 insecticides and repellants containing 37 distinct active ingredients (DoD Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008). Our research program has examined whether certain GW (Gulf War) chemicals could produce a chronic deep tissue pain syndrome in a rat model. Simultaneous molecular and cellular studies determined whether components of the nervous system exhibited molecular and cellular maladaptations that could account for a delayed and persistent pain syndrome. This particular 2 year study focused on changes to certain K^+ channel proteins expressed in muscle and vascular nociceptors (pain sensing neurons).

Objectives:

1) Improve an animal model of GW chronic pain.

2) Characterize molecular changes that occur in nociceptors following exposure to pesticides and pyridostigmine bromide

3) Characterize vascular afferents

Results: Part 1

The first section of the report is a recapitulation of outcomes from year 1. Although it is a completely updated version of the year 1 report, it is mainly the same data that is presented in the attached publication (Nutter et al., 2015). The reader may prefer to read the summary below and then begin with Part 2 (p. 21). An attached manuscript (published) contains a portion of the year 2 studies. A second attached manuscript contains a portion of the year 2 investigations. It will be submitted in late January, 2016.

Summary: Part 1

Significant advances were made during year 1. The rat behavior model was modified by increasing the dosing rate (duty cycle) of anticholinesterases (chlorpyrifos and PB). This resulted in pain-like behavior patterns that emerged 12 weeks after exposure to GW chemicals ended. This finding was replicated in a second group of animals. Only some of this data was reported last year as some these studies were not completed in time for the year 1 report deadline. While this was very encouraging, only one of the two additional groups in year 2 exhibited these pain-like behaviors at the 12 week post exposure test.

Despite some continued weakness in the behavioral model, physiology tests supported the emergent pain finding. We had demonstrated previously that K_v7 channel proteins were perturbed by exposure to GW chemicals; however the altered physiology did not clearly support the presence of a chronic pain condition. With the new 'intensified' GW chemical exposure, shifts in K_v7 and K_{DR} physiology both supported the emergent pain syndrome at 12 weeks post exposure. Cellular studies indicated heightened nociceptor reactivity to ACh at muscarinic receptors. The physiology shifts were limited to muscle nociceptors at 12 weeks post chemical exposure; although vascular nociceptors were affected at the 8 week post period as well. This outcome pattern suggested that the enhanced anticholinesterase dosing regimen produced a delayed myalgia in rats.

Behavioral Studies

Task 5: To examine the development, time course and persistence of a myalgia in rats exposed to combinations of neurotoxicants (permethrin and chlorpyrifos) and pyridostigmine bromide

In a prior study we reported persistent changes in K_v7 channel protein physiology following chronic exposure to GW chemicals (permethrin, chlorpyrifos and PB); however, those alterations in K_v7 activity did not appear in parallel with measurable behavioral indices of pain (Nutter et al., 2013). In order to better assess the relevance of changes in K_v ion channel function to a chronic pain condition, we tested modifications to the exposure protocol with the hypothesis that intensifying the anticholinesterase component would produce a chronic pain condition. In the behavioral studies below, the duty cycle of PB application was increased from 50 to 100%, and the chlorpyrifos duty cycle was increased from 7 to 14%. All doses remained the same as those used previously (Nutter et al., 2013; Nutter and Cooper, 2014). We will refer to this as the 'intensified protocol'. We examined the influence of the intensified protocol, in separate groups of rats, for exposures lasting either 30 or 60 days.

Animals exposed to the intensified protocol for 30 days exhibited significant increases in resting for about 8 weeks after exposure (figure 1). Thereafter, rest measures returned to normal levels, but movement rate was paradoxically increased in weeks 9-12. No other pain measures were affected in the weeks following chemical exposure. Although other measures of pain were not altered, the 8 week increase in rest duration was a substantial improvement over our previous attempts to produce a chronic pain condition with GW chemicals (Nutter et al., 2013; Nutter and Cooper, 2014).

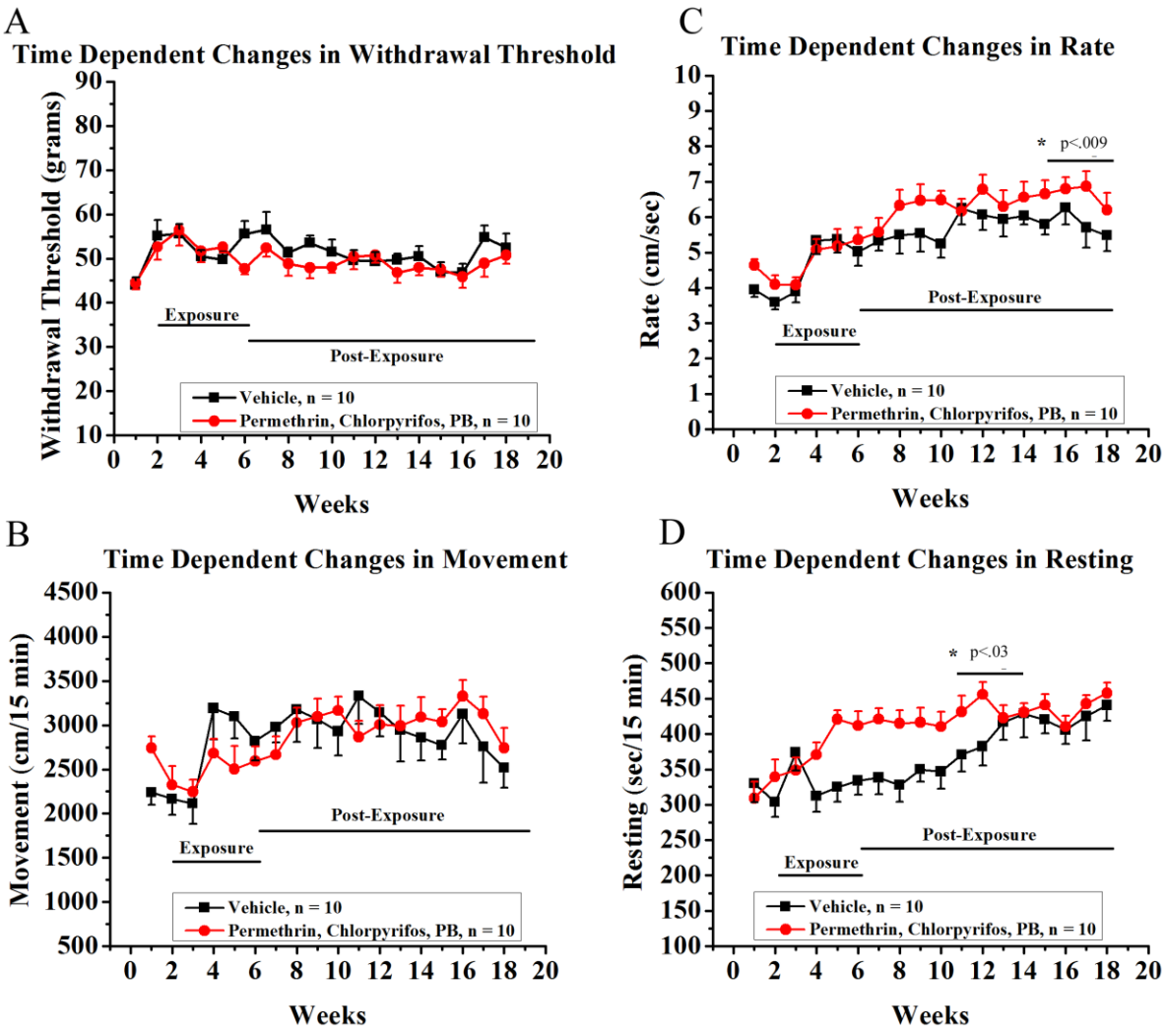

Figure 1. The Intensified 30 day Exposure Produced Some Lasting Behavioral Effects. A and B) Muscle pain pressure thresholds and distance moved were unchanged following the 30 day exposure to GW chemicals. **C and D)** Movement rate and resting were both increased during the post-exposure period. Resting was still significantly increased 5-8 week post-exposure ($F=4.70$; $p<.03$) but returned to normal levels thereafter. The increased resting was replaced by a paradoxical increase in movement rate at post 9-12 weeks ($F= 7.23$; $p<.009$). Statistical tests were not conducted on any measure 1-4 weeks post exposure.

The 60 day exposure group was divided into two independent subgroups that reflected their ultimate use in physiology studies. Group 8WP were animals that were sacrificed for physiology experiments 8 weeks post-exposure (figure 2). Group 12WP were rats sacrificed 12 weeks post-exposure (figure 3). Both groups exhibited similar behavioral effects of the intensified protocol through 8 weeks. Consistent with the presence of a myalgia/arthralgia, rest periods were significantly increased. However, the interpretation of this data, as reflecting a pain condition, was complicated by a significant, but paradoxical, increase in the rate of movement in group 8WP (figure 2). Similar trends were observed in the 30 day exposure group (figure 1). When behavioral studies were carried out to 12 weeks (Group 12WP), resting remained significantly elevated, but movement distance was now significantly decreased and movement rate had normalized (figure 3B and 3D). Although neither the 30 or 60 day exposures produced a change in the muscle pressure pain threshold, it is not clear whether a change in muscle pressure pain thresholds are exhibited by GW veterans (Cook et al., 2010). Animals in pain generally exhibit reduced movement and increased resting; a pattern consistent with myalgia and/or arthralgia due to GW chemical exposure. Our parallel molecular studies on these same animals examined whether the intensified treatment produced molecular adaptations to K_V channels consistent with a chronic pain condition at the 8 and 12 week delays.

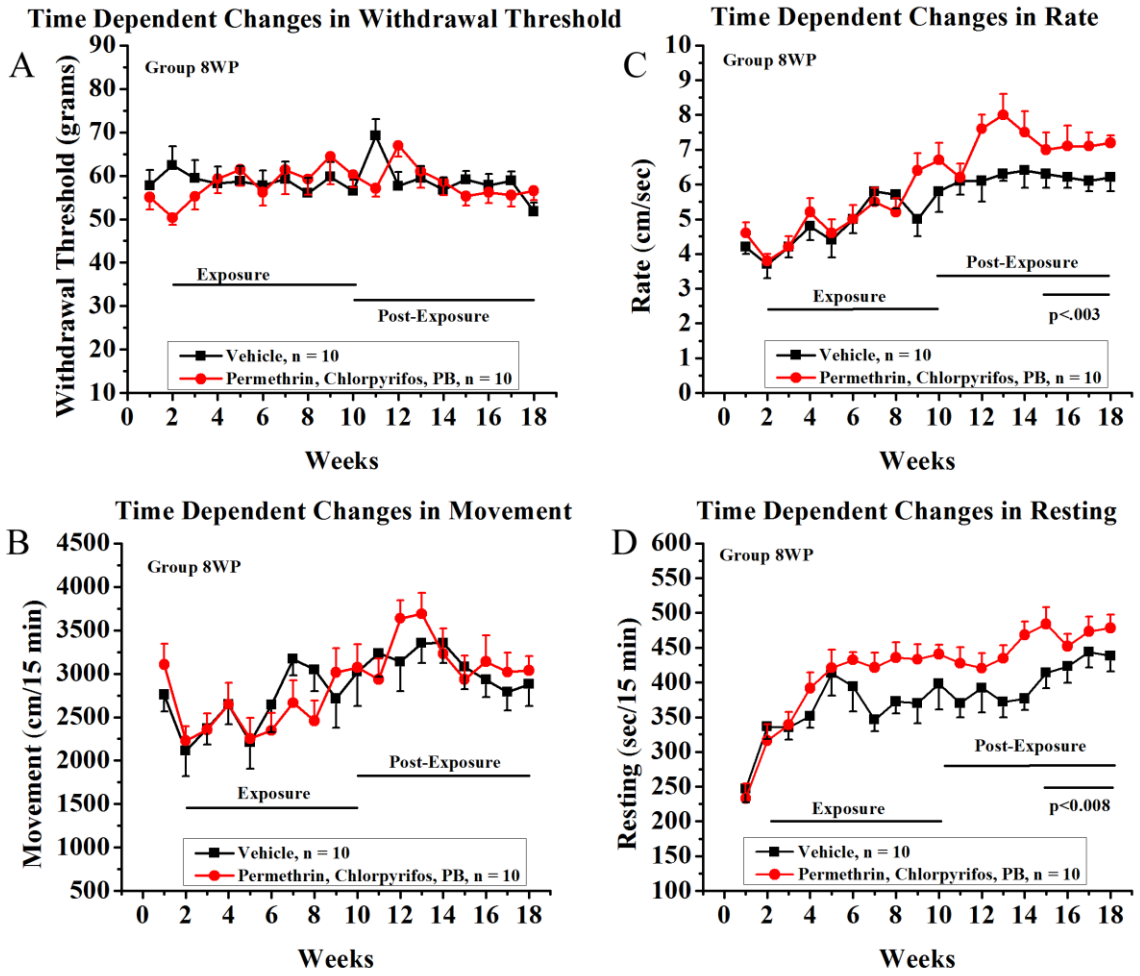

Figure 2. The Intensified Exposure Protocol Produced Some Signs of Lasting Pain Behaviors in the 8 Week Post Exposure Group. A and B) Muscle pain pressure thresholds and distance moved were unchanged following the 60 day exposure to GW chemicals. **C and D)** Movement rate and resting were both increased during the post exposure period. Resting was significantly increased 5-8 weeks post-exposure ($F=7.43$; $p<.008$). Movement rate was significantly increased at the same time delay ($F= 9.23$; $p<.003$). Statistical tests were not conducted on any measure 1-4 weeks post exposure.

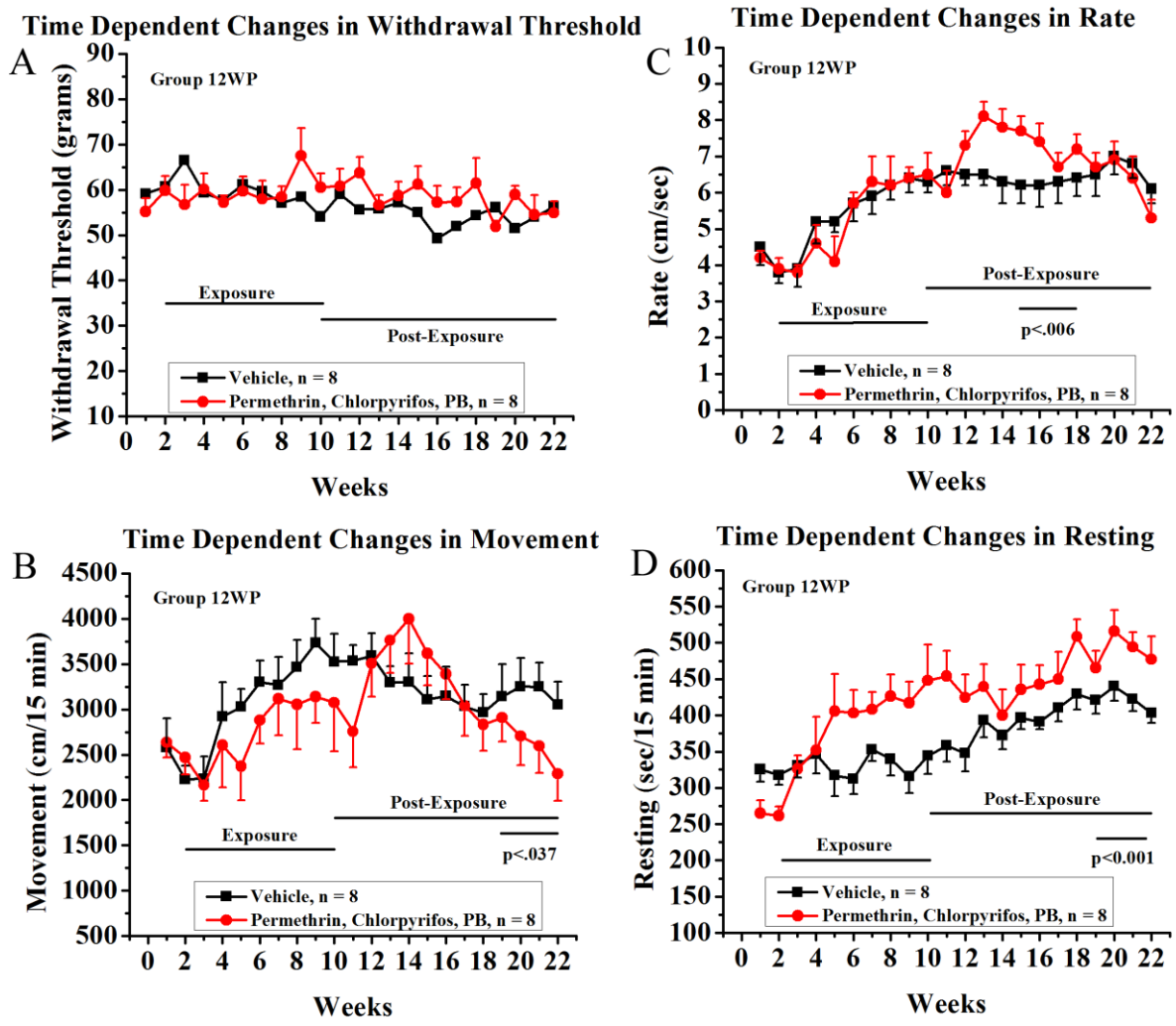

Figure 3. The Intensified Exposure Protocol Produced Consistent Signs of Lasting Pain Behaviors in the 12 Week post Exposure Group. A) Muscle pain pressure threshold was unchanged following the 60 day exposure to GW chemicals. **B)** Consistent with a delayed chronic pain, movement distance was significantly decreased at 9-12 weeks post exposure ($F=5.87$; $p<.04$). **C and D)** Movement rate and resting were both increased during the post-exposure period. Resting was significantly increased at 5-8 and 9-12 weeks post-exposure ($F=7.2$ and $F=10.2$; $p<.008$ and $p<.002$, respectively). Movement rate was significantly increased only at the 5-8 week test period ($F=8.09$; $p<.006$). Statistical tests were not conducted on any measure 1-4 weeks post exposure.

Molecular Studies

Task 2: We will determine whether neurotoxicants/PB produce lasting changes in K_{DR} function that could contribute to chronic and widespread pain

Task 2a: Assess K_{DR} Voltage Dependent Activation 8 Weeks After Treatment.

Task 2b: Assess K_{DR} Voltage Dependent Activation 12 Weeks After Treatment.

Task 2c: Assess K_{DR} Voltage Dependent Deactivation 8 Weeks After Treatment.

Task 2d: Assess K_{DR} Voltage Dependent Deactivation 12 Weeks After Treatment.

Task 2e: Assess K_{DR} Normalized Amplitude 8 Weeks After Treatment.

Task 2f: Assess K_{DR} Normalized Amplitude 12 Weeks After Treatment.

Task 2g: Determine the K_V component Protein that Contributes to Changes in K_{DR} Amplitude, Activation and Deactivation.

Molecular Studies on Chronically Exposed Rats

Eight and 12 weeks after chemical exposures ended, muscle and vascular nociceptors (type 5 and type 8, respectively) were identified in DRG harvested from rats exposed for 60 days to GW chemicals or their vehicles (Petruska et al., 2002; Rau et al., 2007; Cooper et al., 2014). We examined the voltage dependence and amplitude of the K_{DR} and the conductance of the K_v7 current component. We observed persistent changes in both K_v7 and the K_{DR} currents following the intensified exposure protocol. Some of these changes predicted increased neural excitability and pain.

Physiology of K_v7. Consistent with previous reports on K_v7 currents in chemically exposed rats (Nutter et al., 2013), both the normalized average and peak conductance of K_v7 currents were significantly increased 8 weeks following cessation of exposure (figures 4A-B). Both muscle and vascular nociceptors were affected by chemical exposures at the 8 week delay point. The influence on muscle nociceptors was more robust. All significant changes and strong trends ($p<.07$) indicated greater activity of K_v7 and potentially less excitability at 8 weeks post

exposure. These molecular shifts occurred while rats exhibited the unexpected combination of increased resting but faster movement during activity periods.

At 12 weeks post-exposure, K_v7 currents were now significantly decreased in muscle nociceptors, while K_v7 currents in vascular nociceptors had returned to normal levels (figure 4B,D). A decrease in the conductance of K_v7 is consistent with increased nociceptor excitability. The decrease in K_v7 currents corresponded to the time period when the combination of significantly greater resting and significantly decreased movement distance suggested a delayed myalgia and/or arthralgia.

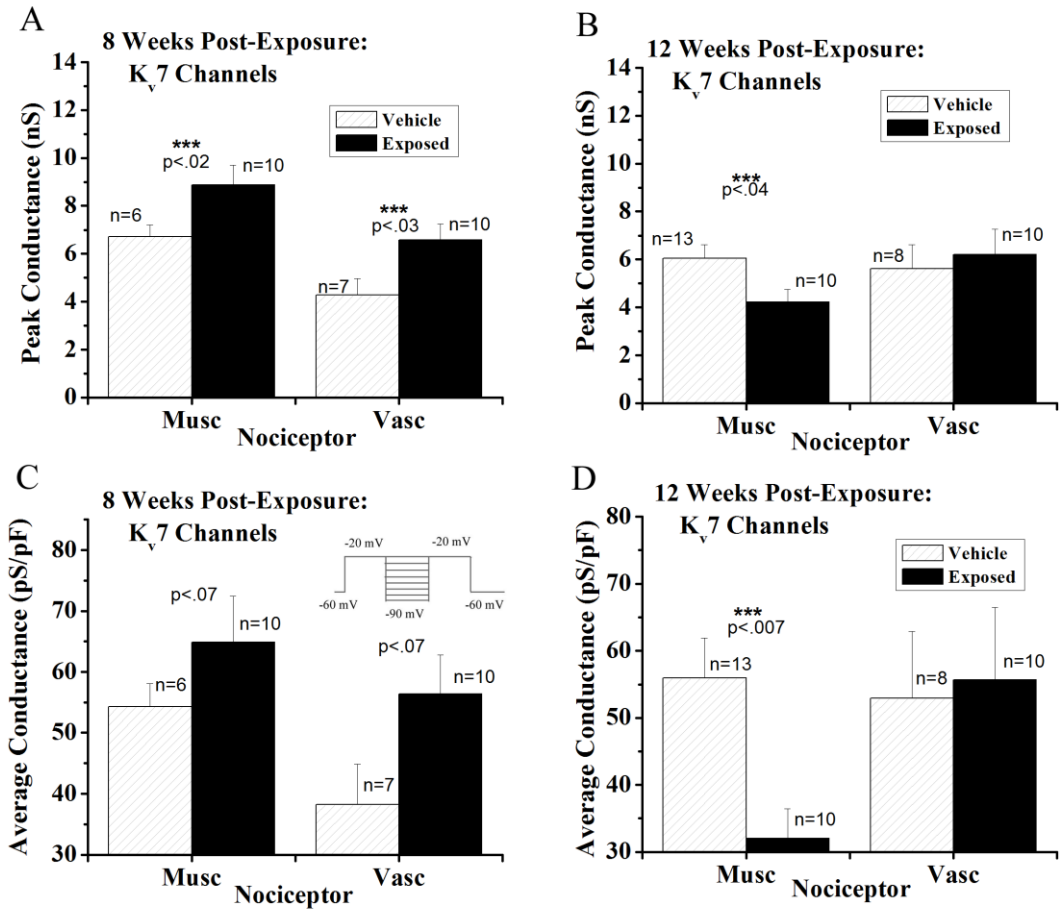

Figure 4. Altered Physiology of K_v7, 8 and 12 Weeks Following a 60 day Exposure to Permethrin, Chlorpyrifos and PB. K_v7 activity was modified at both the 8 week (A and C) and 12 week (B and D) post-exposure periods. Conductances were used as dependent measures in these studies because the peak current did not always occur at the same deactivation voltage step. Peak and average conductances were normalized to cell size. Conductances were averaged over a range of -40 to -70 mV. The voltage protocol used is presented as an inset in 'C.'

Physiology of K_{DR}. In order to determine whether the changes observed in the K_v7 channels were part of a larger, general, influence on K_v channels, we examined the voltage dependence of the K_{DR} channels after inhibition of K_v7 with linopirdine. After exposure to linopirdine for 3 minutes (10 μM), the voltage dependence of the K_{DR} current was assessed. As shown in figure 5, exposure to GW chemicals for 60 days produced significant, if small, hyperpolarizing shifts in the voltage dependence of activation at both the 8 and 12 week examination points. Voltage shifts ranged from 2 to 3.5 mV and were significant in both muscle (8 and 12 weeks; figure 5A and B) and vascular nociceptors (12 weeks; figure 5D). A shift of 3 mV is clinically consistent with the influence of an inflammatory mediator such as prostaglandin E_2.

At 8 weeks post exposure, muscle nociceptors exhibited significant increases in average K_{DR} current that accompanied their hyperpolarized $V_{.50}$ (figure 6C). However, at 12 weeks post exposure, the average and peak amplitude of muscle nociceptors K_{DR} was significantly *decreased* (figure 6B and 6D). Vascular nociceptor K_{DR} amplitudes were unaffected at either time delay. While K_v7 activity is most important for nociceptor excitability, the reduction of the total K_{DR} has special significance. For the purpose of experiment, we isolated K_v7 from the total K_{DR}. K_v7 is a member of the K_{DR} family, and subject to modulation by transcription factors with broad activity against many K_v proteins. Therefore, pathways leading to the development of this chronic pain condition may be traced to those factors with broad control over K_v channel expression, rather than those that specifically target K_v7. These factors are discussed in 'Discussion' (see attached manuscript, as well).

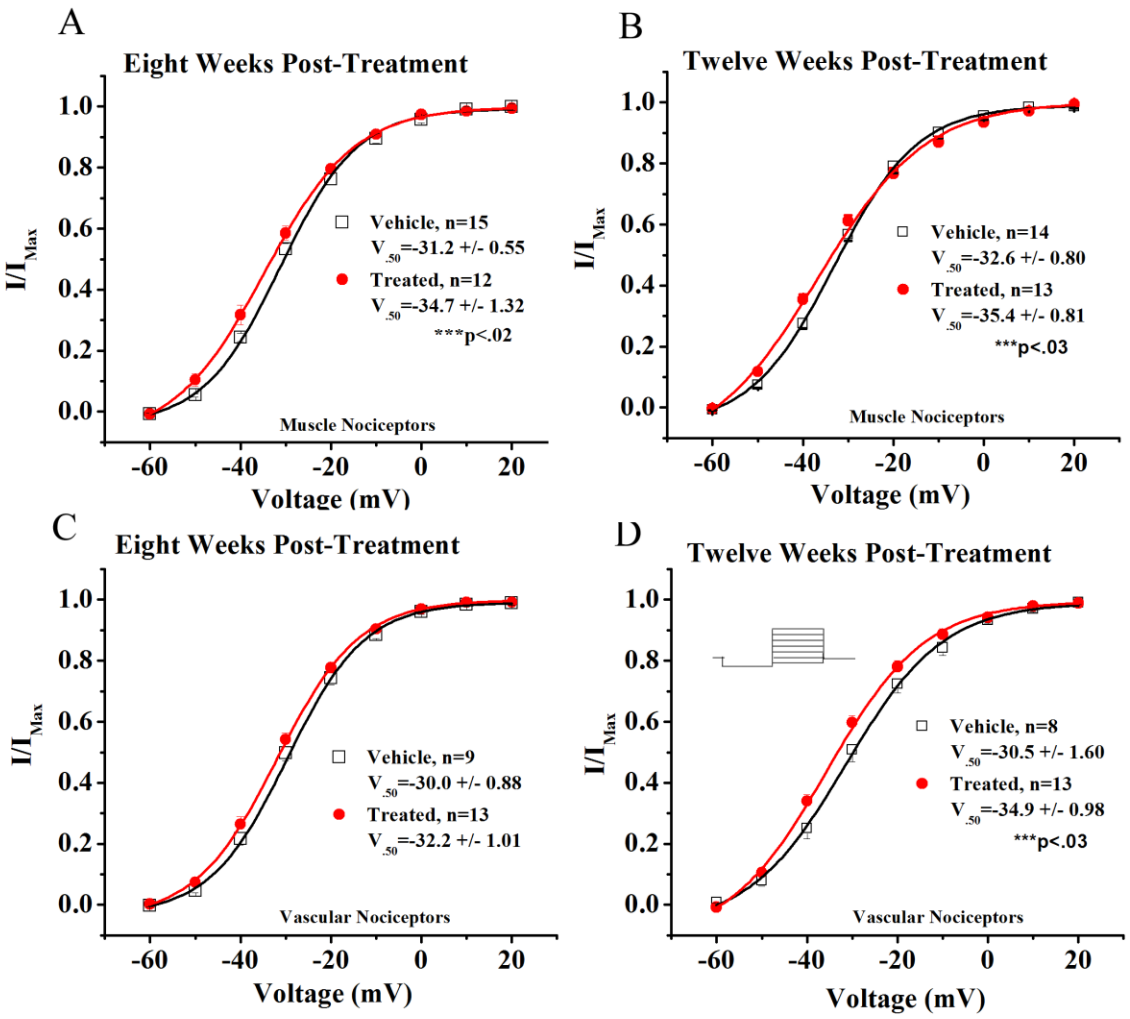

Figure 5. The Voltage Dependence of Delayed Rectifier Channels (K_{DR}) 8 and 12 Weeks Following Exposure to Permethrin, Chlorpyrifos and PB. A and B) In muscle nociceptors, the computed activation voltage constants ($V_{.50}$) were hyperpolarized (leftward shift) at both the 8 and 12 weeks post exposure periods. **C and D)** Vascular nociceptors were shifted only at the 12 week delay. Voltage constants ($V_{.50}$) were derived from Boltzmann fits to individual cells while the curves above reflect the average of all cells. Curves were formed from tail currents evoked by the voltage protocol illustrated in 'D'. From a V_H of -60 mV, cells were hyperpolarized to -100 mV for 2 sec then stepped for 500 msec from -60 to 20 mV in 10 mV steps.

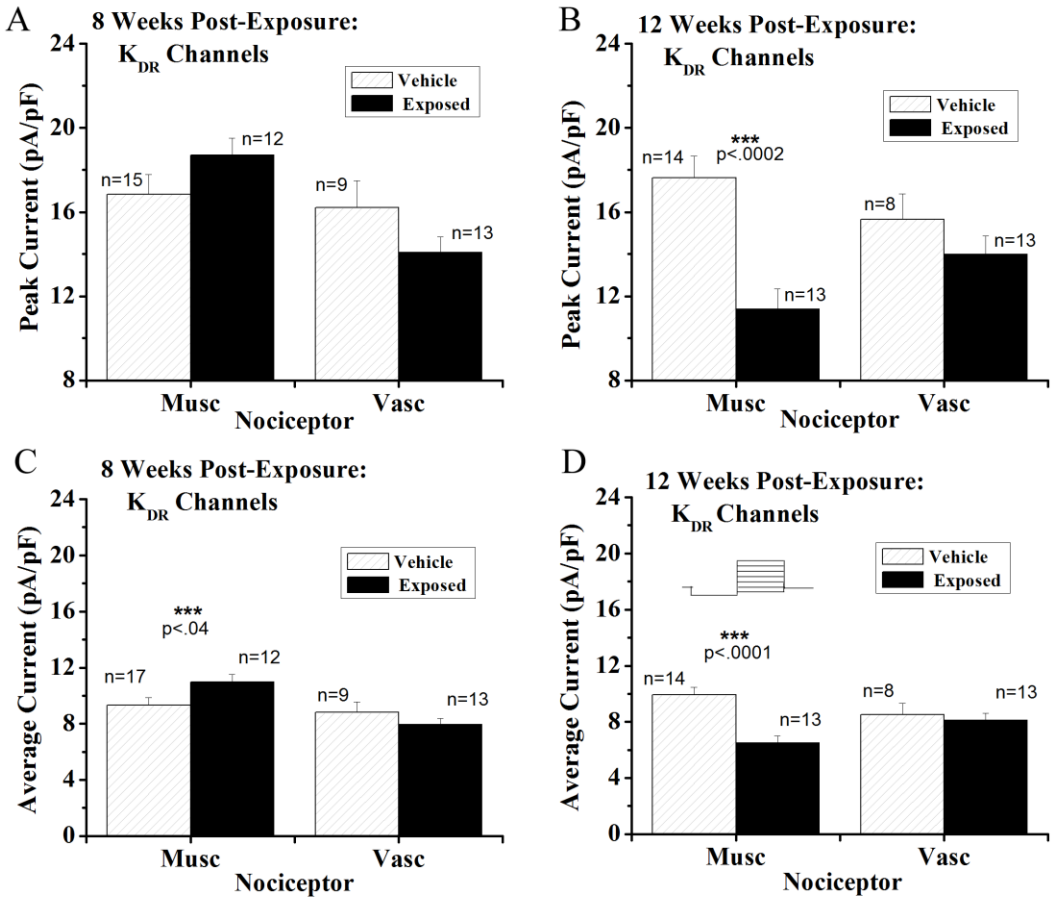

Figure 6. Amplitude of the Delayed Rectifier Channels Currents (K_{DR}) 8 and 12 Weeks Following Exposure to Permethrin, Chlorpyrifos and PB. B, C and D) Muscle nociceptor K_{DR} amplitude measures were affected by GW chemical exposure. At 12 weeks post exposure (D), the influence of GW chemicals reversed from a significant increase at 8 weeks to a significant decreased average amplitude. Peak and average currents were normalized to cell size. Currents were averaged over a range of -60 to 20 mV. The voltage protocol is shown as an insert in panel D. From a V_H of -60 mV, cells were hyperpolarized to -100 mV for 2 sec then stepped for 500 msec from -80 to 20 mV in 10 mV steps.

The total K_{DR} is a combination of a number of molecular subcomponents that differentially contribute to neuronal physiology. In DRG, the total K_{DR} current is a mixture of $K_v1.1$, $K_v1.2$, $K_v1.3$, K_v2 and K_v7 channel proteins. The composition of the total K_{DR} in specific subtypes of DRG nociceptors (e.g., vascular, muscle) is not well detailed (see Gold et al., 1996). We isolated the K_v7 component using the highly specific antagonist linopirdine. These findings were presented above. The K_{DR} data we report is actually the residual K_{DR} after the removal of the K_v7 contribution. For simplicity we refer to it as the K_{DR}. As part of Task 2g, we also attempted to separate the K_v1 components of the K_{DR} using K_v1 channel toxins (margatoxin; dendrotoxin K). These experiments were unsuccessful. The inability to separate the K_{DR} components was probably due the presence of mixed heteromers of the various K_v subunits. Therefore, toxins specific to homomeric channels composed of $K_v1.1$ or $K_v1.2$ or $K_v1.3$ might be ineffective against a heteromeric channel.

While we were unable to separate components of the K_{DR} using toxins, we were able to examine distinct contributions using a deactivation kinetic analysis. This analysis indicated that multiple components (at least 2) were likely to contribute to the residual K_{DR}. Exponential fits to the deactivation tails (at -30 mV) indicated the presence of a fast and a slow kinetic component that could represent distinct molecular entities (i.e., heteromers of $K_v1.1$ or $K_v1.2$, $K_v1.3$, $K_v2.1$, $K_v2.2$). These components were differentially affected by exposure to GW chemicals (figure 7; Task 2c and 2d). The fast component showed little effect at 8 weeks post exposure, but was significantly increased at 12 weeks in both nociceptor classes (figure 7a and 7b). The slow component time constant of decay (tau) was more generally modified at both 8 and 12 weeks post exposure. In year 2, we initiated further studies designed to break down the composition of the residual K_{DR}. These met with greater success and are presented on page 36.

Taken together with the outcomes of experiments on K_v7, the changes we observed to the fast and slow components of the K_{DR} indicate a broad influence of GW chemicals on several other K^+ channel proteins (potentially: $K_v1.1$, $K_v1.2$, $K_v1.3$, $K_v2.1$, $K_v2.2$). Although our measures are limited to nociceptors, these K_v ion channel proteins are widely present throughout the peripheral, central and autonomic nervous systems. They contribute both to neural excitability and the integrity of axonal coding of sensory and motor activity. Depending upon the site,

pattern and character of these influences on neural activity, a wide variety of cognitive, sensory, autonomic and motor dysfunctions could be associated with K_v maladaptations.

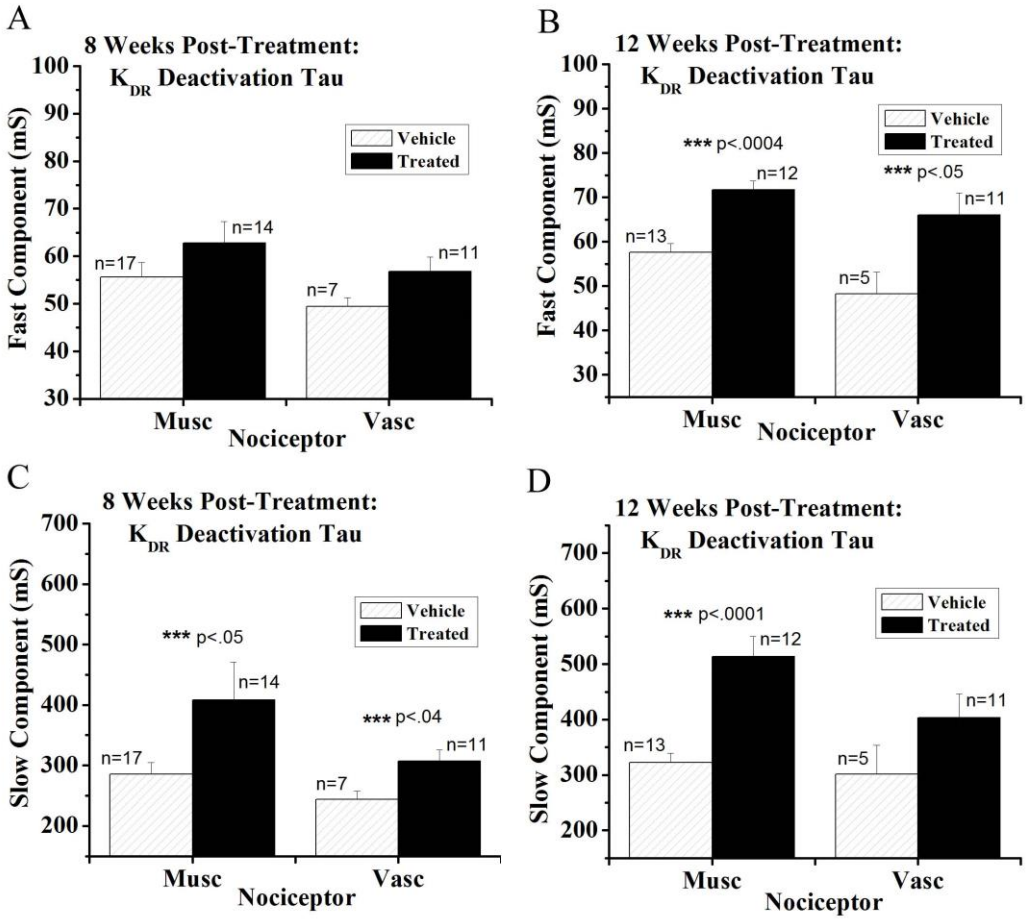

Figure 7. Deactivation Time Constants (Tau) were Increased by Exposure to GW Chemicals. A and B) 8 and 12 weeks after exposure the fast time constants trended longer, but only reached significance at 12 weeks. **C and D)** In contrast, significant changes in the slow decay constants were present at 8 and 12 weeks with some studies still underway (vascular nociceptors).

Results: Part 2

A portion of the data presented here appears in an attached manuscript.

Summary Part 2

Most of the studies reported in part 2 of this report were performed exclusively in year 2. The vascular nociceptor tracing physiology studies, reported below, were carried out mostly in year 1. Experiments on the immunocytochemistry of vascular nociceptors were executed in year 2. For clarity, all of the vascular nociceptor physiology and immunocytochemistry are reported below.

Studies conducted in year 2 investigated the influence of GW chemicals on K2p ion channels, and further examined how intensified anticholinesterase exposure impacted ion channel protein physiology through ACh sensitive muscarinic receptors. Nociceptors harvested from GW chemical exposed rats did not exhibit altered K2p physiology. Apparently, the non-voltage sensitive K^+ channels (K2p) were not a part of the K^+ channel family perturbed by exposure to GW chemicals (K_v7, K_{DR}). This pattern implicates transcription factors with broad influence on K_v channels as key players in the development of GW pain. We suggest that this deranged expression pattern was set into motion by prolonged and excessive Ca^{++} entry under the influence of permethrin (see Discussion). Studies were initiated to understand the critical role played by enhanced anticholinesterase exposure. Muscarinic receptor influences over voltage activated K_v proteins are well known. We investigated how this may have been modified by prolonged exposure to GW chemicals. Studies on muscle nociceptors revealed that the reactivity of K_v7 to muscarinic agonists was enhanced while the reactivity of K_{DR} to muscarinic agonists was decreased by GW agents. These effects would both be expected to increase nociceptor excitability. Attempts to specifically identify that portion of the K_{DR} family that was altered by exposure to GW chemicals (ACh muscarinic agonists) were unsuccessful; although studies did eliminate from consideration those homomeric channels composed of $K_v1.1$, $K_v1.2$, $K_v2.1$ and $K_v2.2$.

Behavior Studies

In year 2, behavioral studies were conducted on groups of rats as part of the molecular experiments of Task 1. Studies were broken into two groups. One group, Y2A (year two A) was made up of 26 rats (13 GW exposed; 13 vehicle treated). A second group Y2B (year two B) was made up of 24 rats (12 GW exposed; 12 vehicle treated). Both groups received the 'intensified protocol' (8 weeks of exposure; see figure 3) that had produced a pattern of decreased movement, increased resting and normalized movement rate 12 weeks after exposure. The goals of Task 1 were to determine whether the influence of GW chemicals on Kv channel proteins (K_v7 and K_{DR}) extended to other important K^+ channels, such as K2p channel proteins (Group Y2B).

Task 1: We will determine whether neurotoxicants/PB produce persistent changes in K2p function that could contribute to chronic and widespread pain.

Task 1a: Assess KCNK Physiology in Muscle, Skin and Vascular Nociceptors 8 weeks After Treatment.

Task 1b: Assess KCNK Physiology in Muscle, Skin and Vascular Nociceptors 12 weeks After Treatment.

The outcomes of the year 2 behavioral studies were similar to, but not identical with those of year 1 (figure 3 above). For ease of comparison, we have reproduced figure 3, below, as figure 9 (Group Y1C). The data from year 2 are shown in figures 8 and 10. In Groups Y2A and Y1C, an 8 week exposure to the GW chemicals (PB, permethrin, chlorpyrifos) produced an increase in resting at weeks 9-12 (figure 8D and 9D). Changes in movement distance and rate (figures 8 and 9, panels B and C) were similar in pattern but movement distance, though declining, failed to reach significance in group Y2A (figure 8B). While both groups showed significant resting increases by 12 weeks post, the increases in resting in group Y2A were more modest. Despite some differences in detail, both of these test groups manifested behavior patterns consistent with

a delayed or emergent myalgia/arthralgia. Due to the very delayed development of pain-like signs, it will be important to extend future observations out to 16 weeks post exposure.

A third group of rats was exposed to the same GW chemicals (group Y2B; figure 10). Changes to movement distance and movement rate were similar to the two previous groups at 8 weeks post-exposure, but these was no evidence that hyperactivity subsided by the 12 week post test period. Moreover, resting times actually *decreased*, relative to the vehicle control group, at all intervals for rats exposed to GW chemicals in Group Y2B (figure 10D). Close inspection of the figures indicates the paradoxical outcome for the rest time data was due to an unusual increase in resting time in the control group, and cannot be attributed to GW exposure (figure 10D). It is not clear why the vehicle treated animals diverged so sharply from their baseline rest levels.

Ultimately, it was clear that increasing the exposure to anticholinesterases greatly improved the behavioral outcomes relative to those of previous studies (Nutter and Cooper, 2013; Nutter et al., 2014). Namely: 1) There were relatively consistent patterns of behavioral changes that long outlasted the exposure periods; and 2) Pain-like patterns emerged in a delayed manner 9-12 weeks after dosing ended. It was less certain that a chronic pain, in the form of a myalgia or arthralgia, was the underlying cause of these behavior patterns. The decreased movement and increased resting revealed in figure 9 (representing year 1) was partially replicated in year 2 where only resting increased at 9-12 weeks post exposure (figures 8 and 10). Although the activity patterns were very similar in all three groups, the resting measure was much more variable. Pain-like resting behavior increased in two groups but actually decreased, relative to controls, in a third group of rats. The latter appeared to be a control group aberration rather than the influence of GW chemicals. The sensitivity of the activity measures to increased anticholinesterase exposure prompted a series of experiments on muscarinic receptor function that are presented below (page 34).

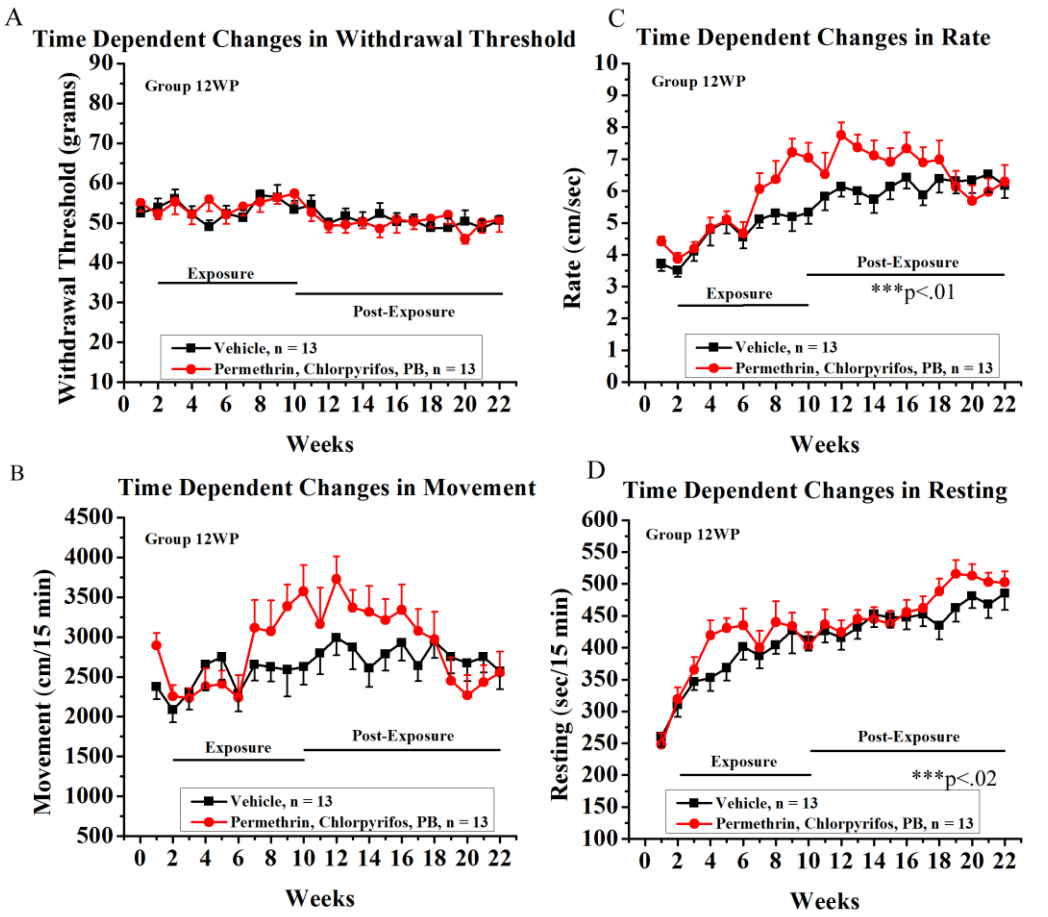

Figure 8. The Intensified Exposure Protocol Produced a Delayed Pain-Like Behavior Pattern in the 12 Week post Exposure Group Y2A. A)-B) Muscle pain pressure threshold, and movement distance were unchanged following the 60 day exposure to GW chemicals. **C)** Movement rate significantly increased during 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 ($F=5.82$; $p<.02$). Tests were not conducted on any measure 1-4 weeks post exposure or during exposure to GW chemicals.

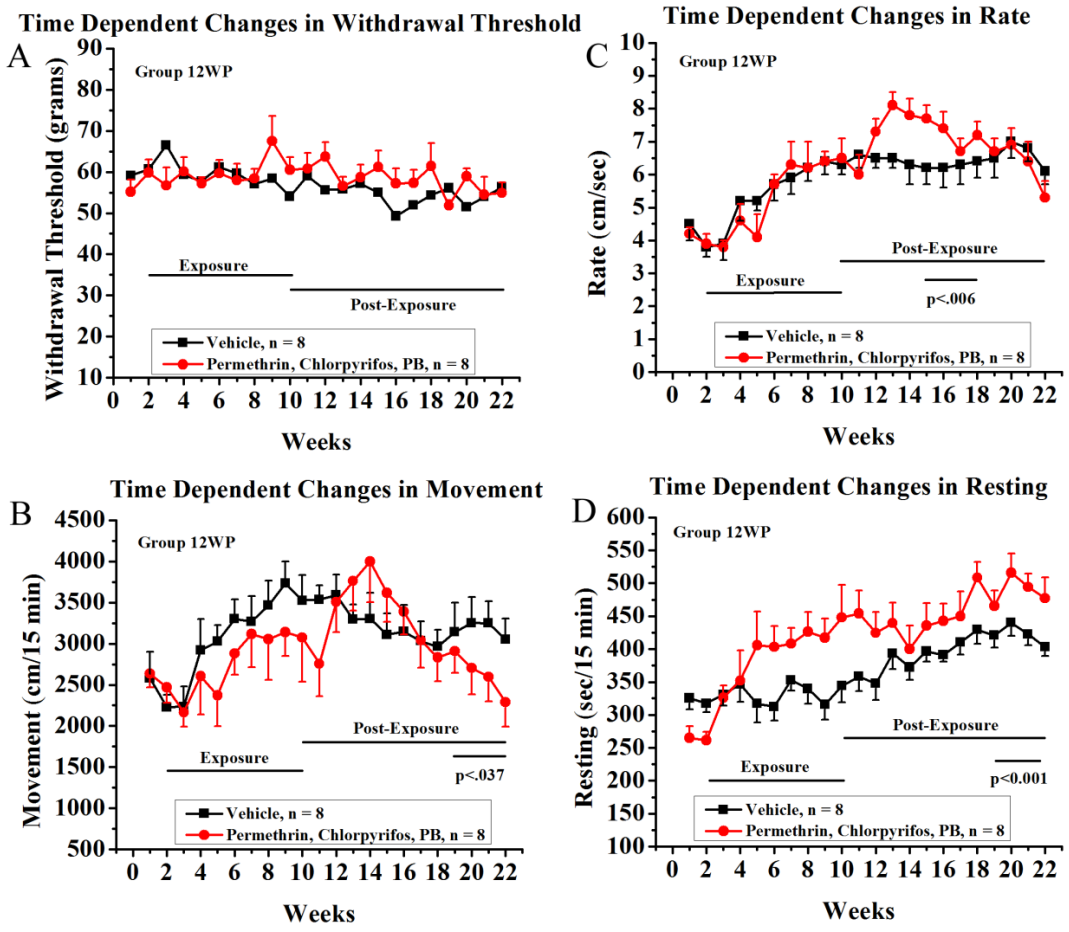

Figure 9. (also Figure 3 above) The Intensified Exposure Protocol Produced Consistent Signs of Lasting Pain Behaviors in the 12 Week post Exposure Group Y1C. Figure 3 is reproduced from page 12 to simplify comparisons from year 1 (group Y1C) and year 2 (groups Y2A and Y2B). **A)** Muscle pain pressure threshold was unchanged following the 60 day exposure to GW chemicals. **B)** Consistent with a delayed chronic pain, movement distance was significantly decreased at 9-12 weeks post exposure ($F=5.87$; $p<.04$). **C and D)** Movement rate and resting were both increased during the post-exposure period. Resting was significantly increased 9-12 weeks post-exposure ($F=10.2$; $p<.001$). Movement rate was significantly increased only at the 5-8 week test period ($F= 8.09$; $p<.006$). Tests were not conducted on any measure 1-4 weeks post exposure.

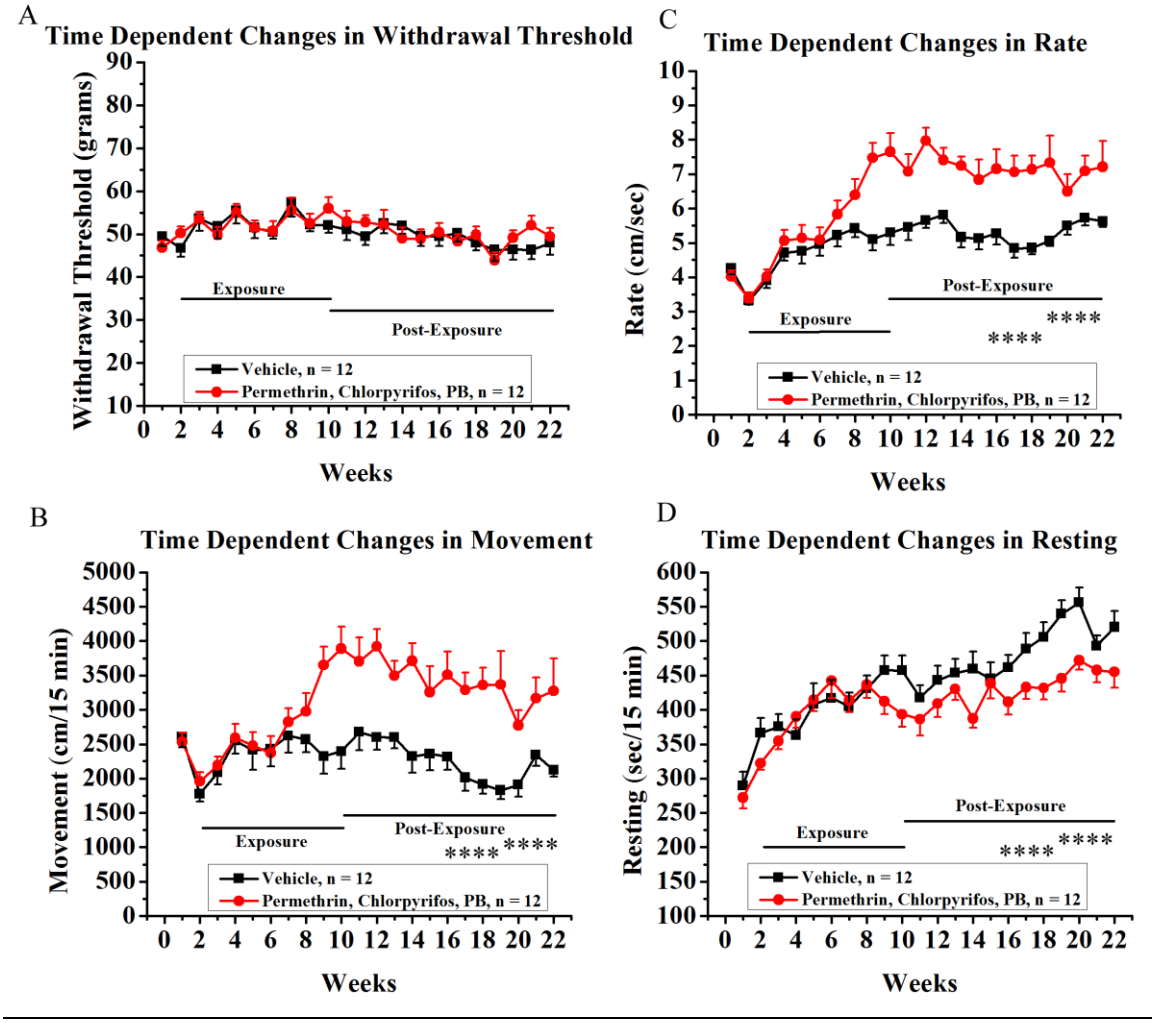

Figure 10. The Intensified Exposure Protocol Did Not Produce a Pain-Like Behavior Pattern in Group Y2B. **A)** Muscle pain pressure threshold was unchanged following the 60 day exposure to GW chemicals. **B)** Movement distance was significantly increased 5-8 and 9-12 weeks post exposure ($F=43.7$ and $F=28.4$, respectively; $p<.0001$). **C)** Movement rate also increased during both of the test periods ($F= 48.1$ and $F=21.3$, respectively; $p<.0001$). **D**) Resting was significantly *decreased* at both the 5-8 and 9-12 weeks post-exposure test periods ($F= 10.5$; $p<.002$ and $F=25.8$; $p<.0001$, respectively). Tests were not conducted on any measure 1-4 weeks post exposure or during exposure to GW chemicals.

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

We demonstrated, in year 1, that K_{DR} currents were modified by GW chemicals (see also Nutter et al., 2013; Nutter et al., 2015, attached). The amplitude of these currents, in muscle nociceptors, co-varied with the appearance of pain-like behaviors (myalgia/arthralgia). During periods when the pain behaviors were increased, the residual K_{DR} was significantly lower in muscle nociceptors.

The total K_{DR} current of the dorsal root ganglion is comprised of a number of distinct functional subcomponents (Gold et al., 1996). We have been studying the residual K_{DR} that is present when the K_v7 component was removed (linopirdine sensitive current). While the K_v7 component is certainly important for cell excitability, the non-K_v7 portion of the K_{DR} may also be important for development and maintenance of chronic pain. Reduced expression of several K_{DR} components have been linked to chronic pain behaviors in animal models (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., 2014; Tsantoulas, 2015). One or more of these currents may have been reduced by the GW chemical exposure protocol, and thereby might play a significant role in the development and maintenance of a GW chronic pain syndrome.

Our previous attempts, in year 1, to separate out the total K_{DR} into distinct K_v subcomponents were unsuccessful (see page 19). Strategies relying on specific toxins targeted against $K_v1.1$ or $K_v1.2$ or $K_v1.3$ (Dendrotoxin-K, Margatoxin) did not produce a rational breakdown of the total K_{DR}. Recent studies, completed in year 2, using Maurotoxin (MTX) or Stromatoxin (StTX), produced better results. MTX is a toxin with potent effects on $K_v1.1$ and $K_v1.2$. Application of MTX to muscle (and vascular) nociceptors produced some reliable inhibition (100 nM; 10-13%; data not shown). However, the amount of inhibition was deemed too slight and too variable to evaluate. In contrast, StTX, a toxin that specifically inhibits $K_v2.1$ and $K_v2.2$ mediated currents, produced reliable and substantial reductions of K_v currents in muscle and vascular nociceptors. In accordance with TASK 2g, we used this toxin to determine whether the $K_v2.1$, $K_v2.2$ components were selectively modified by exposure to GW chemicals. Rats for these studies came from the Y2A group (figure 8)

Task 2g: Determine the K_V component Protein that Contributes to Changes in K_{DR} Amplitude, Activation and Deactivation.

Neurons were identified as muscle or vascular nociceptors as previously described. K_{DR} currents were isolated in the usual manner (see Method; page 89). Ten (10) micromolar linopirdine was added to the isolation solution to block the K_v7 component of the K_{DR}, (3 minute application). Following a pre-pulse to -100 mV, a 500 msec voltage pulse to +20 mV was applied at 30 second intervals. Evoked currents were leak corrected on line using the P/N procedure. Following collection of 6-9 baseline samples, current amplitude stabilized. The average of the final 3 samples served as baseline from which to evaluate the contributions of $K_v2.1$ and $K_v2.2$ to the total K_{DR} current. A neurotoxin, StTX, that specifically targets $K_v2.1$ and $K_v2.2$, was applied for 5 minutes. Studies were conducted in vehicle exposed and GW chemical exposed rats 12 weeks post-exposure (intensified protocol: permethrin, chlorpyrifos, PB; Group Y2A). Studies with MTX are not shown.

The StTX sensitive current was determined as the amount of K_{DR} current that was reduced after a 5 minute application of 100 nM StTX. Once exposed to StTX, K_{DR} currents rapidly declined over time (30 second test intervals). Within 5 minutes after exposure to StTX, the reduction of K_{DR} currents had stabilized. It was apparent that the $K_v2.1$ and $K_v2.2$ currents comprised about 25-35% of the total K_{DR} current present in muscle and vascular nociceptors, respectively (figure 12). There was no indication that exposure to GW chemicals changed the total amount of $K_v2.1$ and $K_v2.2$ current expressed in either muscle or vascular nociceptors (figure 11).

After exposure to StTX a substantial portion of the K_{DR} current remained. We will refer to this as the StTX insensitive current. Consistent with demonstrations in year 1 experiments (see figure 6), the StTX insensitive K_{DR} current was significantly less in muscle nociceptors exposed to GW chemicals (figure 12). Therefore, those specific K_{DR} component currents, down regulated by exposure to GW chemicals were not K_v21 and $K_v2.2$. Nor were they likely to be the maurotoxin sensitive currents ($K_v1.1$ or $K_v1.2$), as these currents were present in only very small amounts in these nociceptors. We cannot rule out Kv1.1 or Kv1.2 heteromers as the influence of Maurotoxin on these heteromeric channels is not known.

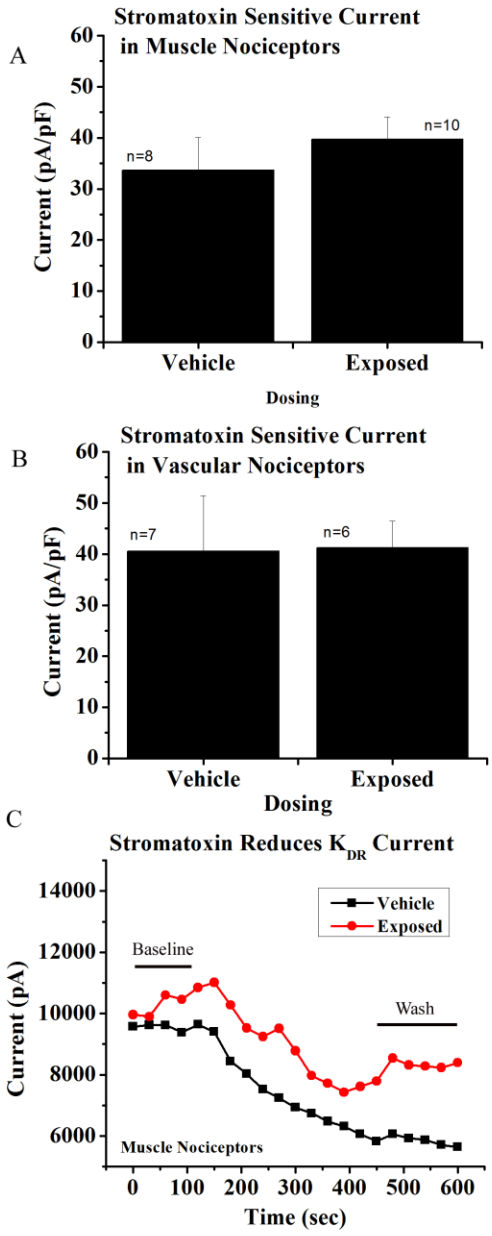

Figure 11. Stromatoxin Sensitive Currents (K_v2.1, K_v2.2) in GW Exposed Nociceptors. The total K_v2.1 and K_v2.2 currents were not modified by chronic exposure to GW chemicals. **(A)** Muscle nociceptors **(B)** Vascular nociceptors. **C)** A representative case showing the time dependent change in peak K_{DR} current after exposure to StTX. Cells were harvested 12 weeks after exposures had ceased.

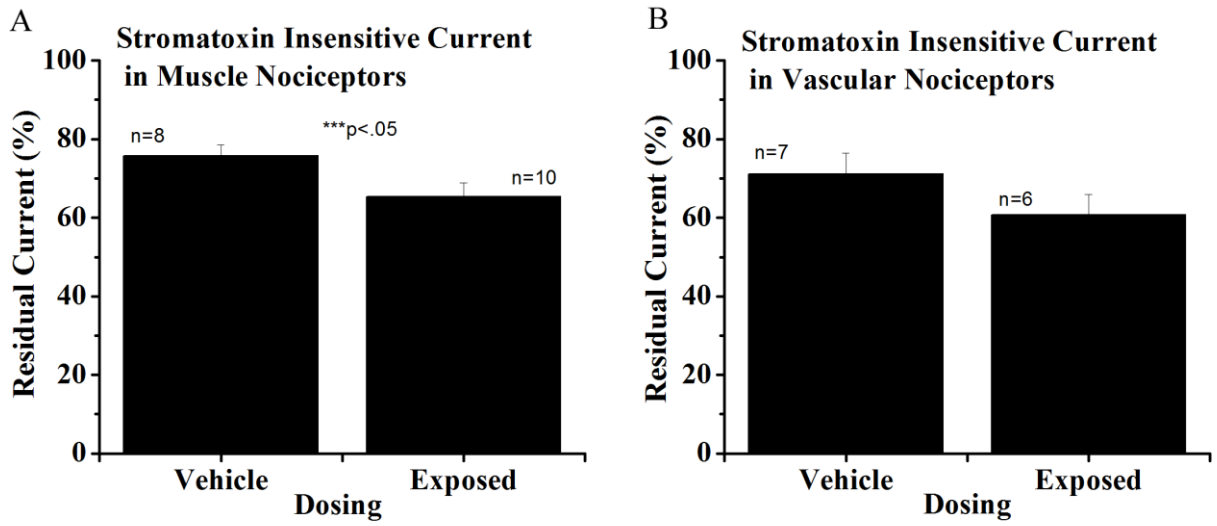

Figure 12. Stromatoxin Insensitive Currents in GW Exposed Nociceptors. StTX sensitive currents ($K_v2.1$ and $K_v2.2$) normally comprised about 25 to 30% of the total K_{DR} current in muscle and vascular nociceptors, respectively (vehicle tested rats). The StTX insensitive current, the proportion of 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.

GW Chemicals and the Role of K2p Channel Proteins

The K2p channel proteins are an important family of K^+ selective channels that play a role in setting membrane resting potential (RMP). The RMP has wide ranging influences on membrane excitability, and therefore, pain sensations. We have shown previously that prolonged exposure to GW chemicals affects multiple voltage sensitive K^+ channels (K_v7, K_{DR}). We now examine whether other families of K^+ channel proteins are modified by GW chemicals.

Task 1: We will determine whether neurotoxicants/PB produce persistent changes in K2p function that could contribute to chronic and widespread pain.

Task 1a: Assess KCNK Physiology in Muscle, Skin and Vascular Nociceptors 8 weeks After Treatment.

Task 1b: Assess KCNK Physiology in Muscle, Skin and Vascular Nociceptors 12 weeks After Treatment.

Task 1c: Separate Persistent Effects on KCNK9 (TASK) from other KCNK Proteins: 12 months

We have demonstrated a broad influence of GW chemicals on voltage sensitive K^+ currents. As part of Task 1, we examined the influence of three GW toxicants on proton sensitive K^+ currents that comprise the TASK family of K2p 'leak currents'. This large family of K2p channels (17 distinct phenotypes) are widely distributed in the CNS and PNS and contribute to the regulation of resting membrane potential (Renigunta et al., 2015; Li and Toyoda, 2015). A pH sensitive subgroup of this family (TASK) are particularly relevant as they are known to be expressed in muscle and vascular nociceptors (Rau et al., 2006; see also Marsh et al., 2012). Tissue acidity is an important signal to nociceptors that innervate muscle and vessels. Tissue acidity can reflect insufficient blood perfusion consequent to a shift from aerobic to non-aerobic metabolism. Channel proteins sensitive to tissue pH detect the drift of proton concentrations toward detrimental, non-physiological, levels. Nociceptors that innervate these tissues express several

pH sensitive channels (ASIC, TRPV1, TASK) that are likely to contribute to proton signaling and regulate local tissue perfusion (Rau et al., 2006; Jiang et al., 2006; Rau et al., 2007).

TASK channel proteins exhibit sensitivity to a range of proton concentrations around the physiological pH of 7.4. Pursuant to Task 1, we used acidic and alkaline pH solutions to characterize TASK-like currents in vascular and muscle nociceptors. Solutions were applied in a K^+ isolation solution to prevent the contribution of other pH sensitive currents that are known to be present in these nociceptors but do not conduct K^+ (ASIC; TRPV1; Jiang et al., 2006; Rau et al., 2007).

After K^+ current isolation (see Appendix, p 89), test solutions at pH 6.0 and pH 8.2 were applied. Acidic solutions close TASK channels that are open at pH 7.4, while alkaline solutions open those that are closed at the physiological pH (Cooper et al., 2004). Subsequently, we added the resting (acid closed) and opened currents (alkaline opened) together to estimate the total TASK K2p current present. Tests were conducted in both vehicle exposed and toxicant exposed rats at 8 and 12 weeks after exposure. As shown in figure 13, there were no changes in total K2p current at either test point or in either the vascular or muscle nociceptor groups. Animals in these studies came from group Y2B. Animals in the Y2B group did not exhibit pain-like behavior patterns (figure 10).

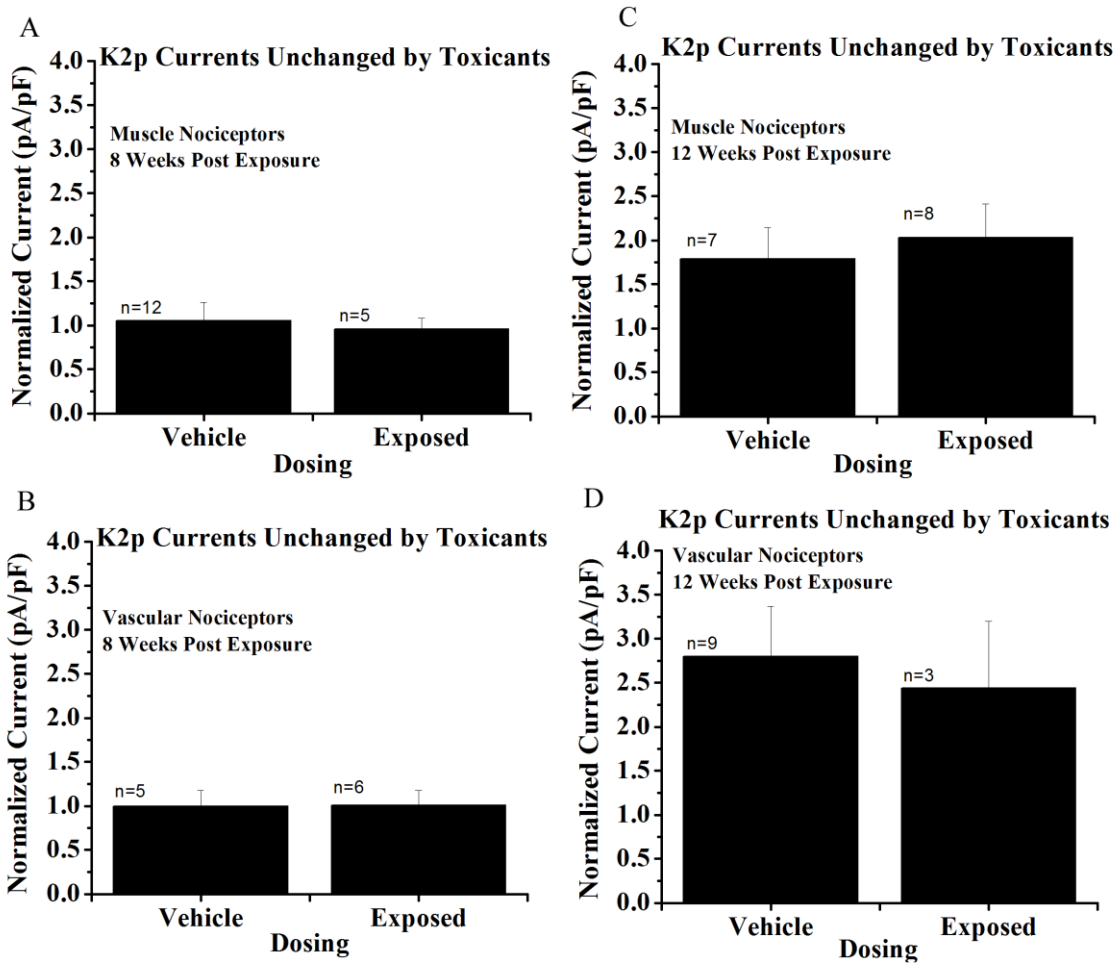

Figure 13. Exposure to GW Chemicals Did Not Modify TASK Mediated Currents. A) and B) Total proton sensitive TASK currents were not altered by GW toxicants 8 weeks after exposures had ended. **C) and D)** Total proton sensitive TASK currents were not altered by GW toxicants 12 weeks after exposures had ended.

Modification of Muscarinic Reactivity by GW Chemicals

Studies executed in year 1 indicated that increased exposure to anticholinesterases (chlorpyrifos, PB) resulted in prolonged behavior changes and sustained perturbations to a number of ion channel proteins (figures 3, 5 and 6). Due to the apparent importance of anticholinesterases to development of pain-like behaviors (figures 8, 9) we examined whether presentation of muscarinic agonists (OXO-M) would distinctly modify the activity of K2p, K_v7 and K_{DR} currents in toxicant and vehicle exposed neurons.

OXO-M, is a broadly active muscarinic agonist. Following isolation of K2p currents in muscle and vascular nociceptors, we exposed neurons to 10 uM OXO-M for 30 seconds. As shown in figure 14, proton sensitive K2p currents were significantly increased in rats exposed to GW chemicals (12 weeks post-exposure). In contrast, K2p currents in vehicle exposed muscle nociceptors trended higher but were not significantly modified by OXO-M. No muscarinic effects on vascular nociceptors were identified in either group. While it appeared that linkages between muscarinic receptors and K2p channels were amplified by GW chemical toxicants, the amount of increase in K2p current caused by OXO-M was not significantly different between the vehicle and toxicant exposed rat muscle nociceptors.

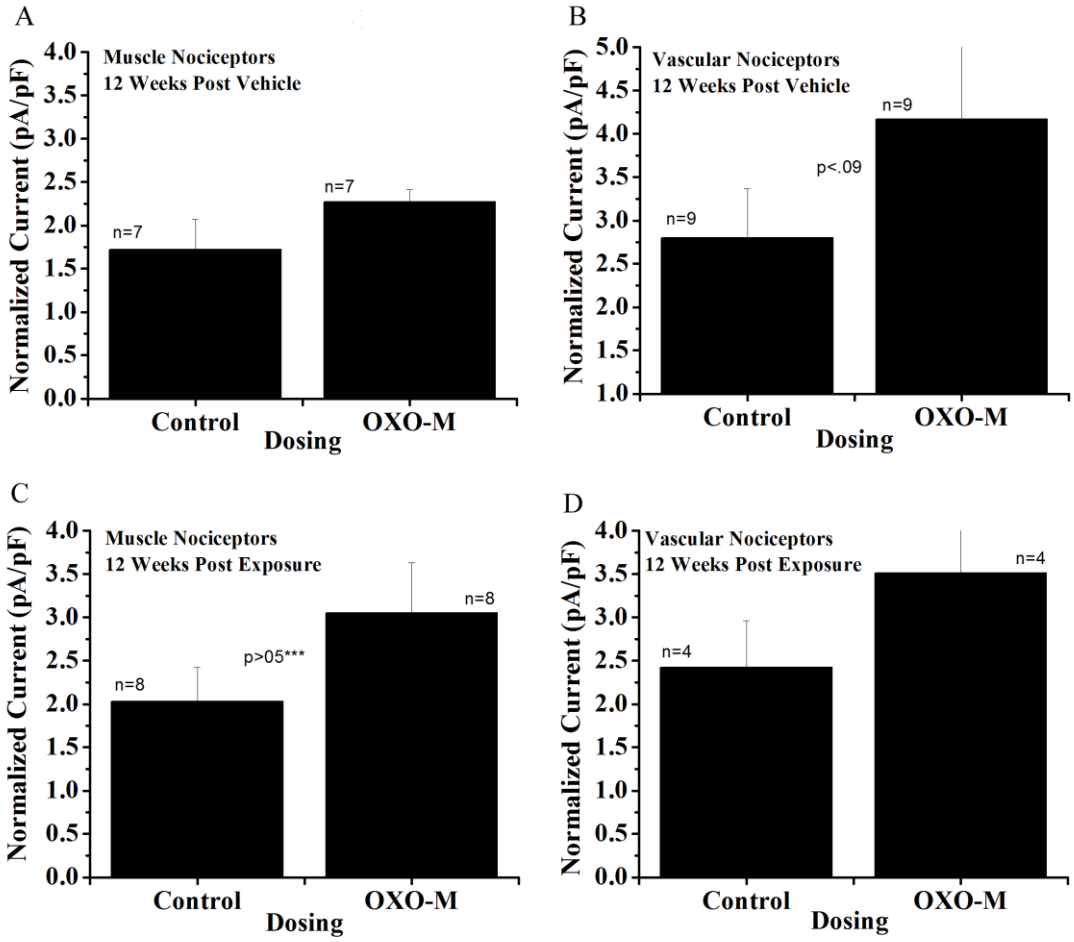

Figure 14. Muscarinic Agonists Increased K2p Mediated Currents in Muscle Nociceptors. **A) and B)** OXO-M did not significantly increase proton sensitive currents in vehicle exposed muscle or vascular nociceptors. **C) and D)** OXO-M significantly increased proton sensitive currents in muscle but not vascular nociceptors. All data from cells collected 12 weeks after GW chemical exposures.

Both muscle and vascular nociceptors express K_v7 channel proteins (Nutter et al., 2013). Cholinergic modulation (inhibition), via the muscarinic receptor activated pathway, is one of the ways by which K_v7 enhances neuronal activity (Brown and Passmore, 2009). We have shown the activity of K_v7 currents are modified by exposure to GW toxicants (Nutter et al., 2013; Nutter et al., 2015, attached). One level of modification could be through the actions of modulatory agents at G-protein couple receptors (e.g., a muscarinic receptor).

A portion of K_v7 channel proteins are open at the resting membrane potential. This allows K^+ to flow out of the cell and hyperpolarize the membrane. Closure of K_v7 depolarizes the membrane and increases neuronal excitability. We examined how exposure to a broadly active muscarinic agonist, OXO-M, altered the resting currents in both muscle and vascular nociceptors. Cells used in these studies were harvested from rats in Group Y2A (figure 8). Studies were conducted in a K^+ isolation solution (Appendix, p 89). OXO-M (10 uM) was applied for 30 seconds. Following exposure to OXO-M (close superfusion), the holding current shifted, in a depolarizing direction, within 10 seconds of OXO-M application (figure 15). The depolarizing shift was consistent with the closure of K_v7 currents coupled to activated muscarinic G-protein coupled receptors. We confirmed this by blocking the depolarizing shift by pretreatment with linopirdine (figure 16). 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 15A). Vascular nociceptors did not exhibit similar effects (figure 15B).

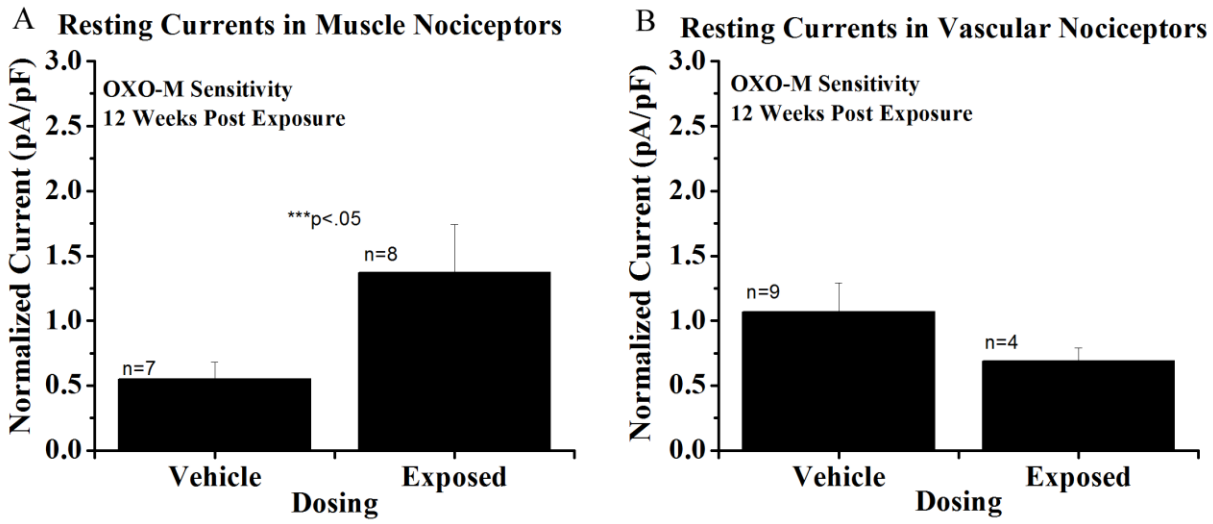

Figure 15. Muscle Nociceptors Exhibit Enhanced Sensitivity to a Muscarinic Agonist. **A**) Shifts in the holding current following a 30 second exposure to OXO-M (10 uM) suggested enhance suppression of Kv7 currents in muscle nociceptors exposed to GW chemicals. **B)** Resting currents in vascular nociceptors were not affected by exposure to GW toxicants.

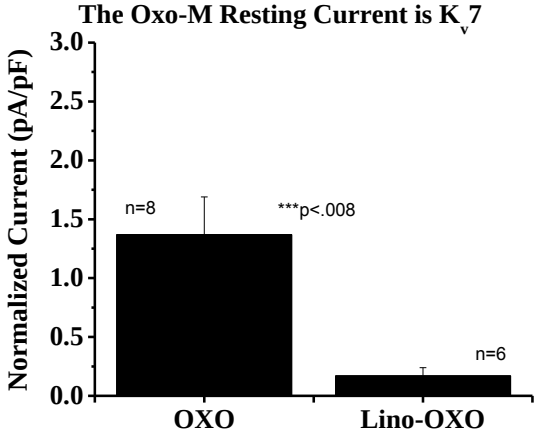

Figure 16. Shifts in the Holding Current are Blocked by Linopirdine. OXO-M induced shifts in the holding current are prevented by pretreatment with linopirdine (10 uM; 3 minutes). Studies conducted on muscle nociceptors taken from rats exposed to the GW chemical protocol.

There was evidence that both K2p and K_v7 channels exhibited enhanced sensitivity to muscarinic agonists. We hypothesized that cholinergic (muscarinic) sensitivity might also be modified in other K_v channels. To assess the functional state of the muscarinic pathway in muscle and vascular nociceptors, we assessed the time dependent sensitivity of K_{DR} channels to the muscarinic agonist OXO-M. Muscle and vascular nociceptors were identified in rats from the Y2A group (figure 8). Following isolation of linopirdine insensitive K^+ currents (3 minutes, 10 uM linopirdine), the baseline K_v reactivity was determined (pre-pulse to -100 mV; 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 17A), but not in vascular nociceptors from these same rats (not shown; 1.17 +/- 0.03 and 1.13 +/- 0.03; n=8 vehicle and 6 exposed, respectively). Some additional studies on vascular afferents are presented in the Appendix (figure A2, p. 85)

In distinct experiments, we used StTX to determine whether the component of the muscarine sensitive K_{DR} was the $K_V2.1$ and $K_v2.1$ portion that was <u>not</u> modified by GW chemicals. In these studies, StTX was presented after a stable baseline current was achieved. Following 5 minutes of StTX exposure, OXO-M was applied for 4 minutes. A separate group of cells were exposed to the StTX vehicle prior to OXO-M. As shown in figure 17B, removal of the StTX sensitive current did not block the influence of OXO-M. 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.

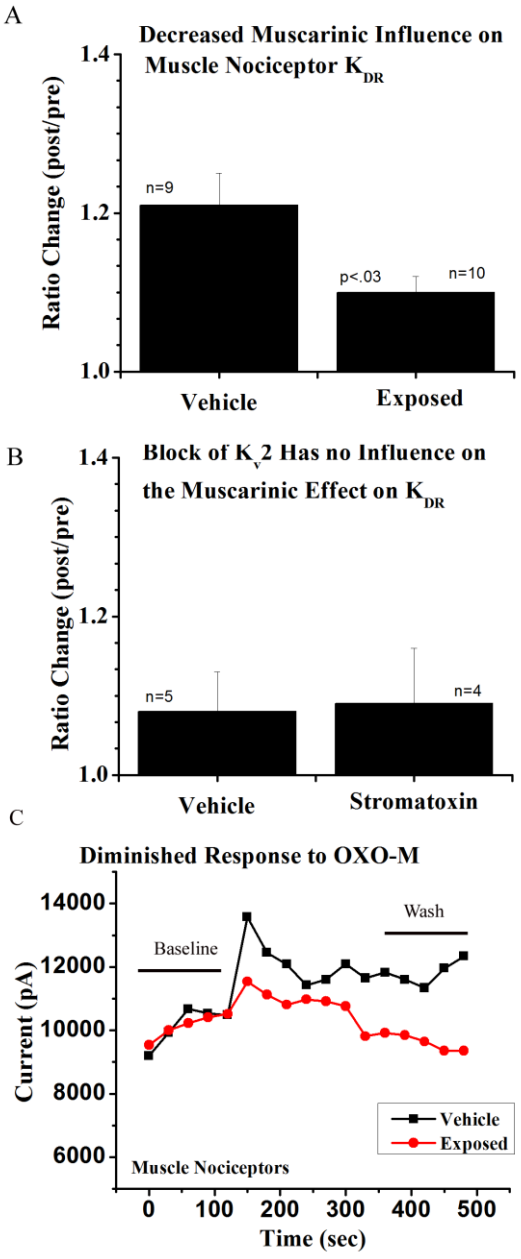

Figure 17. GW Chemicals Decreased the Muscarinic Sensitivity of K_{DR} Currents. A) A broadly active muscarinic agonist (OXO-M) increased K_{DR} currents of muscle nociceptors harvested from both vehicle exposed and GW chemical exposed muscle nociceptors. Muscle nociceptors from exposed rats of group Y2A were significantly less reactive to the muscarinic agonist. **B)** Block of the $K_v2.1$ and $K_v2.2$ current by Stromatoxin did not prevent the increase in K_{DR} current by OXO-M. **C)** A representative case showing the time dependent change in peak K_{DR} current after exposure to OXO-M. Cells pretreated with StTX exhibited a similar OXO-M enhancement as those that were pretreated with a vehicle used for StTX. Measures were taken at 1 minute after the superfusion of OXO-M.

Action Potential Characteristics and Membrane Excitability. Altered muscarinic sensitivity of K_v7 and K_{DR} ion channels suggested that muscle nociceptors might exhibit increased excitability following exposure to GW Chemicals. We examined the properties of muscle nociceptors 12 weeks following a 60 day exposure to the intensified protocol or their respective vehicles. Type 5 muscle nociceptors were identified, in the usual manner, in voltage clamp mode. After membrane properties were assessed (resistance, capacitance), neurons were brought into current clamp mode. The RMP was noted and each neuron and was observed for two minutes for signs of spontaneous activity (20 °C). Subsequently, the RMP was adjusted to -60 mV and action potentials were evoked (1 msec; 3-5 nA; 3 replications at 1 sec intervals). To assess membrane excitability, neurons were tested with an ascending series of current injections (0.1 nA/step; 10 steps; 250 msec). The threshold and total number of action potential were noted. Using close superfusion with a servo controlled heated probe, the same procedure was repeated at 35 °C.

There was little indication that muscle nociceptors, from exposed rats, would exhibit any spontaneous discharge. Only 1 of 24 neurons (20 °C: 14 vehicle and 10 exposed; 35 °C: 14 vehicle and 9 exposed) exhibited any spontaneous activity (2 APs, exposed case; data not shown). In contrast, the exposed group muscle nociceptors manifested significantly longer duration APs and AHPs at 20 °C and longer AP duration at 35 °C (figure 18A and B). Current injection excitability was increased at 35 °C only (5.0 +/- 1.8 vs 1.8 +/- 0.59 total APs; $p<.05$; n=6 and 13 respectively**).** There were no differences in RMP between GW chemical and vehicle exposed neurons (-64.9 +/- 1.7 and -67.3 +/- 1.7 mV respectively).

Following the above tests, muscle nociceptors were exposed to the muscarinic agonist OXO-M (10 μM; 2 minutes; 35 °C). Twelve of 15 muscle nociceptors rapidly depolarized during the exposure. A powerful burst of APs occurred during depolarization. The number of APs evoked was significantly higher in muscle nociceptors harvested from GW exposed rats (figure 18D). The amount of depolarization in response to OXO-M did not differ between the two groups (29.9 +/- 3.6 and 22.8 +/- 4.3 mV; GW exposed and vehicle treated respectively).

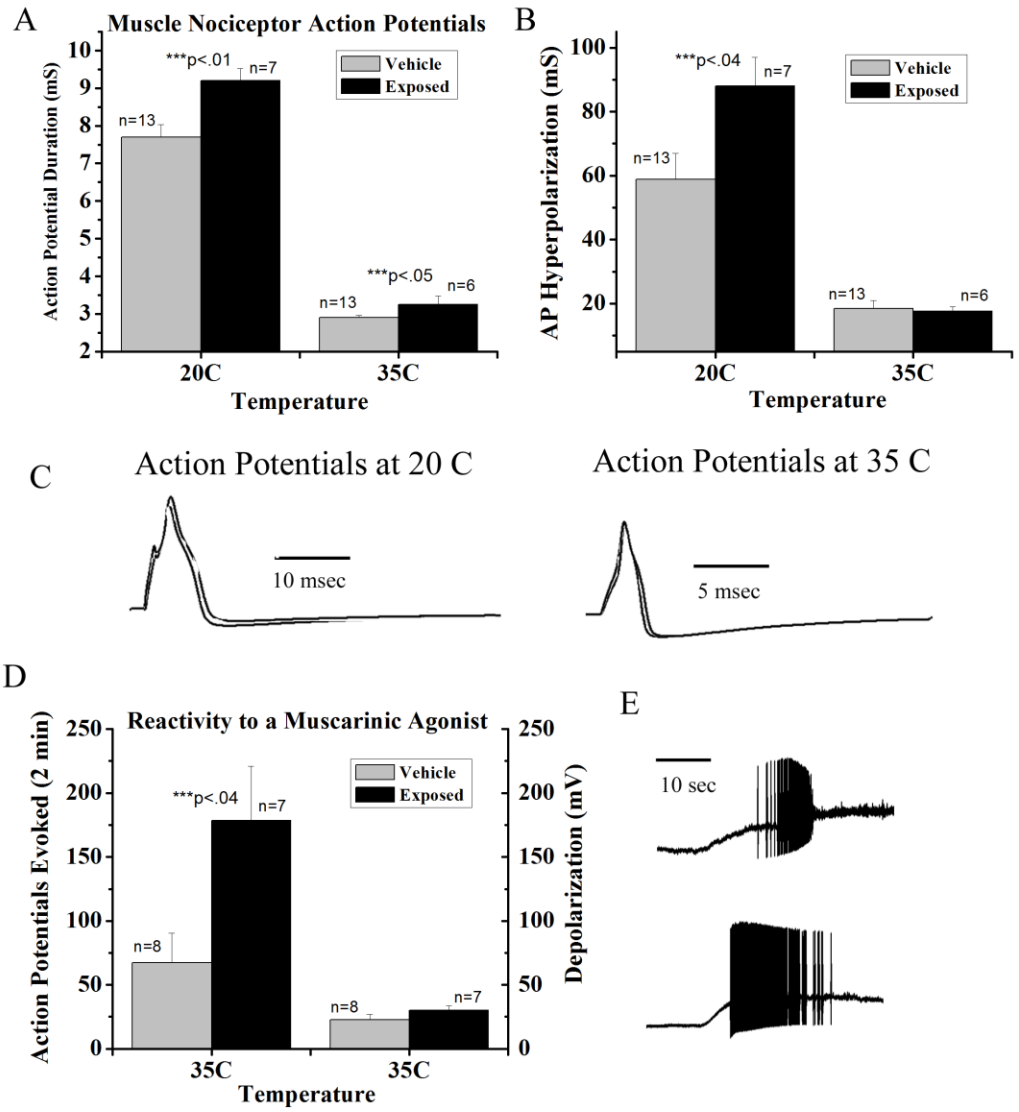

Figure 18. Action Potential Characteristics and Reactivity to Oxotremorine M. A) Action potential duration in vehicle and GW chemical exposed muscle nociceptors. The AP duration was increased at both test temperatures. **B)** Action potential hyperpolarization in vehicle and GW chemical exposed muscle nociceptors. The AHP duration was increased only at 20 °C. **C)** Overlapping AP traces in vehicle and GW chemical exposed rats at each test temperature. GW exposed rat muscle nociceptors had longer traces. **D)** Total action potentials evoked (left axis) and rapid depolarization (right axis) following exposure to the broad muscarinic agonist OXO-M. **E)** Representative AP burst in vehicle (upper trace) and exposed (lower trace) muscle nociceptors.

In summary, we found evidence that the muscarinic sensitivity of several K^+ currents were modified following exposure to GW chemicals. In one instance, a muscarinic agonist was more effective at depolarizing the membrane of muscle nociceptors (actions against K_v7). This would promote cellular excitability. In another instance, a muscarinic agonist was less effective at increasing K_{DR} amplitude; an action generally inhibitory in nature, but with less clear consequences for cellular excitability than the effects on K_v7. In a third instance, muscarinic agonist increased a resting K^+ conductance (K2p) that would hyperpolarize cell membranes, but there were no significant differences between vehicle and GW chemical toxicant exposed groups. When the muscarinic agonist was presented in current clamp mode, action potential bursts could be observed. The number of action potentials discharged was significantly greater in the GW chemical exposed group. Taken together, the heightened excitability and decreased inhibitory action is a compound maladaptation that was persistent selectively in muscle nociceptors, 12 weeks following exposures. This outcome tended to support an interpretation of a chronic myalgia in these rats. The strong AP bursting in muscle nociceptors, initiated by a muscarinic agonist, indicated a net excitatory effect had occurred in this nociceptor pool following a series of persistent adaptations in certain K_v channels and the G-protein coupled receptor pathways that modulate them.

Identify and Characterize Vascular Afferents

A portion of these studies were carried out in year 1 (Task 3a), while other parts were mainly confined to year 2 (Task 3b). Here we present all of the studies in the section devoted to year two progress in order to maintain the logical thrust of the experiments.

Task 3: To determine the anatomical, immunohistochemical, and electrophysiological properties of identified afferent neurons innervating the peripheral vasculature.

Task 3a: Determine electrophysiological properties of patched vascular DRG neurons labeled with DiI from peripheral arterial and venous targets in the tail and hindlimb.

Task 3b: Determine immunohistochemical phenotype for vascular DRG neurons in whole DRG sections (labeled as in Task 3a) using markers that differentiate Type 8 cells (e.g, NF-M, SubP, P2X3, IB4, ASIC1a, ASIC2a, KCNQ9).

Because alterations in the properties of vascular nociceptors have been linked to long term exposure to GW chemicals (Nutter et al., 2013; Nutter and Cooper, 2014), we initiated a series of studies to better identify the family of vascular nociceptors and their specific properties. While type 5 muscle afferents have been traced from muscle tissue, vascular afferents have been only assumed to innervate vessels because they are the only afferents traced from every tissue site examined (skin, muscle, viscera). In these studies (Task 3), we devised a new procedure to selectively trace vascular afferent neurons.

Several centimeters of the left tail vein of rats were isolated, a luminal plug placed at the cranial end, and the vein sutured closed at the caudal end. Two weeks following the injection of DiI-paste (a neuronal tracer), DRGs were excised, digested and dispersed cells were plated on 35 mm Petri dishes. Whole cell patch experiments were conducted on highly fluorescent neurons that represented those innervating the tail vein (figure 19). Rats were examined post-mortem to

confirm that DiI tracer had not leaked into surrounding tissue. Only cases without postmortem evidence of dye leakage outside the vascular compartment were accepted.

Following plating, highly fluorescent neurons were identified by fluorescent microscopy and targeted for whole cell patch experiments. After achieving the whole cell patch mode, classification procedures were applied and cell types were assigned to known and new classes associated directly with vascular innervation. Consistent with our hypotheses, we identified traced neurons that fit the current signature classification of type 8 neurons that were identified following injections of skin, muscle, and viscera (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014; see Table 1; figure 20) and presumed to be vascular due to their universal anatomical representation. This confirms our hypothesis that type 8 neurons are vascular nociceptors. Two additional afferent classes were identified in vascular tracings. Indications were that these were probably non-nociceptive, as they did not respond to capsaicin (Table 2). Caspsaicin reactivity is a widely accepted marker for nociceptors; however capsaicin insensitivity does not rule out a nociceptive function.

Table 1
Physiological Signatures

Type		Cell Size pF		Peak I_H pA/pF		I_A Peaks number		IC Kinetics	IC Base Width msec
8	n=11	76.4+/-4.9		1.06+/-0.34		4		slow	3.38+/-0.40
				p<.003					p<.01
19	n=9	63.0+/-4.2		3.78+/-0.78		4		fast	2.03+/-0.13
				p<.02					p<.01
20	n=11	80.5+/-6.3		6.63+/-0.72		4		fast	1.33+/-0.15
				p<.001					p<.001

IC: Inward Current

Table 1: Cell size and current properties associated with DiI traced vascular afferents. The amplitude of I_H currents and the width of inward currents (IC) form distinct signatures for these afferent types. Only type 8 neurons were nociceptive. 'P' values between measures indicate significant differences between adjacent values. 'P' values below type 20 measures reflect differences between type 8 and type 20.

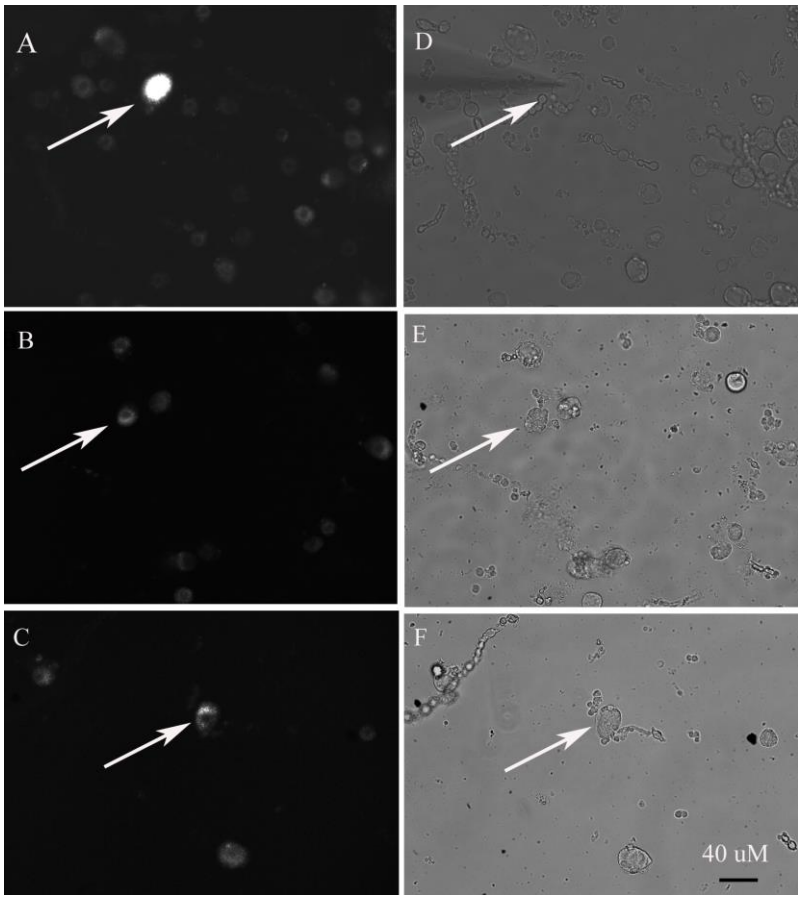

Figure 19. Di-I Fluorescent Labelled Vascular Afferents. A-C) Fluorescent cells traced from tail vein (arrows); **D-F)** Corresponding bright field images. Cells at arrows were patched and subsequently identified as type 8 (A, D), type 19 (B, E) and type 20 (C, F).

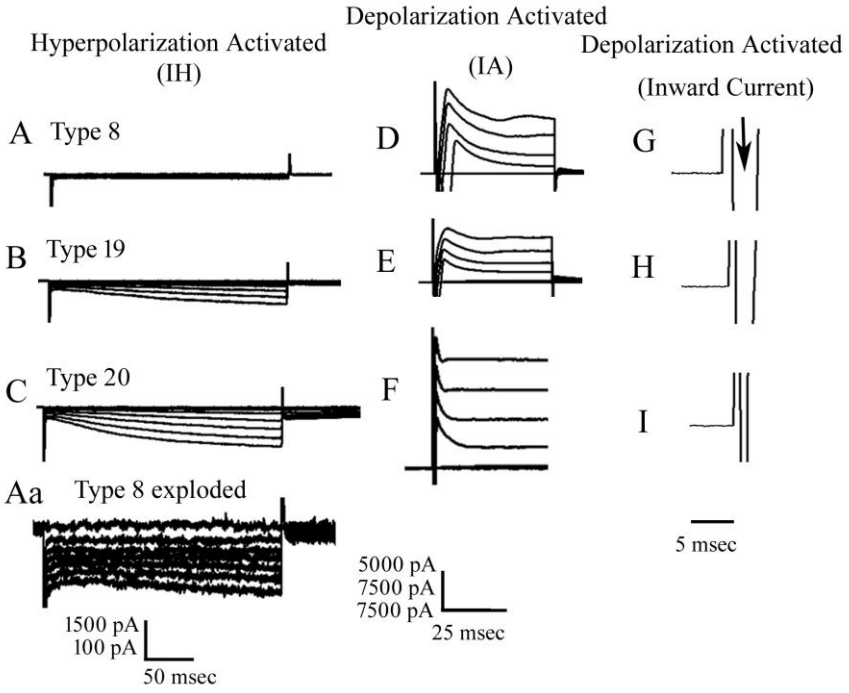

Figure 20. Physiological Signature Identifiers Associated with Vascular Afferents. A-C) Three phenotypes of vascular afferents differed by peak amplitude of hyperpolarization activated currents (I_H). **D-F)** Depolarization activated outward currents (I_A) peaks differed in kinetics but had similar voltage properties. **G-I)** Inward currents during depolarization exhibited significantly different width. See Table 1 for analysis. Classification procedures from Petruska (Petruska et al., 2002). Arrow indicates point of inward current width measurement. Aa) Exploded traces of panel 'A' shows small I_H. Vertical scale bars: A-C: 1500 pA; Aa: 100 pA; D-F: 5000, 7500 and 7500 pA respectively.

DiI traced vascular afferents were sorted by their voltage dependent physiological signatures (figure 20A-C). In order to identify important physiological characteristics of these neurons, they were exposed to ACh (500 μM), capsaicin (1 μM) and pH 6.0 solutions. Presentation of ACh and pH 6.0 were randomized. Capsaicin was always presented last in the sequence.

A portion of fluorescent vascular (tail vein) neurons had physiological signatures associated with type 8 neurons (figure 20A, D, G). We had originally identified and characterized type 8 afferents in skin, viscera and muscle tissue using the DiI tracing technique (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014). The functional properties of vascular afferents we traced from the tail vein exhibited many of the same functional properties we observed in type 8 cells traced from other tissue sites; however a more complex pattern emerged than previously reported (Petruska et al., 2002; Rau et al., 2005). The functional characteristics resembled both type 8a and 8b neurons: 1) capsaicin sensitive (CAPS); 2) fast (Type 8a) or slow (Type 8b) kinetic ASIC responders (Table 2, figure 21b and 21f); and 3) expression of neuropeptides CGRP and SP (substance P; figure 25).

Capsaicin sensitivity is a marker of nociceptive function. The powerful capsaicin evoked currents of DiI traced vascular, type 8 neurons, are consistent with type 8 nociceptors in skin, muscle and viscera (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014). Other consistent properties indicate a high degree of proton sensitivity (ASIC-like responders) that would be required by afferents that monitor tissue pH (Jiang et al., 2006). In contrast, responses to ACh were mixed and exhibited some properties we had not previously reported. Most of the vascular nociceptor population exhibited irreversible baseline shifts consistent with a, muscarinic, G-protein coupled closure of a K_v7 current (figure 21C; Table 2). This is consistent with our observations that NTPB exposed nociceptors express modified K_v7 physiology (Nutter et al., 2013). Others vascular nociceptors exhibited a slow desensitizing, nicotinic, inward current of the sort we have previously characterized in type 8 cells (Rau et al., 2005).

Table 2
Functional Properties

Type		Capsaicin pA/pF		pH 6.0 pA/pF	pH 6.0 Kinetics	ACh pA/pF	ACh kinetics
8	n=11	74.82+/-10.4		25.39+/-7.37	Desen: n=9	7.62+/-6.03	Slow Desen: n=4
					Mixed: n=2	1.83+/-0.54	Base Shift: n=5
				p<.01			
19	n=9	0		2.08+/-0.27	Non-Desen: n=8	33.4+/-5.6	Slow Desen: n=9
				NS			
20	n=11	0		3.92+/-1.09	Non-Desen: n=9	21.05+/-5.27	Slow Desen: n=5
				p<.02			No Response: n=6

Table 2: Chemical response properties of afferents traced from vascular sites. Only type 8 neurons were clearly nociceptive (capsaicin sensitive); although the lack of capsaicin sensitivity does not exclude nociceptive function. Application of pH 6.0 evoked distinct desensitizing (type 8) and non-desensitizing currents (types 19 and 20). Presentation of ACh (500 uM) evoked large slow desensitizing currents in non-nociceptive types 19 and 20, but irreversible base shifts in nociceptive type 8. 'P' values between measures indicate significant differences between adjacent values. 'P' values below type 20 measures reflect differences between type 8 and type 20.

Table 3
Action Potential Properties

Type		AP Duration msec		AP Hyperpol msec
8	n=9	9.02+/-0.53		114.66+/-14.5
		p<.001		NS
19	n=5	4.76+/-0.25		98.08+/-28.6
		NS		NS
20	n=5	4.17+/-0.40		92.83+/-41.3
		p<.001		NS

Table 3: Action potential properties of afferents traced from vascular sites. Type 8 neurons exhibited significantly longer duration APs than Types 19 and 20. The latter two did not differ in this regard. Afterhyperpolarization (AP Hyperpol) duration was similar across the three groups. 'P' values between measures indicate significant differences between adjacent values. 'P' values below type 20 measures reflect differences between type 8 and type 20. NS: non-significant

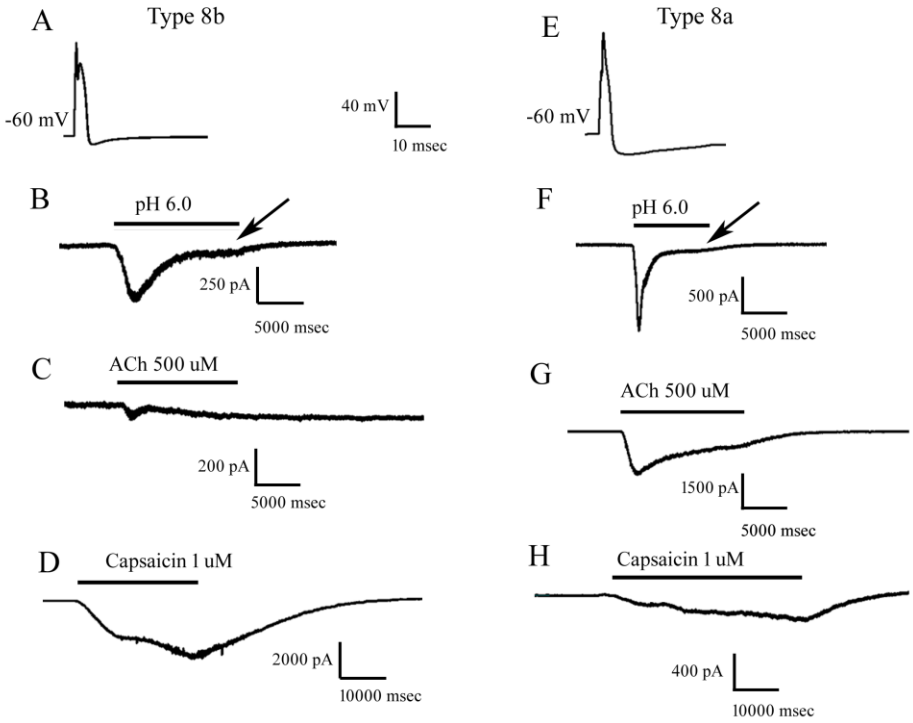

Figure 21. Chemical Response Characteristics of Vascular Nociceptors. A-D) Representative responses of type 8 vascular nociceptor to pH 6.0, ACh 500 uM and Capsaicin 1 um. Five traced afferents exhibited this pattern. Note that the response to ACh (C) is not a true evoked current as the trace never returns to baseline. A baseline shift usually indicates the closing of a resting K^+ current, but that has not been verified by experiment. **E-H)** Representative responses of a type 8 vascular nociceptor to pH 6.0, ACh 500 uM and Capsaicin 1 um. Two traced afferents exhibited this pattern. Note the powerful nicotinic currents of this group (G) that differed from those in 'C' when ACh was applied. Horizontal bars indicate the duration of chemical application. Arrows point to a non-desensitizing component of the response to pH 6.0.

In our tracing studies, we discovered two additional classes of vascular afferents that were distinguished from type 8 by their signature identifiers (Table 1; figure 20), as well as by their distinct functional properties. We have designated these as types 19 and 20. The chemical response properties of these two classes did not differ from each other (figure 22), so it is not certain that the distinction between type 19 and 20 (Table 1), is a worthwhile distinction. Still, many additional properties of these afferents, yet to be discovered, may prove them to be functionally as well as physiologically distinct. Capsaicin insensitive (CAPI) type 19 afferents and type 20 afferents both responded to pH 6.0 with small, non-desensitizing, K2p –like currents (Table 2; figure 22b and f), as well as powerful nicotinic responses to ACh (figure 22c and 10g). Capsaicin reactivity was absent (D and H) and could indicate a non-nociceptive function for types 19 and 20. Possibly these afferents were sensitive to vasolidation or oxygen content via mechanically sensitive and or oxygen sensitive K2p channels. It is not known whether such channels are expressed in these newly identified vascular afferent phenotypes.

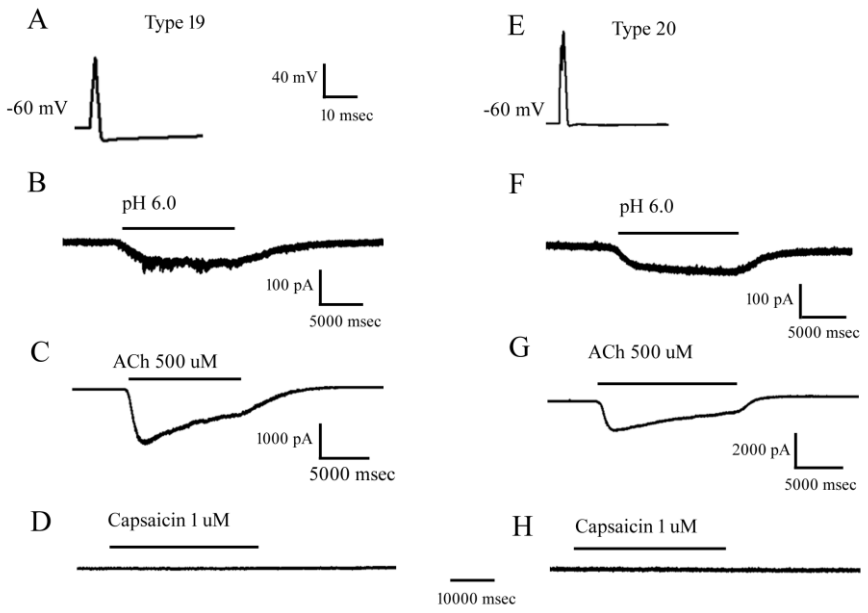

Figure 22. Chemical Response Characteristics of Non-Nociceptive Vascular Afferents. A-D) Representative responses of a type 19 vascular nociceptor to pH 6.0, ACh 500 uM and Capsaicin 1 um. Seven traced afferents exhibited this pattern. **E-H)** Representative responses of a type 20 vascular nociceptor to pH 6.0, ACh 500 uM and Capsaicin 1 um. Eight traced afferents exhibited this pattern. Within the limits of our experiments, these two classes of neurons exhibited identical chemical response characteristics. Horizontal bars indicate the duration of chemical application.

Additional tracing studies examined the immunohistochemical properties of vascular afferents (Tasks 3b and 3d). In these experiments, DiI tracer was injected into the proximal portion of the tail vein as it originates from the internal pudendal vein. Rats were sacrificed and whole DRG were excised and sectioned. DiI-labeled vascular afferents were identified in whole DRG sections (Task 3b). Multiple staining procedures were used to determine the immunohistochemical phenotypes of these neurons. In addition, experiments were performed in sections of vascular tissue to determine the immunohistochemical phenotype of peripheral terminal axons (Task 3d). As shown in figures 24, 25, 26, 27, 28, and 29 vascular afferents were mainly found in the small to medium sized cell body range, consisted of both myelinated and unmyelinated groups and frequently expressed neuropeptides CGRP and/or SP along with the capsaicin-sensitive TRPV1 receptor (Figs. 23-26). The latter is considered a marker for nociceptors.

Of the markers tested, the most predominant was CGRP, but it was seldom co-localized with NF-M, suggesting these afferents are primarily unmyelinated. This interpretation was supported by the co-labeling of CGRP axons in the wall of the vein with Peripherin (another marker for small, unmyelinated DRG neurons). We also found that the skin marker for unmyelinated neurons, IB4, was seldom found in vascular afferent tracings (2%) (Taylor et al 2009). The small-to-medium diameter cell bodies, CGRP/SP and TRPV1 labeling, and a low degree of IB4 lectin binding are consistent with Type 8 neurons previously described in our in vitro patch clamp studies (Rau et al., 2007; Rau et al., 2014). TRPV2+ and TRPV1- neurons did not frequently co-label CGRP and had larger cell diameters, suggesting a non-nociceptive role, and possibly corresponding to the Type 19 and Type 20 neurons described in patch clamp studies. The sensory innervation of the adjacent artery was significantly less than the vein, providing additional evidence that the venous sensory signal is more robust and important in vascular nociceptive mechanisms.

To determine types of stimuli driving vascular nociceptive afferents, we recorded from A- and C-fiber vascular axons during *in vivo* experiments (Task 3c). Response properties of vascular afferents to perfusion of the isolated proximal internal pudendal vein origin of the tail vein were obtained from a small branch of the S1 spinal nerve in urethane-anesthetized rats. Conduction velocity measurements placed the afferents in the unmyelinated C-fiber category (less than 2.0

m/sec) or the A-delta and small A-beta fiber categories (3-35 m/sec). Receptive fields were generally very small and discrete with the exception of some C-fibers which had branching receptive fields spread over a longer section of vessel wall. Very few of the units were spontaneously active although it was more common in smaller fibers. Perfusion of acidified physiological saline (pH of 5.0 or 3.5) produced firing in most units as did the application of very warm (42-45 degree C) saline, both responses consistent with presence of the TRPV1 receptor. Long lasting discharges associated with brief warm saline infusion were reversed with room temperature saline infusion.

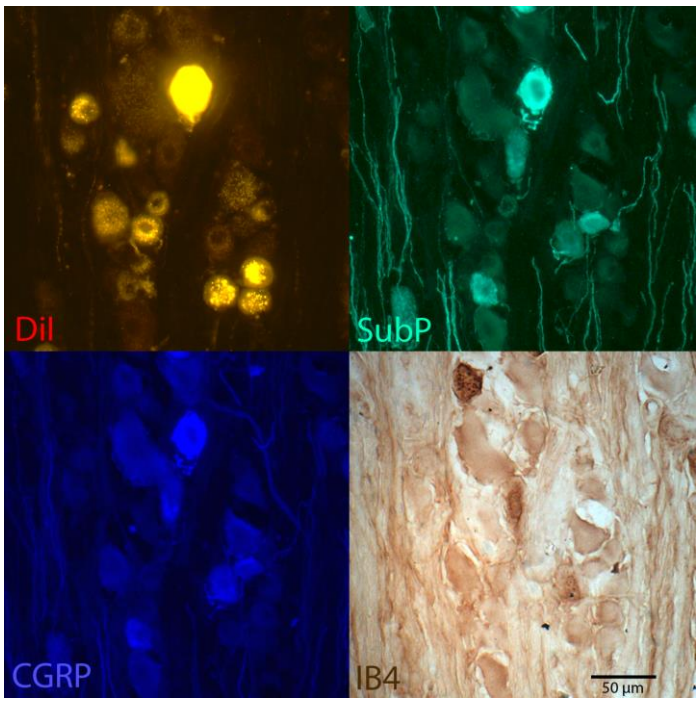

Figure 23. Immunohistochemical (IHC) Phenotype of Labeled Vascular Afferent Nociceptive Neuron. Data from one cryosection taken from an L6 DRG. The four views of the same section under different fluorescence filters/light showing a single vascular DiI-labeled cell (white arrow) containing positive co-labeling for SubP (SP) and CGRP, but negative for IB4 (a marker for unmyelinated neurons).

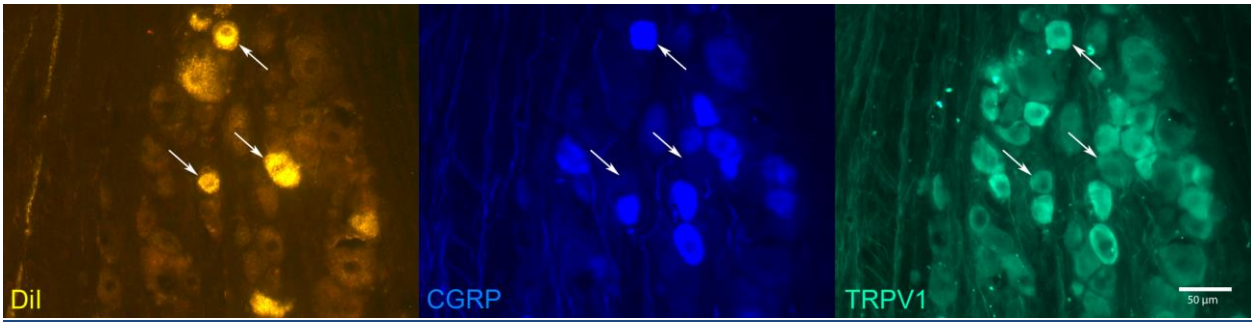

Figure 24. Immunohistochemical (IHC) Phenotype of Labeled Vascular Afferents. Data from one cryosection taken from an L6 DRG. The three views of the same section under different fluorescence filters/light showing a single vascular DiI-labeled cells (white arrows) containing positive (upward arrows) and negative (downward arrows) co-labeling for CGRP and TRPV1.

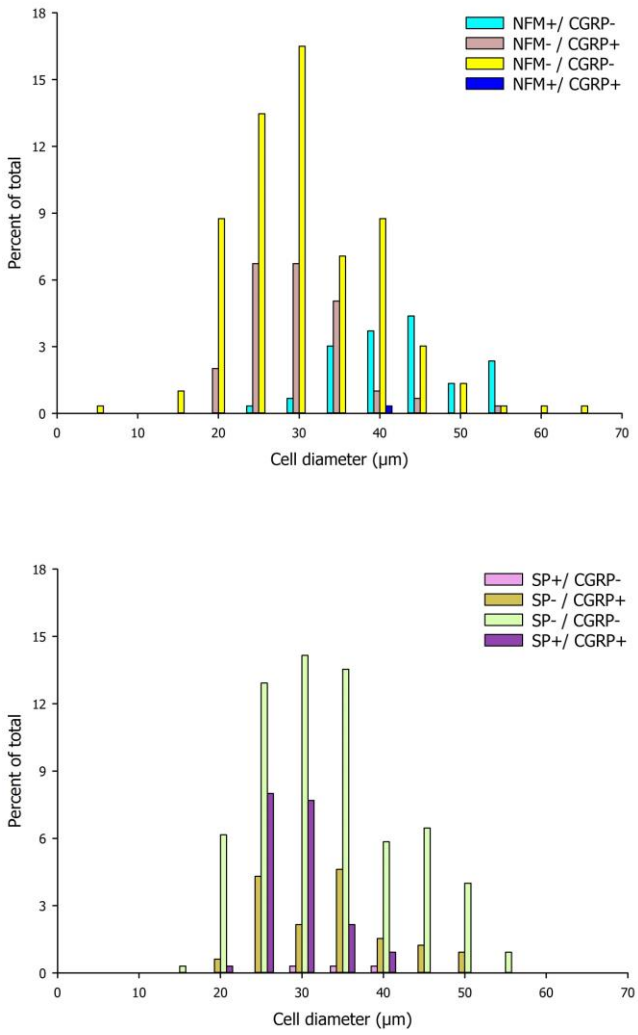

Figure 25. Co-labeling with CGRP, SubP (SP), and Neurofilament-M (NFM). Morphometric and immunohistochemical co-labeling data collected from previously identified DiI+ vascular afferent DRG neurons. A total of 325 and 297 cells were analyzed in A and B, respectively. Cell diameters for NF-M positive venous afferent neurons were significantly bigger (41.4 ± 1.1µm) compared to NF-M negative cells (31.2 ± 0.5µm) and were almost entirely CGRP negative.

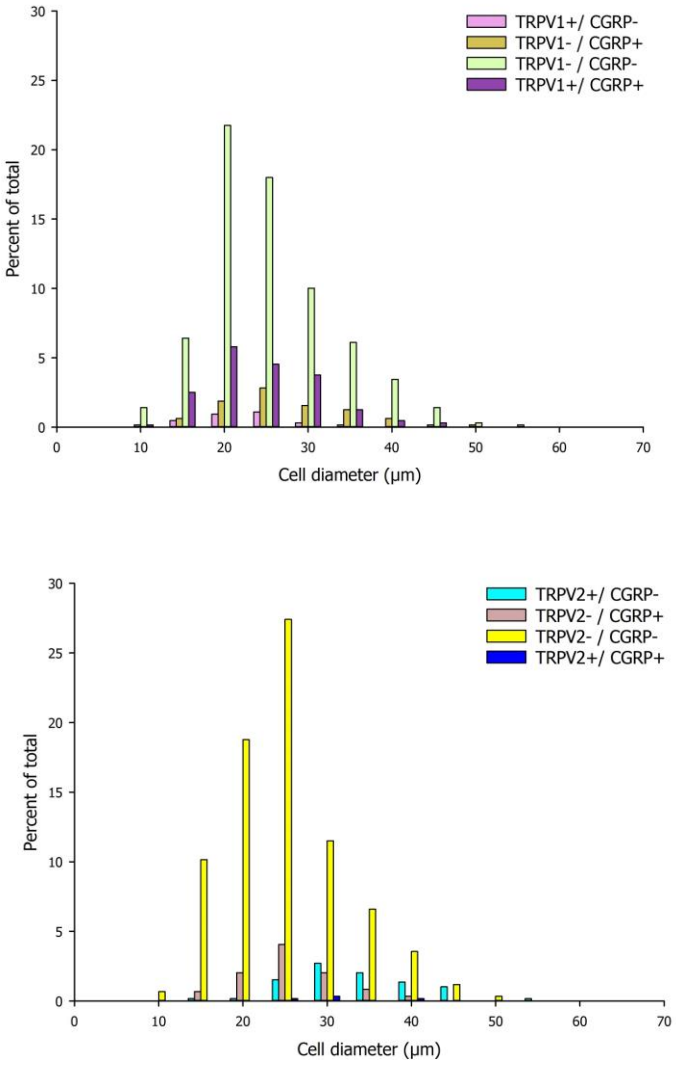

Figure 26. Co-labeling with TRPV1, TRPV2, and CGRP. Morphometric and immunohistochemical co-labeling data collected from previously identified DiI+ vascular afferent DRG neurons. A total of 639 and 591 cells were analyzed in A and B, respectively.

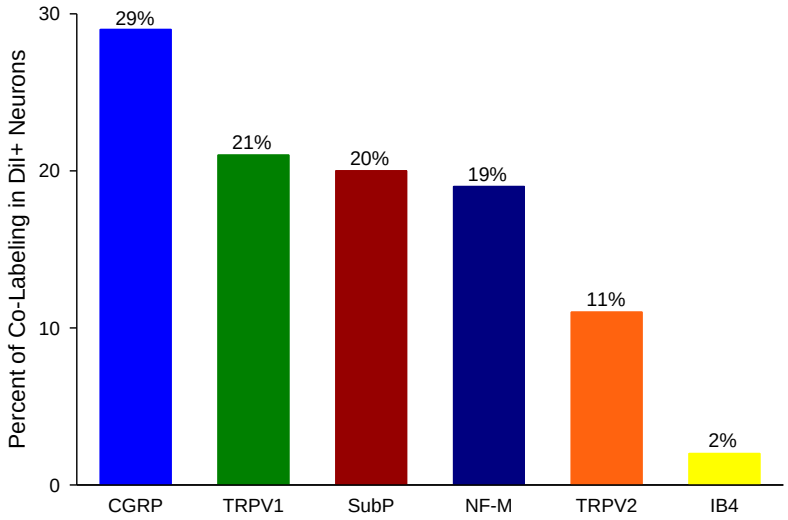

Figure 27. Frequency of IHC marker co-labeling in Di-I+ vascular sensory neurons traced from the lateral tail vein. Of the markers tested, CGRP was found most frequently (29%, 208/622 cells), followed by TRPV1, SubP (SP), NF-M, and TRPV2. Note that IB4 was found in very few vascular sensory neurons

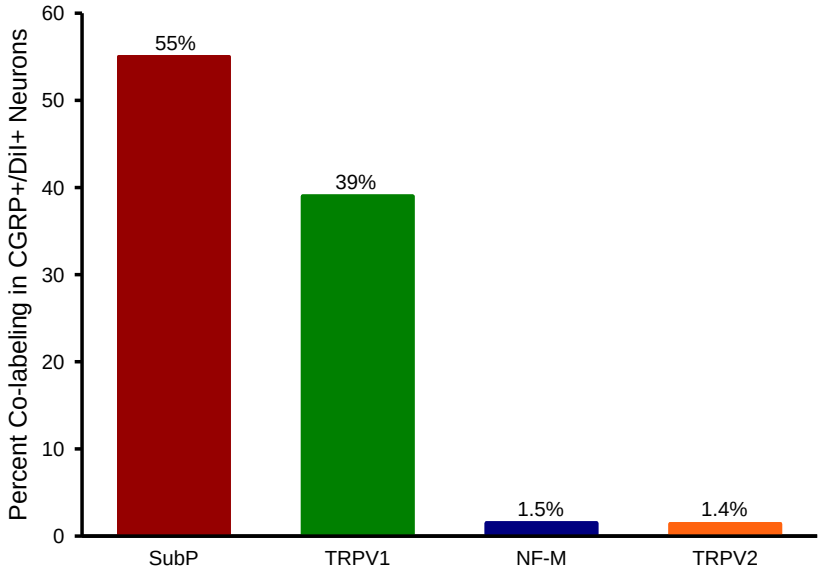

Figure 28. Frequency of IHC marker co-labeling in CGRP+ vascular sensory neurons traced from the lateral tail vein. Of the markers tested, SubP (SP) was co-localized in most CGRP+ neurons (55%, 63/114 cells) and TRPV1 was found in 39% (148/383 cells) suggesting that capsaicin-sensitive neurons are peptidergic, particularly for CGRP. Approximately 90% of the TRPV1+ vascular nociceptors contain CGRP. Note that NF-M and TRPV2 were found in very few CGRP+ vascular sensory neurons suggesting that CGRP+ neurons are mostly unmyelinated with small to medium diameters.

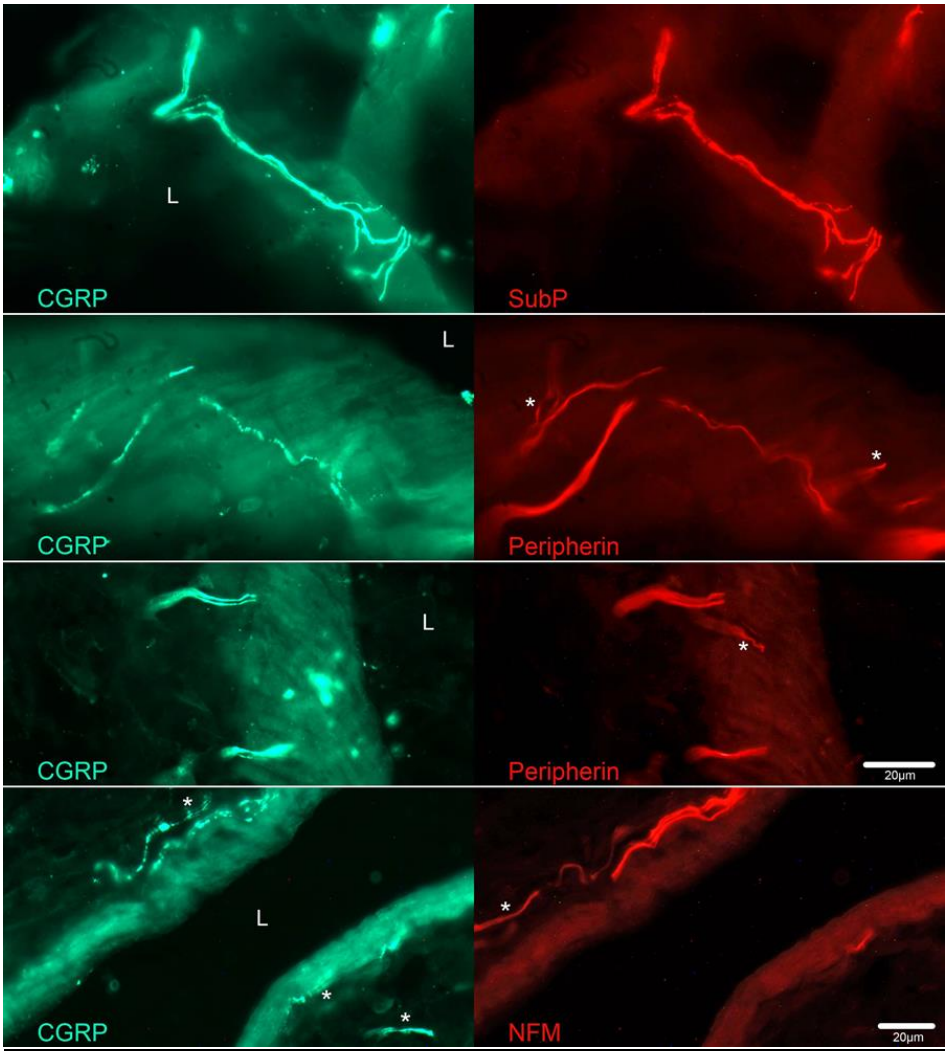

Figure 29. Immunohistochemical profiles of vascular afferent fibers innervating the wall of the proximal tail vein. Four panels, each demonstrating the presence or absence of two markers processed with a green fluorophore secondary antibody (AlexaFluor 488 left) and a red channel fluorophore (AlexaFluor 594 right). Note the frequent co-labeling of CGRP+ axons with SubP (top panel), and peripherin (middle two panels), but only occasionally with NF-M (bottom panel), suggesting that most CGRP+ vascular afferent peripheral terminals are from small diameter unmyelinated DRG neurons. *, axons which contain only one marker; L, location of the vessel lumen.

Acute Studies with Permethrin.

Pyrethroids, like permethrin, produce powerful changes in the physiology of TTXs and TTXr Na_v proteins of DRG neurons (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996; Song and Narahashi, 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi, 2001; Jiang et al., 2013). There have been few published reports documenting the influence of permethrin on K_v channels (Jiang et al., 2013). In order to determine if the chronic changes in K_{DR} current properties might be initiated by the direct influence of permethrin during the time of exposure, we exposed muscle or vascular nociceptors to this pyrethroid. Using a custom-made glass pipette application system (see Methods), we superfused vascular or muscle nociceptors with 10 μM permethrin or .001% ETOH vehicle for 2 minutes. Using a tail current analysis, we did not observe any changes in the voltage dependence (not shown), amplitude or deactivation kinetics of type 5 muscle or type 8 vascular nociceptor K_{DR} (figure 30).

Task 4: To examine the acute effects of permethrin on K2p and K_{DR} proteins expressed in skin, muscle and vascular nociceptors

Task 4a: Assess the Acute Influence of Permethrin on K2p Physiology in Skin, Vascular and Muscle Nociceptors. [Because K2p currents were not modified by GW chemicals, this Task 4a was not executed]

Task 4b: Assess the Acute Influence of Permethrin on K_{DR} Physiology (Activation, Deactivation, Amplitude) in Skin, Vascular and Muscle Nociceptors.

Task 4c: Determine Whether K2p Proteins (KCNK3, KCNK5, KCNK9) Differ with Respect to Acute Permethrin Vulnerability [Because K2p currents were not modified by GW chemicals, this Task 4c was not executed]

Task 4d: Determine K_{DR} Proteins ($Kv_{1.1}$, $Kv_{1.2}$, $Kv_{1.3}$, $Kv_{1.6}$) Differ with Respect to Acute Permethrin Vulnerability. [We were not able to separate these KDR components; Therefore Task 4d was not executed]

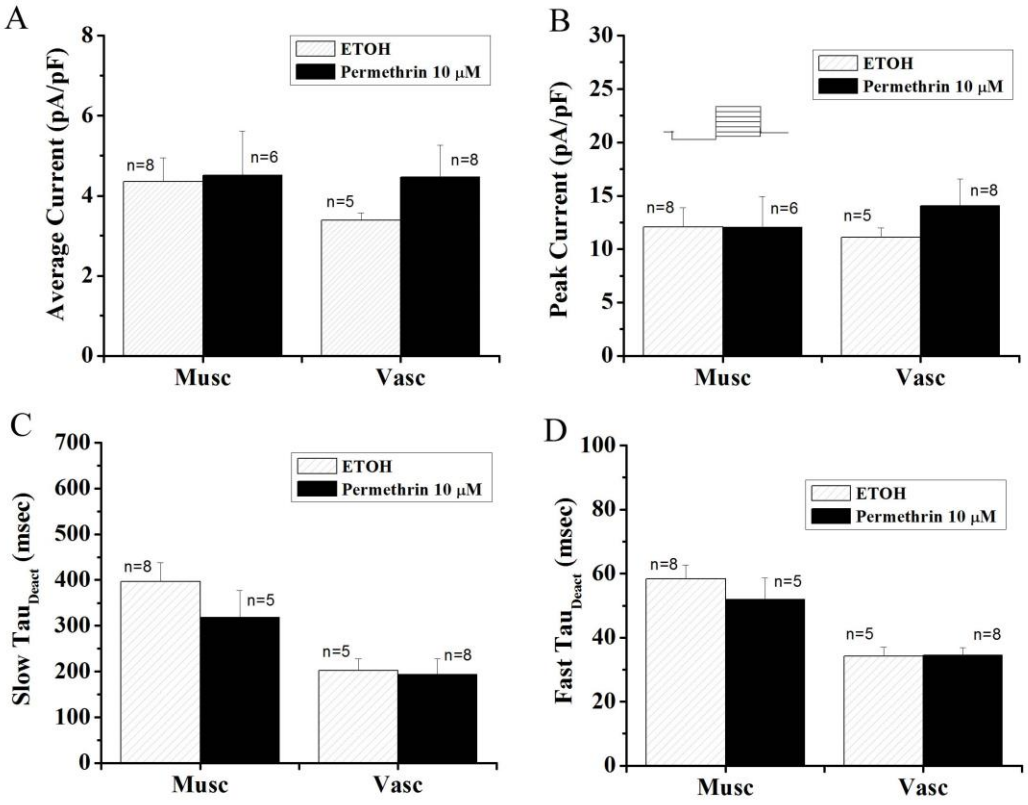

Figure 30. Acute Exposure to Permethrin did not Modify K_{DR} Current Amplitude or Kinetics. The K_{DR} current was evoked in the presence of ETOH or 10 μM permethrin. **A and B)** There were no significant shifts in the peak or average current in either muscle or vascular nociceptors. **C and D)** Permethrin did not induce any significant shifts in the slow or fast component of the Tau_{Deact} in either muscle or vascular nociceptors. Evoked currents were averaged (-60 to 10 mV) and normalized for cell size. Comparisons were made between permethrin (10 μM) and ETOH (.001%) treated cases. The voltage protocol is shown as an insert in panel B. From a V_H of -60 mV, cells were hyperpolarized to -100 mV for 2 sec then stepped for 500 msec from -80 to 20 mV in 10 mV steps.

Discussion

In a series of behavioral studies, we demonstrated that increasing the duty cycle exposure to chlorpyrifos and PB produced signs consistent with a delayed myalgia and arthralgia that mimicked the emergence of GW illness pain reported by many veterans (Haley et al., 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006). Parallel studies in these same GW chemical exposed rats, revealed a pattern of molecular maladaptations in deep tissue nociceptors that were consistent with increased nociceptor excitability and pain 12 weeks after exposure to GW agents had ended.

Behavior Studies.

It was clear that the behavioral model showed substantial improvement. By increasing the frequency of anticholinesterase administration, we began to observe persistent changes in several behavioral indices. In one major test, we observed that a pattern of increased resting and decreased movement, with normalized movement rate, emerged 12 weeks after chemical exposures had ended. This pattern of rat activity measures might represent a myalgia and/or arthralgia, emerging in a delayed manner, much like that reported by most (~75%), but not all, Gulf War veterans (Kroenke et al., 1998). In year two, replications of this procedure produced behavioral outcomes that were similar (Group Y2A; figure 8) or somewhat divergent (Group Y2B; figure 10) from the year one findings (Group Y1C; figure 9). The divergent resting and movement outcomes in Group Y2B appeared to derive from aberrant control animal behavior rather than the behavior of animals' exposure to GW agents. Although the model is headed in the right direction some improvement is definitely desirable.

Molecular Studies on K_v7. Although the activity measures of the behavioral studies were not totally consistent, molecular studies generally supported the interpretation of a delayed myalgia. We hypothesized that increasing the anticholinesterase component of the GW chemical exposure might derange the relationship between depolarizing and hyperpolarizing forces that govern neural excitability in muscle and vascular nociceptors. Increasing the duty cycle of PB exposure

from 50 to 100% and that of chlorpyrifos from 7 to 14% produced a pattern of rat behavior could be interpreted as a myalgia/arthralgia. During time periods in which open field activity measures suggested pain, tests on muscle nociceptor physiology revealed a significant decline in K_v7 (and K_{DR}) activity. This decline in K_v channel activity, in muscle nociceptors, was clearly consistent with the presence of a myalgia (Du and Gamper, 2013; Tsantoulas and McMahon, 2014). It is noteworthy that K_v7 (and K_{DR}) ion channel activity was elevated at 8 weeks post-exposure before it eventually declined at the 12 week delay.

Intracellular pathways that produce long term changes in the expression of K_v7 are not thoroughly understood, but have been linked to the development of neuropathic-like pain behaviors exhibited in some animal models of chronic pain (Rose et al., 2011; Cai et al., 2013). Neural activity can promote the expression of K_v7, through the Ca^{2+}/calcineurin dependent induction and nuclear translocation of members of the NFAT family of transcription factors (nuclear factor of activated T cell; Rao et al., 1997; Graef et al., 1999; Smith, 2009; Zhang and Shapiro, 2012). There is evidence that this pathway functions as an excitation driven negative feedback loop through an 'activity reporter' that is sensitive to action potential enabled entry of Ca^{2+} via the L-type voltage activated Ca^{2+} channel ($Ca_v1.3$; Mucha et al., 2010; Zhang and Shapiro, 2012). Exposure to pyrethroids, such as permethrin, could contribute substantially to drive this regulatory pathway due to their influence on Na_v inactivation and deactivation (Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006; Jiang et al., 2013). The permethrin induced broadening of the Na_v component of the nociceptor action potential would enhance Ca^{2+} entry through all voltage dependent Ca^{2+} channels to produce a false report of excessive activity. Pyrethroid dependent enhancement of Ca^{2++} entry has been shown to promote the expression of BDNF (brain derived neurotrophic factor) and other neurotrophins (Imamura et al., 2006; Cao et al., 2011; Ihara et al., 2012; Matsuya et al., 2012; but see Imamura et al., 2000). BDNF is one of several neurotrophins that drive expression of NFAT transcription factors (Groth and Mermelstein, 2003; Groth et al., 2007; Kim et al., 2014).

Calcium enters neurons during action potential depolarization. Ca^{2+} entry into nociceptors tends to be greater due to their broad action potentials; this is due, in part, to the slow inactivation kinetics of $Na_v1.8$. If exposure to permethrin induced a disproportionate entry of Ca^{2+} into DRG nociceptive neurons, relatively minor activity of nociceptors could result in gene transcriptions

that are normally initiated by the intense activity associated with inflammatory or post-traumatic discharge. Muscle nociceptors would be a prime candidate for such a mechanism as, unlike other nociceptor populations that are mainly inactive, and would not provide a path for Ca^{2+} entry, muscle nociceptors are activated by ischemic pain related to muscle exertion. Ischemic muscle pain would be a frequent experience for the deployed warfighter. Accordingly, we can hypothesize a scenario where exaggerated Ca^{2+} entry, under the influence of permethrin exposure, could drive the expression of NFAT transcription factors to substantially elevate expression of K_v7 (as we observed at 8 weeks post exposure). There are also indications from our studies that the maladaptive entry of excessive calcium might persist into the post exposure period. Following a 60 day exposure to permethrin, chlorpyrifos and PB, we have documented a slowing of $Na_v1.8$ inactivation kinetics that persisted for 8 weeks and coincided with the upregulation of K_v7 (Nutter et al., 2014). The slowing of $Na_v1.8$ kinetics implies a continuing dysregulation of Ca^{2+} entry that could falsely report excessive discharge rates and spur BDNF/NFAT expression long after exposures had ended (Cao et al., 2010; Cao et al., 2011). In the present study, we found that both action potential duration and afterhyperpolarization were significantly longer in muscle nociceptors 12 weeks after GW chemical exposure. Because the family of Ca^{2+} activated K^+ channels govern the AHP, the longer AHP in muscle nociceptors implies that enhanced, Ca^{2+} entry persists 12 weeks after exposure. The known acute and possible chronic impact of pyrethroids on voltage activated Ca^{2+} channels could further magnify AP dependent Ca^{2+} entry (Shafer and Meyer, 2004; Neal et al., 2010).

Ultimately, it is the down regulation of K_v7, in muscle nociceptors, that we detected 12 weeks post exposure, which is likely to be a key event in the delayed emergence of the pain-like behavior syndrome. NRSF/REST (neuron-restrictive silencer factor; repressor element 1–silencing transcription factor) has been identified as a transcription factor that suppresses the expression of several K_v channels, including K_v7 ($K_v4.3$, $K_v3.4$, $K_v7.2$, $K_v7.3$**;** Chien et al., 2007; Uchida et al., 2010; Mucha et al., 2010; Rose et al., 2011). We have demonstrated the expression of $K_v7.3$ in both type 5 muscle and type 8 vascular nociceptors (Nutter et al., 2013). In a nerve injury model of neuropathic pain, the appearance of chronic pain-like behaviors was accompanied by the elevated expression of NRSF/REST (Uchida et al., 2010; Rose et al., 2011). Interestingly, significant REST expression was delayed 15-30 days following neural insult and well past the onset of pain behaviors (Rose et al., 2011). Delayed expression of REST could

play a key role in the delayed decline of K_v7 activity and the appearance of pain-like activity patterns in our model. Factors contributing to the time dependent expression of REST have not been determined.

Molecular Studies on $K_{DR.}$ While decreased expression of K_v7 is likely to be an important event leading to increased neural excitability, we also observed down regulation of total K_{DR} channel activity, in muscle nociceptors, during those periods when rats exhibited pain-like behaviors. It is possible that the reduction of K_v7 activity may have only been part of a broader decline of K_v channels expressed in muscle nociceptors. The lack of any functional alterations of the K2p TASK channel family indicates that K_v channels are the focus of important molecular maladaptations induced by GW chemicals.

Recent studies have identified an antisense mRNA that induces broad reductions of total K_v current in DRG neurons. Expression of the long non-coding antisense mRNA *kcna2* (AS mRNA *kcna2*) has been shown to decrease total K_v current in DRG neurons, increase neuronal excitability and produce neuropathic pain-like behaviors (Zhao et al., 2013; Li et al., 2015). Long antisense mRNA *kcna2* expression is very low in normal DRG, but rises sharply after nerve injury (Zhao et al., 2013). It is expressed only in select populations of DRG neurons that make up the medium and large diameter pools (Zhao et al., 2013; Fan et al., 2014). Both the type 5 muscle and type 8 vascular nociceptors come from the medium diameter pool of DRG neurons (Petruska et al., 2002). While the decline in the activity of K_{DR} and K_v7 ion channel proteins might be due to increased expression of long antisense mRNA *kcna2*, there is no demonstrated linkage between permethrin, chlorpyrifos or PB and the expression of *kcna2.*

Muscarinic Influences on K_v Activity. Over relatively long time periods, 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_v channels are also regulated acutely by pathways leading to rapid changes in membrane excitability arising from events such as protein phosphorylation/dephosphorylation and/or PIP_2

expression/cleavage. These events often result from activation of G-protein coupled receptors that are set into motion by extracellular agents (e.g., ACh, bradykinin, serotonin, proteinases, ATP; Brown and Passmore, 2009; Du and Gamper, 2014). For our purposes, the action of ACh at muscarinic receptors is particularly relevant. Given the potential disturbance to ACh sensitive receptors that are known to occur secondary to chronic exposure to anticholinesterases (Liu et al., 1999; Huff et al., 2001; Zhang et al., 2002; Abou-Donia et al., 2003;; Abdel Rahman et al., 2004a; Abdel Rahman et al., 2004b; Abou-Donia et al., 2004; Pung et al., 2006; Zou et al., 2006; Proskocil et al., 2010), it would not be surprising that our GW exposure protocol would alter the balance of muscarinic activity. The persistence of this modulation, 12 weeks after exposures had ceased, suggests it may be an important component of the molecular basis of GW pain. As we have now demonstrated that muscarinic agonists are not only capable of increasing excitability, via inhibition of K_v7, but are also capable of directly activating muscle and vascular nociceptor discharge (figure 18; see also the attached manuscript; 'Muscarinic Receptor Maladaptations following Exposure to Gulf War Illness Chemicals'), the persistent and disturbed influences of GW toxicants on muscarinic receptors may be especially important.

Our studies have demonstrated that chronic exposure to GW agents disturbs the normal relationship between ACh muscarinic receptors and certain K_v proteins. Twelve weeks after exposure to GW toxicants, the muscarinic up-regulation of an inhibitory K_{DR} was diminished; simultaneously the muscarinic down regulation of inhibitory K_v7 channel proteins was enhanced. Action potential burst discharges, following muscarinic agonists, indicated not only a net pro-excitatory shift in inhibitory influences on nociceptor membranes (see figure 18), but also that muscarinic activation was itself a route to nociceptor discharge and pain. While we were unable to identify the specific K_{DR} target(s) of muscarinic modulation, it was shown that it was not $K_v2.1$ or $K_v2.2$. Highly specific inhibitors of these K_v proteins (StTX) could not block or reduce muscarinic modulation. The composition of the StTX insensitive K_{DR} is not known, but candidates include $K_v1.1$ and $K_v1.2$. There is evidence that $K_v1.1$ currents are amplified by activation of the M_1 muscarinic receptor (Kruse et al., 2012); while other evidence indicates that $K_v1.2$ is typically depressed by M_1 activation (Cachero et al., 1998; Tsai et al., 1999). Moreover there is overlap between those transcription factors regulating expression of K_v channels and those regulating M receptors (M_4 and REST; Mieda et al., 1997; Saffen et al., 1999) However, our experiments using the $K_v1.1$ and $K_v1.2$ toxin, Maurotoxin, did not demonstrate a substantial

Maurotoxin sensitive current in either muscle or vascular nociceptors. Therefore, that particular K_{DR} that was amplied via one or more ACh muscarinic receptors is uncertain; nor is it clear which M receptors were modified by the GW chemical exposure.

Our discovery of a mAChr dependent burst discharge (MDBD) mechanism in nociceptive neurons suggests that the convergence of the GW chemical disturbed K_v7, K_{DR}, and mAChr coupling pathways may come to be expressed as a maladaptive high frequency discharge in muscle and vascular nociceptors. MDBD could play a key role in the generation of GWI pain. In sensory neurons, MDBD could contribute to postsynaptic spinal cord neuroplasticity (i.e., central sensitization) that is associated with some chronic pain syndromes (Willis, 2001; Latremoliere and Woolf, 2009). Central sensitization, can be initiated by high frequency discharge dependent release of the paracrine vasoactive neuropeptide SP from primary afferent nociceptors. In the spinal cord, afferent derived SP can produce prolonged depolarizations of post-synaptic relay neurons that relieve Mg^{++} blockade of the NMDA receptor. The activation of the NMDA receptor is essential for central sensitization (Dougherty et al., 1993; Willis, 2001). Moreover, central release of the coexpressed neuropeptide CGRP activates resident microglia that synthesize and/or release of a number of cytokines (IL-1β, IL-6) chemokines (CCL2), nitric oxide and other pro-inflammatory agents (Li et al., 2008; De Corato et al., 2011; Malon et al., 2011) in the CNS. In DRG, SP, CGRP, $K_v7.3$ and $Na_v1.9$ are known to be expressed in type 5 and type 8 nociceptors (Petruska et al., 2002; Nutter et al., 2013; Nutter and Cooper, 2014).

An eight week exposure to permethrin, chlorpyrifos and PB, suppresses K_v7 activity, increases $Na_v1.9$ amplitude, enhances muscarinic coupling to K_v7, K_{DR} and potentiates an MDBD burst to a broadly active muscarinic agonist (Nutter and Cooper, 2014; Nutter et al., 2015). It is likely the SP and CGRP would be released by the high frequency discharges observed in MDBD (Holz et al., 1988). By shifting the balance of muscarinic modulation of K_v7, a GW chemical induced maladaptation functionally expressed as an exaggerated MDBD could contribute to the development and chronicity of Gulf War Illness pain syndromes through central sensitization. We have shown that increasing the exposure to anticholinesterases played an important role in the development of a delayed myalgia. The consequence of heightened exposure to anticholinesterases is consistent with the pattern of a modified mAChr dependent phenomena (K_v7, K_{DR}) and its functional expression through amplification of MDBDs.

4. Key Research Accomplishments

- Intensified Exposure to Anticholinesterases Produces Pain-like Behaviors 12 Weeks Post-Exposure
- Exposure to GW Chemicals Modulates K_{DR} Channel Activity 8 and 12 Weeks Post-Exposure
- Exposure to GW Chemicals Modulates K_v7 Channel Activity 8 and 12 Weeks Post-Exposure
- Altered Channel Activity Occurs in Both Vascular and Muscle Nociceptor Phenotypes
- The Decline of K_v7 Activity in Muscle Nociceptors at 12 weeks post Exposure is consistent with myalgia
- Traced and Characterized Vascular Nociceptors Exhibit Currents with K_v7 Current-like Properties
- Non-Nociceptive Vascular Afferents Were Identified and Exhibited K2p-like Currents
- K2p Currents were not Modified by Exposure to GW Chemicals
- K_{DR} Currents Exhibit Decreased Modulation by Muscarinic Agonists in GW Exposed Groups
- K_v7 Currents Exhibit Increased Modulation by Muscarinic Agonists in GW Exposed Groups
- Muscarinic Agonists Produce Powerful Action Potential Discharges in Muscle Nociceptors in GW Exposed Groups

5. **Conclusion:**

We have now shown that an intensified exposure to GW chemicals (PB, permethrin, chlorpyrifos) can produce pain-like behaviors that last at least 12 weeks after exposure. Although all indices of pain were not consistently replicated by subsequent experiments, the parallel changes in molecular status was consistent with an emergent myalgia. Some additional efforts along the lines of the present approach to dosing may bring about a more consistent pain syndrome. By this we mean not only increased resting, but also reduced movement during activity measures. Based upon recent pilot studies, we plan to add DEET to the exposure group (Appendix figure A1; p 84). In a newly funded project (GW10066), we will contrast the influence DEET with the other components that are currently a part of our GW agent protocol (PB, permethrin, chlorpyrifos).

At the molecular level, we observed that K_v proteins expressed in vascular and muscle nociceptors exhibited long lasting changes in their activity following exposure to GW chemicals. In our previous studies, using a less intense dosing schedule, we observed increases in the activity of K_v7 (a K_{DR} subtype) and $Na_v1.9$ proteins that persisted for 8 weeks (Nutter and Cooper, 2013; Nutter et al., 2014). We have now revealed changes in the physiology of K_v7 proteins, with the intensified protocol, consistent with an arthralgia, and in the presence of some positive behavioral outcomes. With the new dosing procedure, molecular adaptations now persist out to 12 weeks after the termination of chemical exposure (K_{DR}, K_v7). We have further demonstrated that the decline in K_v7 activity is part of a broader decline of K_v activity that includes some additional members of the K_{DR} family. We eliminated $K_v2.1$ and $K_v2.2$, but were not able to identify that particular K_{DR} isoform(s) diminished by GW toxicant exposures. Other, voltage insensitive, K^+ currents were not affected by GW chemicals (K2p). Broad reductions of K_v current expression were accompanied by changes in muscarinic modulation of GW perturbed currents (K_v7 and the unknown K_{DR} component) that govern a unique burst discharge mechanism in DRG nociceptors. This burst discharge could be initiated by the release of ACh from vascular endothelial cells or other epithelial cells that release this agent. Our studies indicate that these bursts are of sufficiently high frequency as to promote forms of neurogenic inflammation, both in the periphery and as central sensitization. These outcomes are consistent with a role for the transcription factor REST. The REST transcription factor exhibits broad influences on K_v ion

channels and also on muscarinic receptor expression. Manipulation of this transcription factor could be developed into a novel approach to treatment.

6. Publications, Abstracts, And Presentations:

A. Peer Reviewed Scientific Articles:

Nutter, T.J., Johnson, R.D. and Cooper, B.Y. A Delayed Chronic Pain Like Condition with Decreased K_v Channel Activity in a Rat Model of Gulf War Illness Pain Syndrome. NeuroToxicology 51 (2015) 67–79.

B. Abstracts and Presentations

Cooper, B.Y., Nutter, T.J., Dugan, V.P., Johnson, R.D. Classification and characterization of vascular afferents in the rat. An abstract accepted by the Society for Neuroscience, Washington, D.C. November, 2014.

Cooper, B.Y., Johnson, R.D and Nutter, T.J. Persistent Changes in Pain Behaviors and K^+ Channel Physiology in Rats Chronically Exposed to Gulf War Neurotoxicants. American Pain Society, 2015.

Henao, V., Nguyen, H.D., Dugan, V.P., Cooper, B.Y., Johnson, R.D. Vascular afferents innervating lumbosacral veins have distinct immunohistochemical phenotypes in DiI-traced DRG neurons in the rat. Society for Neuroscience, 2015.

C. Manuscripts in Preparation

Cooper, B.Y., Nutter, T.J. and Johnson, R.D. Muscarinic Receptor Maladaptations following Exposure to Gulf War Illness Chemicals, Manuscript attached; to be submitted late January, 2015.

Cooper, B.Y. and Johnson, R.D. Electrophysical and Immunohistochemical Characterization of Vascular Afferents innervating the Lumbosacral Vein.

7. Inventions, Patents and Licenses

none

8. Reportable Outcomes:

1) An intensified dosing procedure that exposed rats to permethrin, chlorpyrifos and pyridostigmine bromide produced some lasting pain-like behaviors that outlasted the chemical application period by 12 weeks.

2) Pain-like behavioral effects were accompanied by decreased activity of muscle nociceptor expressed voltage sensitive K_v7 and K_{DR} ion channels. No changes were observed in non-voltage sensitive K^+ channel proteins (K2p)

3) Muscarinic reactivity of K_v7 and K_{DR} channel proteins were altered, in muscle nociceptors, 12 weeks after exposures. This contributed to an increase in action potentials evoked by muscarinic agonists through a newly discovered mAChr instigated burst discharge in DRG.

4) Vascular nociceptors sensitive to GW chemicals were shown to belong to a family peptidergic neurons that express paracrine, pro-inflammatory, mediators CGRP and SP, the highly proton sensitive ASIC channels; and exhibit K_V7-like membrane current shifts (M-current) in response to ACh.

5) Additional, non-nociceptive, phenotypes of vascular afferents were identified (types 19 and 20). The muscarinic reactivity of type 19 was not changed 12 weeks after GW exposure.

9. Other Achievements:

Based, in part, upon data collected during this funding period, we applied for new funding from GWIRP:

Neurovascular and Autonomic Dysfunction Associated with Gulf War Illness Pain

GW140066

This proposal was funded with a start date of Sept. 29, 2015

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11. Appendices

Supplementary Figures

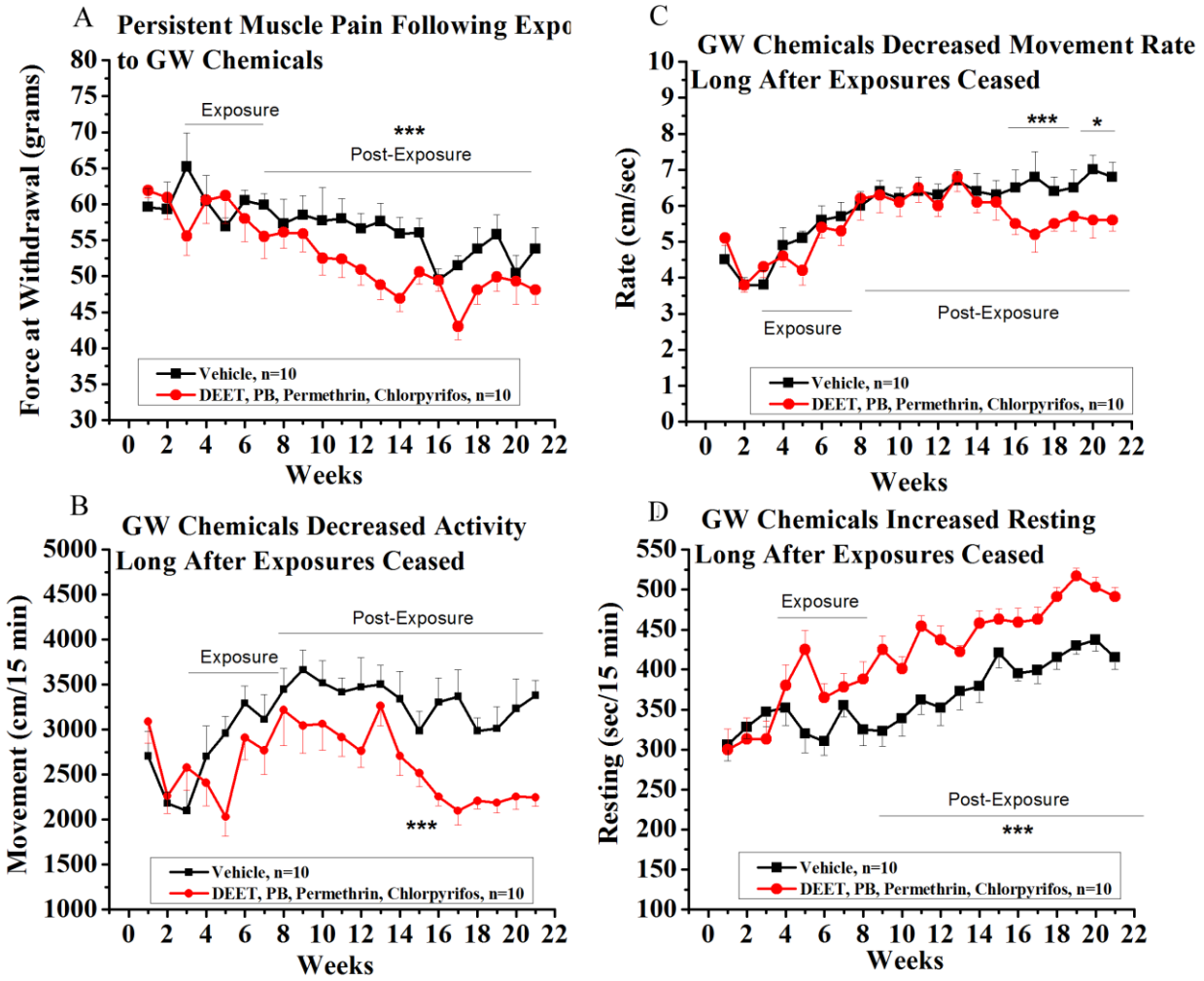

Figure A1. Adding DEET to the Exposure Protocol Produces Signs of Persistent Pain Behavior Effects. Adding DEET to the intensified 30 day exposure produced a broad range of pain behaviors for up to 16 weeks post exposure. Decreased pressure threshold withdrawal, decreased movement distance, increased resting periods and slower movement rate were all consistent with deep muscle and joint pain. All tests were blinded to the experimenter or collected by an automated activity box. * P<.05; *** P<.001

As part of the SOW we indicated that, time permitting, studies would be conducted on newly characterized vascular afferents. Accordingly, we identified type 19 vascular afferents that had been harvested from rats exposed to GW chemicals and examined their reactivity to the broad muscarinic agonist OXO-M. Using the same procedure as the experiments in figure 17A (p. 39), we documented rapid up regulation of KDR currents in type 19 vascular afferents. Currents were increased about 15% after OXO-M (10 uM). There were no differences between neurons taken from vehicle or GW chemical exposed rats (figure A2). These outcomes indicate that changes that occurred in muscle nociceptors, 12 weeks after dosing ended, were confined to that group of afferents. They did not appear in vascular nociceptors or in other identified vascular afferents.

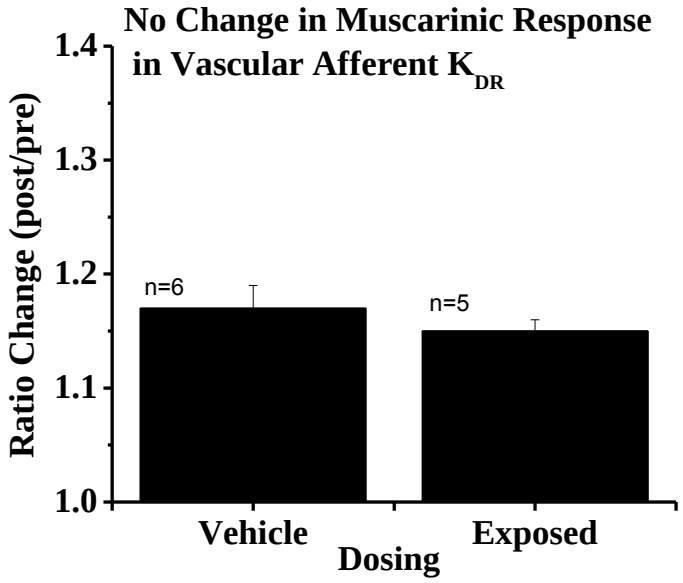

Figure A2. Muscarinic Responses of Type 19 Vascular Afferents Were Unchanged by GW Chemicals. A presumed non-nociceptive vascular afferent was identified in tracing studies. Althouth the KDR currents were increased by application of OXO-M (10 uM, 4 minutes), there were no difference between GW exposed and vehicle exposed cells. All cases collected 12 weeks after GW agent exposure had ceased. Data represents the responses at 1 minute post OXO-M.

Methods

Exposure Protocol. Juvenile male rats weighing between 90 and 110 g were used in all studies (Sprague-Dawley; Harlan). Over a period of 60 days, 16 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). 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 (20) additional rats received only vehicle exposures using an identical administration schedule. Rats were sacrificed for electrophysiological studies 8 and 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; n=20, Vehicle; and 430.8 +/- 8.6 g. n=16, Exposed; $p<.28$). Rats were weighed once per week throughout the studies and doses were adjusted accordingly.

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.

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 (right 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 chemically exposed (permethrin, chlorpyrifos, PB) and vehicle treated (ETOH, corn oil, water) animals over an identical time course. Rats were tested once per week on the behavioral tasks. Most tests were conducted in 'blinded' conditions. Only 'blinded' testing scores were used in the analysis.

Electrophysiological Studies

Preparation of Cells. Dorsal root ganglion neurons (DRG) were harvested from chemically and vehicle exposed rats 8 and 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 10 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 1000 Hz (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 were conducted at room temperature (21 ° 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 studies on K^+ 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. 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 Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 65-70% 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) 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). 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). Cells not fitting the classification criteria were discarded.

Current Clamp Experiments. Studies were conducted on type 5 muscle nociceptors. Following cell identification in voltage clamp mode, the cell was brought into current clamp mode for experiments on action potential characteristics, membrane excitability and reactivity to a muscarinic agonist. Current was injected to bring the cell to -60 mV. Subsequently, an action potential was evoked by a brief, high amplitude, current injection pulse (1 ms, 3-5 nA; 3 replications). Excitability was then assessed by a series of stepped current injections. Increasing currents (0.1 to 1 nA) were injected for 250 ms in 10 consecutive steps. Tests were conducted at both 20 and 35 ºC. The superfusion temperature was controlled by a heated probe positioned ~1 mm from the target cell (Cell Microsystems; HPRE probe).

Measures of action potential duration (APD), action potential afterhyperpolarization (AHP), and membrane excitability were made. The duration of the action potential was scored as the value (in ms) from the first upswing of the potential above the resting membrane potential until the potential again fell below the original RMP (average of 3 tests). AHP was scored as the time (ms) from the maximum dip of the AP below the baseline until the magnitude of the AHP returned to within 80% of the original baseline (average of 3 tests). Membrane excitability was quantified as the number of action potentials evoked during the 10 stepped depolarizations. Following the above tests, the cell was exposed to 10 μM Oxotremorine-M for 2 minutes (Sigma-Aldrich). The total number of action potentials evoked was noted. Cells with membrane potentials more negative than -70 mV or more positive than -40 mV were excluded from the study.

Isolation and Characterization of K_{DR} and K_v7 Channel Currents. K^+ currents were characterized in a 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 KOH. 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.

K_{DR} Currents. For the purpose of this study, the K_{DR} current was defined as the residual total K^+ current following removal of the K_v7 component. Attempts to remove other components using K_v1 inhibitors Margatoxin or Dendrotoxin K were unsuccessful. The voltage dependent activation of the residual total K_{DR} current, was assessed, as a tail current, after a 3 minute application of the K_v7 inhibitor linopirdine (10 uM). From a holding potential of -60 mV, a 2000 msec conditioning pulse (-100 mV) was followed by 11 consecutive command steps to 20 mV (10 mV increments; returning to -60 mV). The amplitude of the tail current at -60 mV was measured from the peak relative to the baseline current recorded 2500 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 $V_{1/2}$ determined for each individual cell. Student's T-test was used to compare the pooled $V_{1/2}$ values for vehicle and GW chemical treated cells. To assess average and peak 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 (-50 to 0 mV) to obtain a mean current amplitude. The normalized peak current was determined by inspection. Student's T-tests were performed on peak and average amplitude as described above. The alpha level was set at .05.

The kinetics of deactivation of K_{DR} tail currents were assessed using current traces evoked at –30 mV ($V_{.50}$). A time constant for deactivation (tau_{deact}) was determined by fit of a function of the form: $A_1exp(-(t-k)/tau_1)$ $A_2exp(-(t-k)/tau_2 \ldots + C$, to the falling phase of the current trace over a range representing points from 90% of the peak to 10% of the base current. It was determined that the K_{DR} deactivation tail current were best fit by a two parameter model that included a slow time constant (tau_1) and a fast time constant (tau_2). The time constants for each neuron were pooled for fast and slow components. Student's T-tests were performed as described above on the vehicle treated and GW chemical exposed cells. The alpha level was set at .05.

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 1000 ms step command to -20 mV was followed by a series of repolarizing 10 mV steps from -10 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 2 minutes later 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 cell size normalized amplitudes (pA/pF) were converted into a conductance (G). 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.

Stromatoxin and Maurotoxin Sensitive K_{DR} Currents. Tests were conducted to isolate those portions of the K_{DR} that were modified by GW chemicals. The K^+ isolation (Kiso) solution was used to isolate all K_{DR} current as described above. Ten micromolar linopirdine was added to Kiso to block K_v7 components of the current. Bovine serum albumin (BSA, 0.1%) was also added to all solutions as a carrier for the toxins and for vehicle test contrasts against toxin testing. 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 ($K_v2.1$, $K_v2.2$; Escoubas et al., 2002) or Maurotoxin ($K_v1.1$, $K_v1.2$; Kharrat et al., 1996) 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. The evoked current at 20 mV was leak corrected, on line, using the P/N procedure utility of Clampex 9.0. 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 Maurotoxin (100 nM) or Stromatoxin (100 nM) was applied for 5 minutes. Toxins were applied continuously by close superfusion. 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 the toxin could be washed out in 3 minutes.

The Maurotoxin or Stromatoxin sensitive current was determined as the amount of current that decreased after 5 minutes of toxin application. Leak corrected 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 for cell capacitance. The Stromatoxin insensitive current was determined from the currents remaining after the 5 minute application 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 (itox) was the average of the last 3 tests evoked after the 5 minute toxin presentation. The percentage of toxin insensitive current in the K_{DR} was then determined as a ratio: 1-(itox/base).

Muscarinic Influences on K_{DR}. Tests were conducted to determine if the K_{DR} currents were sensitive to a muscarinic agonist (OXO-M) and if that sensitivity was altered by GW chemicals. The K^+ isolation (Kiso) solution was used to isolate K_{DR} current as described above. Ten micromolar (10 uM) 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 uM oxotemorine-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 from leak corrected data (P/N). 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 (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). There was little indication that the influence of OXO-M changed over the 4 minute application period or after a 3 minute washout test that followed.

mAChr Initiated Burst Discharges. Type 5 muscle, type 8 and type 19 vascular neurons were identified and brought into current clamp mode (see also Nutter et al., 2015). The recorded cell was exposed to 10 uM oxotremorine-M for 30 seconds (OXO-M). In some tests, linopirdine (10 uM, ethanol), HC-030031 (2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamid; 10 uM), or TTX (1 uM) were presented for 2-3 minutes prior to application of OXO-M. All compounds were purchased from Sigma-Aldrich. When an antagonist preceded OXO-M, the antagonist (or its vehicle) was included in the OXO-M test. Tests were conducted at 35 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.

K2p Currents. The K^+ isolation solution, described above, was used to study K2p currents expressed in muscle and vascular nociceptors. $CsCl_2$, linopirdine and 4AP were not used in studies on K2p currents. Following identification of the targeted nociceptor subtype, the isolation solution was applied for 3 minutes. We used the pH sensitivity of the TASK channel to characterize the pH sensitive K2p currents that were present in each neuronal class. To determine those K2p currents open at the holding potential, (V_H=-60mV, pH 7.4) a K^+ isolation solution at pH 6.0 was applied for about 10 seconds. This produced a small inward current. After 1 minute, another Kiso solution (pH 8.2) was presented for about 10 seconds. This produced a small outward current that represented those K2p TASK currents that were present but not open at pH 7.4. After a 1minute interval, the broad muscarinic antagonist OXO-M (10

uM) was applied (Kiso solution, pH 7.4). Subsequently the pH 6.0 and pH 8.2 tests were repeated.

Tracing Vascular Afferents. All animal procedures used for this study was approved by the Institutional Animal Care and Use Committee in accordance with USDA regulations. Under aseptic procedures in seven male mature adult Sprague-Dawley rats, the proximal segment of the left lateral tail vein (from its origin from the internal pudendal vein) was surgically isolated (1.5-2 cm), a glass microsphere luminal plug placed at the cranial end behind a 9-0 circumferential monofilament suture, and the vein sutured closed at the caudal end. This closed-end venous luminal space was then filled with a fluorescent DiI-paste (1,1'-182 dilinoleyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate) delivered through a 26-gauge angiocatheter and the vein sutured closed just cranial to the dye injection hole to prevent the dye from leaking into other tissue. After 12-14 days of tracer transport time, the animals were euthanized and used for either (i) patch clamp experiments on isolated DRG cells or (ii) immunohistochemical experiments on whole DRG cryosections. Rats were examined post-mortem to confirm that DiI tracer had not leaked into surrounding tissue. Only cases without postmortem evidence of dye leakage outside the vascular compartment were accepted.

For patch clamp experiments on vascular traced DRG neurons, the DRGs were excised, digested and dispersed cells were plated on 35 mm Petri dishes. Whole cell patch experiments were conducted on highly fluorescent neurons that represented those innervating the proximal portion of the tail vein. Fluorescent and bright field images were taken using the NIS Elements Software package (Nikon Instruments). Cells were observed through a 20X Plan Fluor objective (0.45 NA). Fluorescence was captured by a RET-2000R camera (Quantitative Imaging; Melville, NY).

For immunohistochemical experiments on DRG cryosections, animals were euthanized and perfused transcardially with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. The left and right L5-S2 DRGs were dissected free, post-fixed overnight and cryoprotected in a 30% sucrose solution. Serial cryosections at 14 µm were thaw-mounted onto two alternating slides. The section thickness of 14 µm and exclusion of cell images that were not nucleated or fell on the border of the image, avoided the double counting of the cells. Nucleated DiI-positive DRG neurons were directly visualized via multi-label

fluorescence microscopy, digitally imaged with Zeiss Axiophot optics, and measured with morphometric software (ImagePro). Using our previously published techniques (e.g. Petruska et al, 2000), slides were immunohistochemically processed for various cellular markers including CGRP, Substance P (SP), Neurofilament-M (NF-M), IB4, TRPV1 (VR1), and TRPV2 (VRL-1) described in detail below.

Immunohistochemistry. Immunohistochemical and morphometric studies of Di-I positive vascular afferents were performed in serial cryosections. DiI applied to the vascular endothelium and wall of the proximal tail vein was transported to the sensory neuronal cell bodies in the DRG. DRGs were dissected free, post-fixed in 4% paraformaldehyde overnight, and cryoprotected in a 30% sucrose solution until equilibrated. DRGs were embedded in OCT compound (Baxter), serially sectioned on a cryostat at a thickness of 14 µm, and thaw-mounted on alternating poly-L-lysine double-subbed slides. Nucleated DiI-positive DRG neurons were directly visualized via fluorescence microscopy and digitally imaged with Zeiss Axiophot/ImagePro software. Slides were immunohistochemically processed for various cellular markers. Nucleated DiI-positive cells innervating the vascular tissue were first visualized (red channel) on cryosections of left L6 and S1 DRGs (S2 contained very few Di-I labeled cells and L5 contained none). Images of labeled cells were digitized for subsequent retrieval following multi-fluorescence IHC processing. Using cryosections placed on alternate slides, four animals were processed for co-labeled markers for NF-M/CGRP and SP/CGRP using the following primary antibodies, 1:500 mouse anti-NFM (Sigma-Aldrich), 1:200 mouse anti-SP (Neuromics), and 1:200 rabbit anti-CGRP (Neuromics), followed by the appropriate anti-mouse and anti-rabbit secondary antibodies, green channel (AlexaFluor 488) and blue channel (AlexaFluor 350). Similarly, three animals were processed for TRPV1/CGRP and TRPV2/CGRP using the following primary antibodies, 1:1000 guinea pig anti-TRPV1 (Neuromics), 1:200 rabbit-CGRP (Neuromics), 1:750 rabbit anti-TRPV2 (EMD Millipore), 1:200 Mouse-CGRP (Sigma-Aldrich). All primary antibodies were applied to the tissue for 16 hours, and secondary antibodies (Invitrogen) were applied for 3.5 hours. After quantitation and digital storage of the multi-labeled images, we detected binding to the lectin IB4 in four animals as follows: incubation for 12-18 hours in biotinylated lectin IB4 followed by an avidin-biotin complex for 1 hour prepared at half concentration (Elite standard Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and detected using DAB (diaminobenzidine) as the chromogen (20 mg in 50 mls PBS; Sigma).

Following application of DEPEX mounting media, the slides were coverslipped and the Di-I labeled cells were retrieved, identified as positive or negative for IB4, and their diameters/circumferences measured with stereology software tools in bright field. In some animals, cell diameters were measured in toluidine blue counterstained bright field images.

Similar immunohistochemical methodologies were applied to the imaging of afferent axons innervating the proximal portion of the tail vein. For these experiments, samples of the vein/artery complex were taken from fixative-perfused animals, and cryoprotected/equilibrated/mounted as described above. Both 14 and 20um cryosections were obtained from these samples and processed for multi-label fluorescence of cellular markers as described above for DRG neurons.

In vivo venous afferent recordings: Rats were anesthetized with urethane for terminal electrophysiological recording experiments in which peripheral nerve filaments were dissected for multi- and single unit recordings from vascular afferents innervating the proximal portion of the tail vein near its origin from the internal pudendal vein (same site used for DiI injections and vascular innervation studies). Recordings were obtained from a small branch of the S1 spinal nerve in the proximal portion of the ischiorectal fossa. The tail vein was isolated from surrounding tissue and positioned so that (i) perfusates containing either normal saline or algesic chemicals could be injected without adding them to the general body circulation and (ii) a mechanical glass probe (for luminal mechanical stimulation of the vessel wall) or an indwelling stimulating electrode (for conduction velocity determination) could be easily slid into and out of the lumen.

Immunohistochemistry. Immunohistochemical and morphometric studies of vascular afferents were performed in serial cryosections. DiI applied to the vascular endothelium and wall of the lateral tail vein was transported to the sensory neuronal cell bodies in the DRG. After 13 days of tracer transport time, the animals were euthanized and perfused transcardially with 4% paraformaldehyde. The left and right L5-S2 DRGs were dissected free, post-fixed in 4% paraformaldehyde overnight, and cryoprotected in a 30% sucrose solution until equilibrated. DRGs were embedded in OCT compound (Baxter), serially sectioned on a cryostat at a thickness of 14 µm, and thaw-mounted on alternating poly-L-lysine double-subbed slides. Nucleated DiI-

positive DRG neurons were directly visualized via fluorescence microscopy and digitally imaged with Zeiss Axiophot/ImagePro software. Slides were immunohistochemically processed for various cellular markers. For example, to detect neurofilament-M (NF-M) in the DRG cell bodies, a marker for myelinated neurons, mouse anti-neurofilament-M antibody, diluted 1:500, was used. The neurofilament-M primary antibody was labeled with the fluorescent secondary antibody anti-mouse-350 (blue), diluted 1:100. A second primary/secondary process to detect CGRP with anti-rabbit primary (1:200) and anti-rabbit-488 (green) was performed on the same slide. Alternate slides were similarly processed with the same fluorophore to detect substance P (SP) in the cell bodies, using mouse anti-substance P antibody diluted 1:200. All primary antibodies will be applied to the tissue for 12-18 hours, and secondary antibodies were applied for 4 hours. After quantitation and digital storage of the images, we detected binding to the lectin IB4 (a marker for cells with unmyelinated axons) as follows: incubation for 12-18 hours in biotinylated lectin IB4 followed by an avidin-biotin complex for 1 hour prepared at half concentration (Elite standard Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and detected using DAB (diaminobenzidine) as the chromogen (20 mg in 50 mls PBS; Sigma). Following application of DEPEX mounting media, the slides were coverslipped and the Di-I labeled cells re-imaged, identified as positive or negative for IB4, and their diameters/circumferences measured with morphometric ImagePro software tools. The section thickness of 14 μm and exclusion of cell images that are not nucleated, or those which fell on the border of the image, will avoid the double counting of the cells.

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Classification and Characterization of Vascular Afferents in the Rat

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Introduction. A series of studies have revealed that current signature classified afferents exhibit tissue specific innervation and distinct expression patterns of protein subunits mediating nicotinic (α_7, $\alpha_3\beta_4$; $\alpha_3\beta_4\alpha_5$), protonergic (ASIC1-3), heat sensing TRP (V1, V2), and 2 pore potassium channels (K2p; TASK-1, 2, 3). In contrast, the type 8 nociceptor could be traced from multiple tissues (hairy skin, glabrous skin, muscle, distal colon, penile mucosa). We have recently shown that type 8 neurons exhibit persistent molecular alterations following an 8 week exposure to insecticides linked to Gulf War Illness (Nutter and Cooper, 2014). We hypothesized that type 8 neurons might also be tissue specific, but represent a class of nociceptors that innervate tissues common to all injection sites. In the present studies we examined whether type 8 could be traced specifically from venous tissues.

Methods. Experiments were conducted on young adult male Sprague-Dawley rats (n=6). Several centimeters of the left tail vein were isolated, a luminal plug placed at the cranial end, and the vein sutured closed at the caudal end. Two weeks following the injection of DiI-paste, DRGs were excised and plated on 35 mm Petri dishes. Whole cell patch experiments were conducted on highly fluorescent neurons. Only cases without postmortem evidence of dye leakage outside the vascular compartment were accepted. Following current classification procedures (Petruska et al., 2002), neurons were exposed to ACh (500 μM), capsaicin (1 μM) and pH 6.0 solutions.

Results. Both known and previously unidentified type 8 neurons were labeled by DiI. Many vascular afferents manifested characteristics of kinetically distinct type 8a and 8b neurons (capsaicin sensitive (CAPS), fast and slow kinetic ASIC responders; n=7). Capsaicin insensitive (CAPI) type 8 afferents (n=7) exhibited significantly higher H-current (4.1 +/- 1 vs 1.3 +/-.5 pA/pF; $p<.02$), smaller cell size (59.5 +/- 2.4 vs 78.3 +/- 5.5; $p<.02$) and narrower action potentials (4.7 +/- 0.2 vs 8.1 +/- .6 msec; $p<.0003$). CAPI type 8 neurons responded to pH 6.0 with small, non-desensitizing, K2p –like currents (127 +/- 24.2 pA; n=7). Two distinct cholinergic response forms were represented in CAPS neurons: slow desensitizing, $\alpha_3\beta_4\alpha_5$-like currents (n=4), and irreversible holding current shifts suggesting closing of a resting current (156 +/- 52 pA; n=3).

Conclusions. Type 8 nociceptors innervate vascular tissue. These include previously identified type 8a and 8b CAPS responders and a new type 8c CAPI subclass. Cholinergic response patterns suggested further specializations among vascular afferents.

Persistent Changes in Pain Behaviors and K^+ Channel Physiology in Rats Chronically Exposed to Gulf War Neurotoxicants

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Following their return from deployment, Gulf War (GW) veterans reported widespread joint and muscle pain at rates that far exceeded those of soldiers returning from other conflicts. The Research Advisory Committee on Gulf War Veterans' Illnesses (GWI) identified neuroactive insecticides, repellants and nerve agent prophylactics used during the war that might have contributed to the development of GWI. In our previous work, we identified a distinction between membrane nociceptor proteins affected by acute exposure to neurotoxicants ($Na_v1.8$) and those affected by chronic exposure ($Na_v1.9$, K_v7). Despite persistent alterations in the physiology of $Na_v1.9$ and K_v7, behavioral assessments were not consistent with either an arthralgia or myalgia. In the present studies, we intensified the exposure to neurotoxicants NTPB ((13 mg/kg PB (oral gavage), 2.6 mg/kg permethrin (topical) and 120 mg/kg chlorpyrifos (subcutaneous)) in an attempt to improve the behavioral outcomes (pain threshold, movement, resting) and then examined changes that occurred on the molecular level. Animals receiving the intensified protocol for 30 days exhibited significant increases in resting for about 8 weeks after exposure. Thereafter, all measures were comparable to controls. Animals treated with intensified NTPB for 60 days exhibited increased resting and reduced movement that persisted at least 12 weeks post-exposure (434+/-11 vs 487+/-13 sec; $p<.002$; n=8 and 8). In whole cell patch studies, muscle and vascular nociceptor K_{DR} and K_v7 ion channels exhibited increased amplitude relative to controls (e.g., normalized current and/or peak conductance) at 8 weeks post, however at 12 weeks post-exposure, the amplitude of these currents were significantly decreased (11.4 +/-1.0 vs 17.6=/- 1.0 pA/pF; 4.2+/-0.5 vs 6.1+/-0.6 nS; $p<.001$ and $p<.001$, n=10 and 13 respectively). The decline in activity of muscle and vascular nociceptor K^+ channel proteins was consistent with increased excitability and a delayed pain syndrome involving deep tissue nociceptors.

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Vascular afferents innervating lumbosacral veins have distinct immunohistochemical phenotypes in DiI-traced DRG neurons in the rat.

Henao, V., Nguyen, H.D., Dugan, V.P., Cooper, B.Y., Johnson, R.D.

Input from vascular sensory neurons has been implicated in painful conditions including migraine headache and deep tissue pain. Our previous work on DRG neurons used a combined in vitro electrophysiology and immunohistochemical phenotyping approach utilizing target-specific tracing from the periphery (skin, muscle, viscera, mucocutaneous, etc.) in the rat lumbosacral region. We have found distinct target-specific phenotypic patterns, and in the present report, extend this investigation to the vasculature.

Under aseptic procedures in adult male Sprague-Dawley rats, the proximal segment of the left lateral tail vein was surgically isolated (1.5-2 cm), a glass microsphere luminal plug placed at the cranial end, and the vein sutured closed at the caudal end. The closed-end venous luminal space was filled with a fluorescent DiI-paste delivered through a 26-gauge catheter. After 13 days of tracer transport time, the animals were euthanized and perfused transcardially with 4% paraformaldehyde. The left and right L5-S2 DRGs were dissected free, post-fixed overnight and cryoprotected. The Di-I injection site was examined postmortem to verify that no dye leaked into non-vascular structures. Serial cryosections at 14 µm were thaw-mounted on alternating slides. Nucleated DiI-positive DRG neurons were directly visualized via multi-label fluorescence microscopy, digitally imaged with Zeiss optics, and measured with morphometric software. Using our previously published techniques (e.g. Petruska et al, 2000), slides were immunohistochemically processed for various cellular markers including CGRP, SubP, Neurofilament-M (NF-M), IB4, TRPV1, TRPV2.

Di-I positive cells neurons (n=929) traced from the proximal tail vein were found almost exclusively in the ipsilateral L6 and S1 DRGs (some in S2) and exhibited small to medium cell diameters (96% between 15-45 µm). Only 19% were NF-M positive but had a significantly greater cell diameter (41.5 ± 1.1 µm) compared to NF-M negative cells (31.2 ± 0.5 µm; $p<.01$). The latter were mostly IB4 negative. While only 21% were typical peptidergic cells containing both CGRP and SubP, those containing only one peptide were CGRP+ (92%). Combined with our finding that one third of the total Di-I cells contained CGRP, our data support the known importance of CGRP in vascular control. Consistent with our in vitro patch clamp data from capsaicin-sensitive and -insensitive Di-I traced vascular DRG neurons (type 8, type 19 and type 20; Cooper et al., 2014), a portion of the vascular afferent population were positive for TRPV1 and TRPV2 with most CGRP+ cells co-labeled with the capsaicin-insensitive TRPV2 receptor (63%).

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A delayed chronic pain like condition with decreased K_v channel activity in a rat model of Gulf War Illness pain syndrome

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ABSTRACT

Following their return from deployment, Gulf War (GW) veterans reported widespread joint and muscle pain at rates that far exceeded those of soldiers returning from other conflicts. It is widely believed that exposure to insecticides, repellants and nerve gas prophylactics contributed to the symptoms of Gulf War Illness (GWI), but an animal model of GW pain has been elusive. In our previous work, we observed that 4–8 weeks exposure to pyridostigmine bromide (PB), permethrin and chlorpyrifos could produce persistent alterations in the physiology of $Na_v1.9$ and K_v7 expressed in deep tissue nociceptors of the dorsal root ganglion. However, behavioral assessments from these same rats were not consistent with a delayed pain syndrome similar to that of GWI pain. In the present studies, we intensified the exposure to anticholinesterases PB and chlorpyrifos while retaining the same dosages. Animals receiving the intensified protocol for 30 days exhibited significant increases in resting for about 8 weeks after exposure. Thereafter, all measures were comparable to controls. Animals treated with intensified anticholinesterases for 60 days exhibited increased resting and reduced movement 12 weeks post-exposure. In whole cell patch studies, muscle and vascular nociceptor K_{DR} and K_v7 ion channels exhibited increased amplitude relative to controls (*e.g.*, normalized current and/or peak conductance) at 8 weeks post-exposures; however, at 12 weeks post-exposure, the amplitude of these currents was significantly decreased in muscle nociceptors. In current clamp studies, muscle nociceptors also manifested increased action potential duration, afterhyperpolarization and increased discharge to muscarinic agonists 12 weeks post-exposure. The decline in activity of muscle nociceptor K_{DR} and K_v7 channel proteins was consistent with increased nociceptor excitability and a delayed myalgia in rats exposed to GW chemicals.

1. Introduction

Many soldiers returning from the Persian Gulf War suffered from unusual complexes of headache, joint, muscle and abdominal pains associated with Gulf War Illness (GWI; Haley Syndrome 3; Haley and Kurt, 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013; see also Gopinath et al., 2012). While it is well recognized that veterans returning from service overseas often report lingering musculoskeletal pain, the relative likelihood of joint, muscle, and/or abdominal pain were 3-fold greater in GW veterans than in those that were deployed elsewhere (Thomas et al., 2006; Kelsall et al., 2004; Stimpson et al., 2006; see also Kang et al., 2000). The appearance of these symptoms was typically delayed, but up to 25% of those stationed in the Persian Gulf exhibited GWI related pain prior to their return to the States (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).

The Research Advisory Committee on Gulf War Illness (GWI) concluded that pesticides could have contributed to the development of the symptoms of GWI (Binns et al., 2008; RAC, 2014). During the brief course of the Gulf War, soldiers were potentially exposed to 67 insecticides and repellants containing 37 distinct active ingredients (DoD Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008). These included a variety of organophosphate, organochlorine, dialkylamide, carbamate and pyrethroid pesticides and repellants. Our laboratory has identified

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molecular adaptations in pain system neurons that result from exposure to GWI suspected pesticide neurotoxicants (permethrin, chlorpyrifos) and the nerve gas prophylactic, pyridostigmine bromide (PB; Jiang et al., 2013; Nutter et al., 2013; Nutter and Cooper, 2014). These chemicals have direct interactions with important proteins expressed in the peripheral pain (nociceptor) system. Permethrin acutely modifies the physiology of central and peripheral nervous system Na^+ channels that are essential for pain coding ($Na_v1.6$, $Na_v1.7$, $Na_v1.8$, $Na_v1.9$; Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006; Nutter et al., 2013; Nutter and Cooper, 2014). Chronic exposure to anticholinesterases chlorpyrifos and PB upregulate expression of muscarinic acetylcholine receptor proteins that couple to important voltage sensitive K^+ channels that control neuronal excitability (K_v7; Abou-Donia et al., 2003, 2004). Following an 8-week exposure to permethrin, chlorpyrifos and PB (rats), we were unable to demonstrate a pattern of behavior consistent with a chronic pain condition. Nevertheless, we observed upregulation of ion channel proteins $Na_v1.9$ and K_v7 (Nutter et al., 2013; Nutter and Cooper, 2014). These molecular adaptations persisted 8 weeks after neurotoxicant exposure had ceased.

The K_v7 family of ion channel proteins belongs to the large family of K_v (voltage sensitive K^+) proteins that contribute to neural excitability and conduction in multiple ways (Du and Gamper, 2013; Tsantoulas and McMahon, 2014). The adaptations exhibited by K_v7 channels, following GW chemical exposure, could represent a general adaptation affecting K_v channels or a more specific adaptation of the K_v7 family through their linkages to muscarinic receptors whose expression may have been perturbed by anticholinesterases (Abou-Donia et al., 2003, 2004; Passmore et al., 2012). Because a variety of K_v channels have been implicated in the development of chronic pain, determining the scope of this defect could aid in the interpretations of determinant factors contributing to the development and treatment of GWI pain syndromes (Du and Gamper, 2013; Tsantoulas and McMahon, 2014). In the experiments presented below, we used an intensified anticholinesterase exposure protocol to induce a delayed pain behavior syndrome, and examined whether K_v7 and K_{DR} currents in deep tissue nociceptors were modified in a manner consistent with delayed chronic pain.

2. Methods

2.1. Behavioral studies

2.1.1. Subjects

Young adult male rats weighing 229.4 ± 2.6 g (~6 weeks old) were used in the pesticide exposure studies (Sprague-Dawley; Harlan; $n = 61$). 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). No animals perished or exhibited signs of acute pesticide toxicity during the execution of these studies.

2.1.2. Chronic exposure protocol

Over a period of 30 or 60 days, rats ($n = 30$) 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). 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. 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 a couple of days (Smith et al., 2009). 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 ($n = 31$).

Rats were sacrificed for electrophysiological studies 8 and 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 (vehicle: 443.4 ± 9.1 and exposed: 430.8 ± 8.6 g; $p < .35$).

2.1.3. Assessment of pain behaviors

On arrival, rats were acclimated to the behavioral procedures for 2 weeks before dosing began. Testing continued weekly 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 (right 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; Fusion System, AccuScan Instruments Inc.). Behavior tests were conducted on both chemically exposed (permethrin, chlorpyrifos, PB) and vehicle treated (ETOH, corn oil, water) animals over an identical time course. Rats were tested once per week on the behavioral tasks. Tests were conducted in 'blinded' conditions.

2.2. Electrophysiological studies

2.2.1. Preparation of cells

Dorsal root ganglion neurons (DRG) were harvested from chemically and vehicle exposed rats 8 and 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 (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 min 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 min, and then spun at 1000 RPM (30 s). 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 were conducted at room temperature (21 °C) within 10 h 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). 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 65–70% 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). Anatomical targets of type 5 and type 8 neurons were determined by a series of anatomic tracing experiments (Jiang et al., 2013; Rau et al., 2007, 2014; Cooper et al., 2014). Cells not fitting the classification criteria were discarded.

2.2.3. Current clamp experiments

Studies were conducted on type 5 muscle nociceptors. Following cell identification in voltage clamp mode, the cell was brought into current clamp mode for experiments on action potential characteristics, membrane excitability and reactivity to a muscarinic agonist. Current was injected to bring the cell to −60 mV. Subsequently, an action potential was evoked by a brief, high amplitude, current injection pulse (1 ms, 3–5 nA; 3 replications). Excitability was then assessed by a series of stepped current injections. Increasing currents (0.1–1 nA) were injected for 250 ms in 10 consecutive steps. Tests were conducted at both 20 and 35 °C. The superfusion temperature was controlled by a heated probe positioned ~1 mm from the target cell (Cell Microsystems; HPRE probe).

Measures of action potential duration (APD), action potential afterhyperpolarization (AHP), and membrane excitability were made. The duration of the action potential was scored as the value (in ms) from the first upswing of the potential above the resting membrane potential until the potential again fell below the original RMP (average of 3 tests). AHP was scored as the time (ms) from the maximum dip of the AP below the baseline until the magnitude of the AHP returned to within 80% of the original baseline (average of 3 tests). Membrane excitability was quantified as the number of action potentials evoked during the 10 stepped depolarizations. Following the above tests, the cell was exposed to 10 μM oxotremorine-M for 2 min (Sigma–Aldrich). The total number of action potentials evoked was noted. Cells with membrane potentials more negative than −70 mV or more positive than −40 mV were excluded from the study.

2.2.4. Isolation and characterization of K_{DR} and 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.

2.2.5. 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. The voltage dependent activation of the total K_{DR} current, was assessed, as a tail current, after a 3 min application of the K_v7 inhibitor linopirdine (10 μM). From a holding potential of −60 mV, a 2000 ms conditioning pulse (−100 mV) was followed by 11 consecutive command steps from −80 to 20 mV (10 mV increments; 500 ms duration). The amplitude of the tail current at −60 mV was measured from the peak relative to the baseline current recorded 2500 ms 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. Student's *t*-test was used to compare the pooled $V_{.50}$ values for vehicle and GW chemical treated cells. To assess average and peak 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 10 mV) to obtain a mean current amplitude. The normalized peak current was determined by inspection. Student's *t*-tests were performed on peak and average amplitude as described below.

The kinetics of deactivation of K_{DR} tail currents were assessed using current traces evoked at −30 mV ($V_{.50}$). A time constant for deactivation (tau_{deact}) was determined by fit of a function of the form: $A_1\exp(-(t-k)/\text{tau}_1)\ A_2\exp(-(t-k)/\text{tau}_2)\ldots + C$, to the falling phase of the current trace over a range representing points from 90% of the peak to 10% of the base current. It was determined that the K_{DR} deactivation tail currents were best fit by a two parameter model that included a slow time constant (tau_{slow}) and a fast time constant (tau_{fast}).

2.2.6. 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 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.

2.2.7. Acute studies on K_{DR} with permethrin

An all glass superfusion system was used to avoid complications due to the known binding of highly lipophilic pyrethroids to plastics (Tatebayashi and Narahashi, 1994; Shafer and Hughes, 2010). Ten ml glass syringe reservoirs were coupled to a small Plexiglas manifold by a flexible glass tubing system terminating in a 'sewer pipe' that was positioned within 2 mm of the targeted cells. The glass tubing consisted of 3 rigid sections of 150 mm glass (.86 mm inner diameter, 1.5 mm outer diameter; Sutter Instruments). Each rigid piece was linked by a 3 mm section of silastic tubing. Only about 1 mm of each silastic linker was in contact with

the permethrin containing solution. The Plexiglas manifold was not exposed to permethrin until the actual application occurred. Following each application of permethrin, the manifold was rinsed for at least 2 min with 100% ETOH. Stock solutions of permethrin were prepared and maintained in glass bottles. Whole cell voltage clamp studies were performed on muscle and vascular nociceptors that were identified as previously described (type 5 and 8 respectively). Cell capacitance, access resistance, membrane resistance, and series resistance corrections were performed as described above. Following a 3 min application of a K-ISO solution containing 1 mM CsCl, 2 mM 4-AP and 10 μM linopirdine (above) the remaining total K_{DR} currents were evoked by a series of depolarizing command steps (−80 to 20 mV; 10 mV steps; 500 ms duration). To remove inactivation, each command step was preceded by 2000 ms step to −100 mV. Permethrin 10 μM (or .001% ethanol) in the K-ISO solution was applied for 2 min by close superfusion. The K_{DR} was again evoked as described. Statistical comparisons were made between the activation, $V_{.50}$, normalized average, peak amplitude, and deactivation time constants in the presence of either permethrin or ETOH.

2.2.8. Statistics

A repeated measures ANOVA was used to assess the influence of chemical exposure and weeks on dependent measures of muscle pain threshold (g), movement distance (cm/15 min), movement rate (cm/s) and rest (s/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 (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* tests were used to contrast amplitude, kinetics and voltage measures of K_{DR} and K_v7 activity expressed in exposed, permethrin and vehicle treated animals. 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

In a prior study we reported persistent changes in K_v7 channel protein physiology following chronic exposure to GW chemicals (permethrin, chlorpyrifos and PB); however, those alterations in K_v7 activity did not appear in parallel with measurable behavioral indices of pain (Nutter et al., 2013). In order to better assess the relevance of changes in K_v ion channel function to a chronic pain condition, we tested modifications to the exposure protocol with the hypothesis that intensifying the anticholinesterase component would produce a chronic pain condition. In the behavioral studies below, the duty cycle of PB application was increased from 50 to 100%, and the chlorpyrifos duty cycle was increased from 7 to 14%. All doses remained the same as those used previously (Nutter et al., 2013; Nutter and Cooper, 2014). We will refer to this as the 'intensified protocol'. We examined the influence of the intensified protocol, in separate groups of rats, for exposures lasting either 30 or 60 days.

Animals exposed to the intensified protocol for 30 days exhibited significant increases in resting for about 8 weeks after exposure (Fig. 1). Thereafter, rest measures returned to normal levels, but movement rate was paradoxically increased in weeks 9–12. No other pain measures were affected in the weeks following chemical exposure. Although other measures of pain were not altered, the 8-week increase in rest duration was a substantial improvement

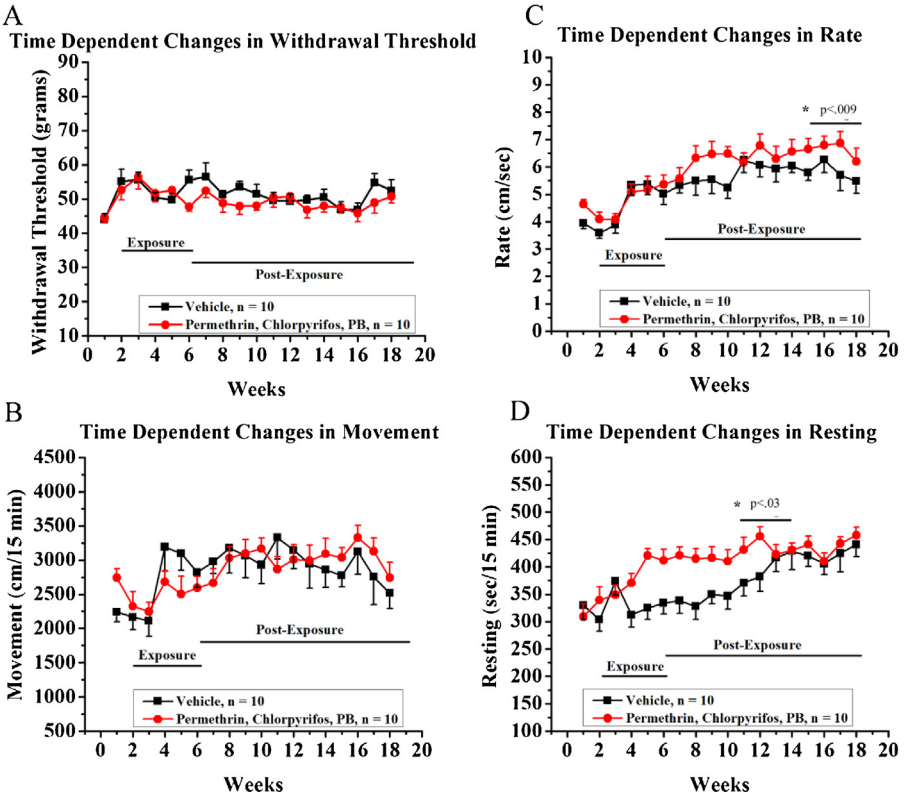

Fig. 1. The intensified 30-day exposure produced some lasting behavioral effects. (A, B) Muscle pain pressure thresholds and distance moved were unchanged following the 30-day exposure to GW chemicals. (C, D) Movement rate and resting were both increased during the post-exposure period. Resting was still significantly increased 5–8 week post-exposure ($F = 4.70$; $p < .03$) but returned to normal levels thereafter. The increased resting was replaced by a paradoxical increase in movement rate at post 9–12 weeks ($F = 7.23$; $p < .009$). Tests were not conducted on any measure 1–4 weeks post-exposure.

over our previous attempts to produce a chronic pain condition with GW chemicals (Nutter et al., 2013; Nutter and Cooper, 2014). None of these animals were sacrificed for physiological studies.

The 60-day exposure group was divided into two independent subgroups that reflected their ultimate use in physiology studies. Group 8WP (8 weeks post) were animals that were sacrificed for physiology experiments 8 weeks post-exposure (Fig. 2). Group 12WP (12 weeks post) were rats sacrificed 12 weeks post-exposure (Fig. 3). Both groups exhibited similar behavioral effects of the intensified protocol through 8 weeks. Consistent with the presence of a myalgia/arthralgia, rest periods were significantly increased. However, the interpretation of this data, as reflecting a pain condition, was complicated by a significant, but paradoxical, increase in the rate of movement in group 8WP (Fig. 2). Similar trends were observed in the 30-day exposure group (Fig. 1). When behavioral studies were carried out to 12 weeks (Group 12WP), resting remained significantly elevated, but movement distance was now significantly decreased and movement rate had normalized (Fig. 3B, D). Neither the 30 or 60-day exposures produced a change in the muscle pressure pain threshold, but it is not clear whether a change in muscle pressure pain thresholds is exhibited by GW veterans (Cook et al., 2010). Animals in pain generally exhibit reduced movement and increased resting; a pattern consistent with myalgia and/or arthralgia due to GW chemical exposure. Our parallel molecular studies on these same animals examined whether the intensified treatment produced molecular adaptations to K_V channels consistent with a chronic pain condition at the 8 and 12-week delays.

3.2. Molecular studies on chronically exposed rats

Eight and 12 weeks after chemical exposures ended, muscle and vascular nociceptors (type 5 and type 8, respectively) were identified in DRG harvested from rats exposed for 60 days to GW chemicals or their vehicles (Petruska et al., 2002; Rau et al., 2007; Cooper et al., 2014). Using a tail current analysis, we examined the voltage dependence and amplitude of the K_{DR} and the conductance of the K_v7 current component (isolated as the linopirdine sensitive deactivation tail current). These currents were isolated from other voltage dependent currents using conventional procedures (see Section 2; also see Nutter et al., 2013). We observed persistent changes in both K_v7 and the K_{DR} currents following the intensified exposure protocol. Some of these changes predicted increased neural excitability and pain.

3.2.1. Physiology of K_v7

Consistent with previous reports on K_v7 currents in chemically exposed rats (Nutter et al., 2013), both the normalized average and peak conductance of K_v7 currents were significantly increased 8 weeks following cessation of exposure (Fig. 4A, B). Both muscle and vascular nociceptors were affected by chemical exposures at the 8-week delay point. The influence on muscle nociceptors was more robust. All significant changes indicated greater activity of K_v7 and potentially less excitability at 8 weeks post-exposure. These molecular shifts occurred while rats exhibited the unexpected combination of increased resting but faster movement during activity periods.

At 12 weeks post-exposure, K_v7 currents were now significantly decreased in muscle nociceptors, while K_v7 currents in vascular nociceptors had returned to normal levels (Fig. 4B, D). A decrease in the conductance of K_v7 is consistent with increased nociceptor excitability. The decrease in K_v7 currents corresponded to the time period when the combination of significantly greater resting and significantly decreased movement distance suggested a delayed myalgia and/or arthralgia.

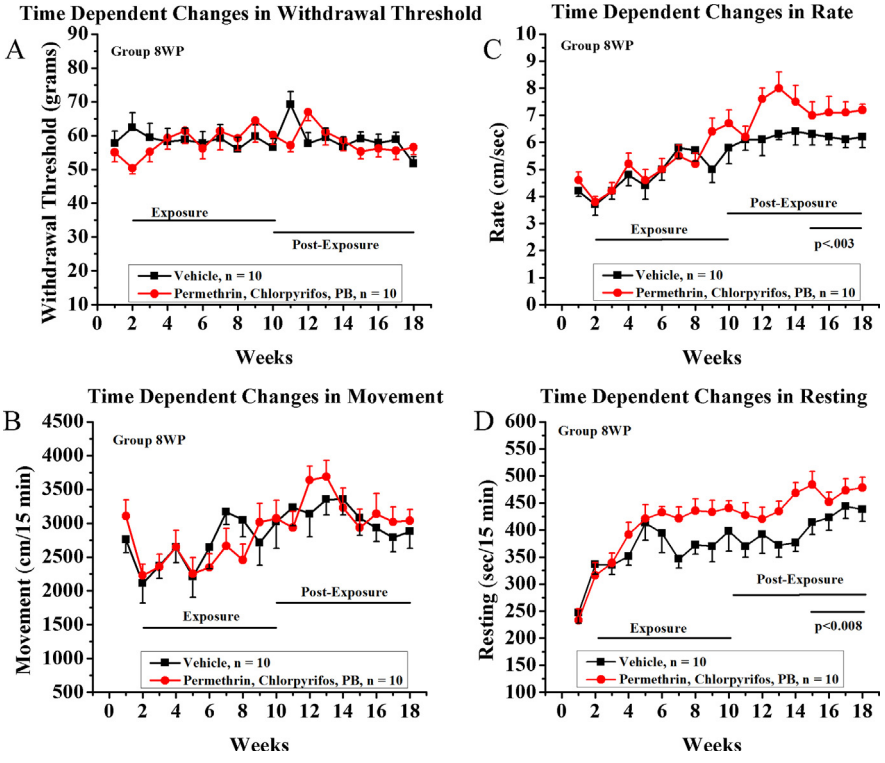

Fig. 2. The intensified exposure protocol produced some signs of lasting pain behaviors in the 8-week post-exposure group. (A, B) Muscle pain pressure thresholds and distance moved were unchanged following the 60-day exposure to GW chemicals. (C, D) Movement rate and resting were both increased during the post-exposure period. Resting was significantly increased 5–8 weeks post-exposure ($F = 7.43$; $p < .008$). Movement rate was significantly increased at the same time delay ($F = 9.23$; $p < .003$). Tests were not conducted on any measure 1–4 weeks post-exposure.

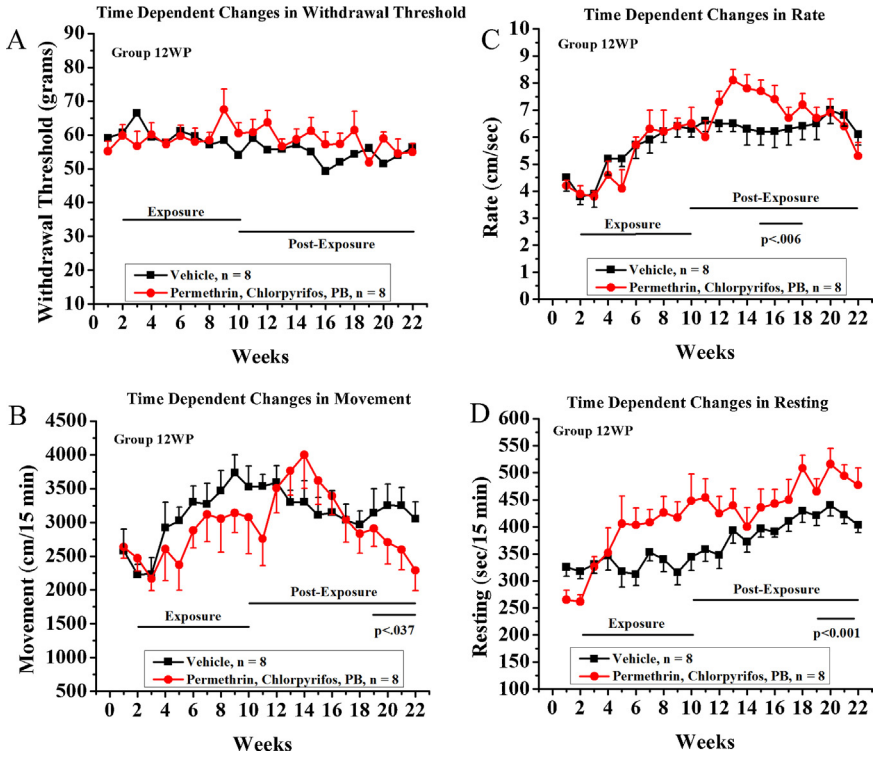

Fig. 3. The intensified exposure protocol produced consistent signs of lasting pain behaviors in the 12-week post-exposure group. (A) Muscle pain pressure threshold was unchanged following the 60-day exposure to GW chemicals. (B) Consistent with a delayed chronic pain, movement distance was significantly decreased at 9–12 weeks post-exposure ($F = 5.87$; $p < .04$). (C, D) Movement rate and resting were both increased during the post-exposure period. Resting was significantly increased 9–12 weeks post-exposure ($F = 10.2$; $p < .001$). Movement rate was significantly increased only at the 5–8 week test period ($F = 8.09$; $p < .006$). Tests were not conducted on any measure 1–4 weeks post-exposure.

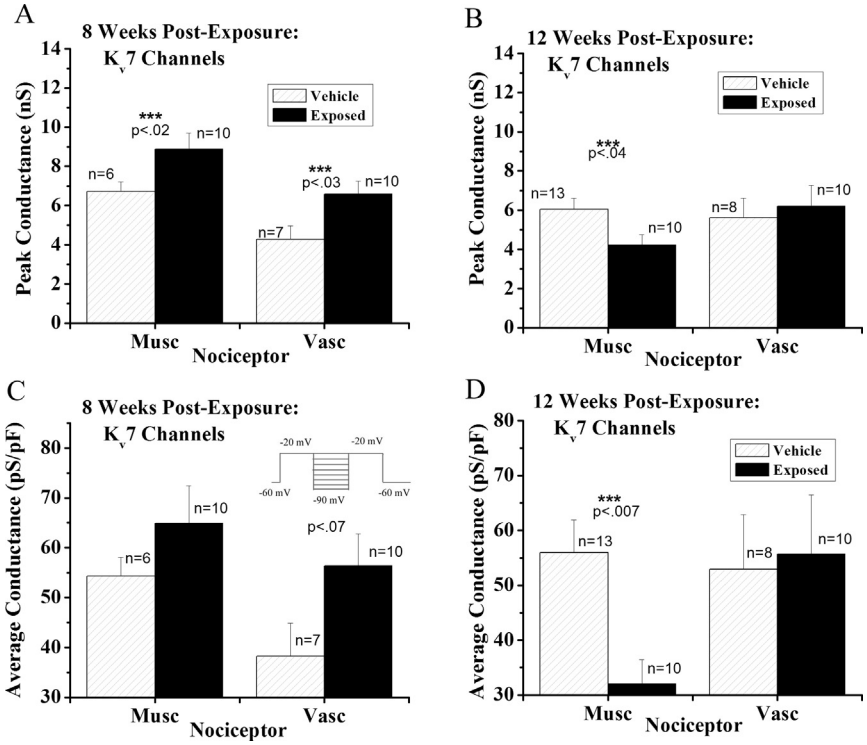

Fig. 4. Altered physiology of K_v7, 8 and 12 weeks following a 60-day exposure to permethrin, chlorpyrifos and PB. K_v7 activity was modified at both the 8-week (A, C) and 12-week (B, D) post-exposure periods. Conductances were used as dependent measures in these studies because the peak current did not always occur at the same deactivation voltage step. Peak and average conductances were normalized to cell size. Conductances were averaged over a range of −40 to −60 mV. The voltage protocol used is presented as an inset in (C).

3.2.2. Physiology of K_{DR}

In order to determine whether the changes observed in the K_v7 channels were part of a larger, general, influence on K_v channels, we examined the voltage dependence of the K_{DR} channels after inhibition of K_v7 with linopirdine. After exposure to linopirdine for 3 min (10 μM), the voltage dependence of the K_{DR} current was assessed by a tail current analysis. Tail current amplitude was measured, normalized to the maximum and plotted as a Boltzmann function. As shown in Fig. 5, exposure to GW chemicals for 60 days produced significant, although small, hyperpolarizing shifts in the voltage dependence of activation at both the 8 and 12-week examination points. Voltage shifts ranged from 2 to 3.5 mV and were significant in both muscle (8 and 12 weeks; Fig. 5A, B) and vascular nociceptors (12 weeks; Fig. 5D).

The total K_{DR} current was assessed in muscle and vascular nociceptors. Because voltage dependence and current amplitude are interdependent, we minimized the influence of a hyperpolarized voltage dependence by averaging currents across the full range of test voltages (−60 to 10 mV). At 8 weeks post, shifts in the average and peak K_{DR} currents (Fig. 6A, C) paralleled changes in voltage dependence (Fig. 5A). At 8 weeks post-exposure, muscle nociceptors exhibited significant increases in average K_{DR} current that accompanied their hyperpolarized $V_{.50}$. However, at 12 weeks post-exposure, and despite a hyperpolarized $V_{.50}$, the average and peak amplitude of muscle nociceptors K_{DR} was significantly *decreased*. Regardless of the significantly hyperpolarized voltage dependence (Fig. 5D), vascular nociceptor K_{DR} amplitudes were unaffected at either time delay. The divergence of voltage dependent and amplitude measure outcomes suggested the amplitude of these currents were being altered by factors aside from any shifts in voltage dependence.

The K_{DR} is likely to be composed of multiple K_v phenotypes. We attempted to examine how GW chemicals might have influenced distinct components of the K_{DR} by using a deactivation kinetics analysis. In order to derive deactivation time constants (tau_{deact}), exponential fits were made to the K_{DR} deactivation tails (at −30 mV; see Section 2). Fits were optimized by a two component model. The presence of two deactivation components (fast and slow) could represent two separate molecular entities. Statistical comparisons between fast and slow tau_{deact} of exposed and vehicle treated rats revealed significant influences of GW chemicals on type 5 muscle nociceptor K_{DR} channel deactivation (Fig. 7). Trends toward slowed deactivation occurred in both nociceptor groups at both delays. Significant changes in the slow tau_{deact} were observed at both the 8 and 12-week delays in muscle nociceptors (Fig. 7C, D). These shifts paralleled significantly increased tail current amplitude at 8 weeks (Fig. 6C), but diverged from those at 12 weeks (Fig. 6D). Because tail currents are measured following deactivation, slowed deactivation kinetics could account, at least in part, for the increased amplitude of K_{DR} currents observed during the 8-week period. However, the significant slowing of deactivation kinetics at week 12 (Fig. 7D) could not account for a significant decrease in the K_{DR} at this time delay (Fig. 6D). A significant slowing of the fast component of deactivation was only observed at the 12-week delay (Fig. 7B).

3.2.3. Action potential characteristics and membrane excitability

The decline in activity of K_v7 and K_{DR} ion channels suggested muscle nociceptors might exhibit increased excitability following exposure to GW Chemicals. We examined the properties of muscle nociceptors 12 weeks following a 60-day exposure to the intensified protocol or their respective vehicles. Type 5 muscle

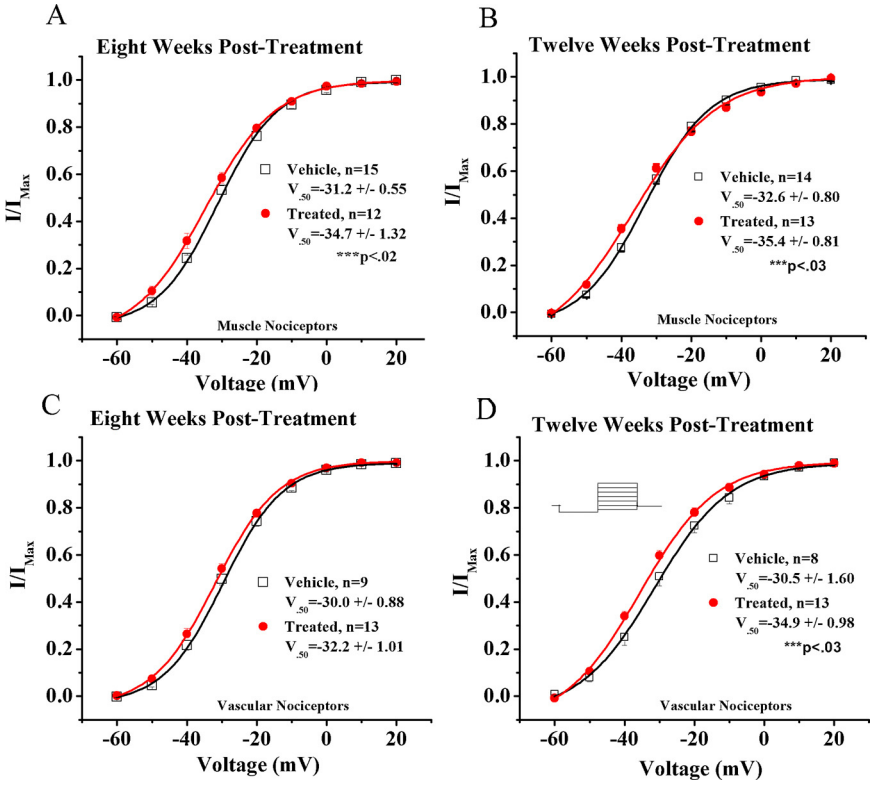

Fig. 5. The voltage dependence of delayed rectifier channels (K_{DR}) 8 and 12 weeks following exposure to permethrin, chlorpyrifos and PB. (A, B) In muscle nociceptors, the computed activation voltage constants ($V_{.50}$) were hyperpolarized (leftward shift) at both the 8 and 12 weeks post-exposure periods. (C, D) Vascular nociceptors were shifted only at the 12-week delay. Voltage constants ($V_{.50}$) were derived from Boltzmann fits to individual cells while the curves above reflect the average of all cells. Curves were formed from tail currents evoked by the voltage protocol illustrated in (D). From a V_H of −60 mV, cells were hyperpolarized to −100 mV for 2 s then stepped for 500 ms from −60 to 20 mV in 10 mV steps.

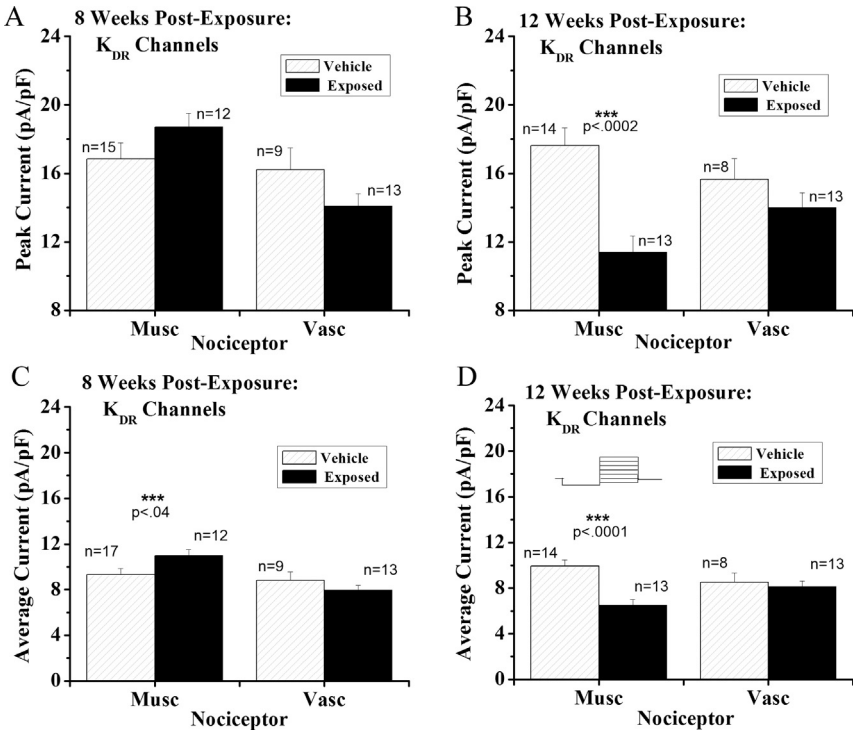

Fig. 6. Amplitude of the delayed rectifier channels currents (K_{DR}) 8 and 12 weeks following exposure to permethrin, chlorpyrifos and PB. (B–D) Muscle nociceptor K_{DR} amplitude measures were affected by GW chemical exposure. At 12 weeks post-exposure (D), the influence of GW chemicals reversed from a significant increase at 8 weeks to a significant decreased average amplitude. Peak and average currents were normalized to cell size. Currents were averaged over a range of −60 to 10 mV. The voltage protocol is shown as an insert in panel (D). From a V_H of −60 mV, cells were hyperpolarized to −100 mV for 2 s then stepped for 500 ms from −80 to 20 mV in 10 mV steps.

nociceptors were identified, in the usual manner, in voltage clamp mode. After membrane properties were assessed (resistance, capacitance), neurons were brought into current clamp mode. Each neuron was observed for 2 min for signs of spontaneous activity (20 °C). Subsequently, the RMP was held, by current injection, at −60 mV and action potentials were evoked (1 ms; 3–5 nA; 3 replications at 1 s intervals). To assess membrane excitability, neurons were tested with a series of depolarizing current

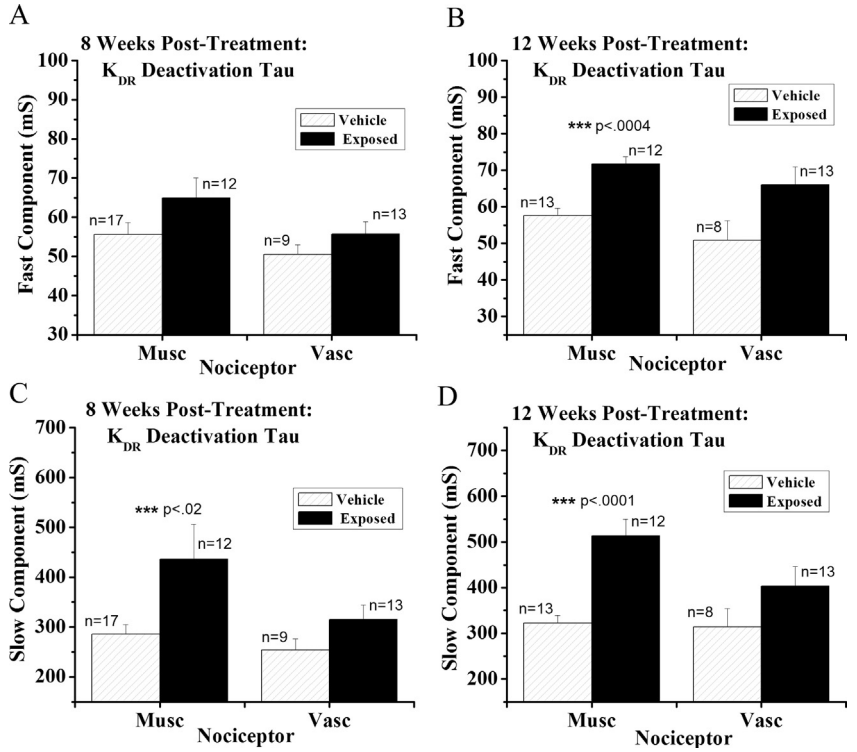

Fig. 7. Deactivation time constants (tau) were increased by exposure to GW chemicals. (A, B) Eight and 12 weeks after exposure, the fast time constants of deactivation trended longer, but only reached significance in muscle nociceptors at the 12-week delay. (C, D) Significant changes in the slow decay constants were present at 8 and 12 weeks. All significant increases were in muscle nociceptors.

injections (0.1 nA/step; 10 steps; 250 ms). The total number of action potential evoked by the series was determined by inspection. Using close superfusion with a heated probe, the same procedure was repeated at 35 °C (see Section 2).

There was little indication that muscle nociceptors, from exposed rats, would exhibit spontaneous discharge. Only 1 of 24 neurons (20 °C: 14 vehicle and 10 exposed; 35 °C: 14 vehicle and 9 exposed) exhibited any spontaneous activity (2 APs, exposed case; data not shown). In contrast, the exposed group muscle nociceptors manifested significantly longer duration APs and AHPs at 20 °C and longer AP duration at 35 °C (Fig. 8A, B). Current injection excitability was increased at 35 °C only (5.0 ± 1.8 *vs* 1.8 ± 0.59 total APs; $p < .05$; $n = 6$ and 13 respectively). There were no differences in RMP between GW chemical and vehicle exposed neurons (-64.9 ± 1.7 and -67.3 ± 1.7 mV respectively).

Following the above tests, muscle nociceptors were exposed to the nonspecific muscarinic agonist oxotremorine-M (10 μM; 2 min; 35 °C). Twelve of 15 muscle nociceptors rapidly depolarized during the exposure. A powerful burst of APs occurred during depolarization. The number of APs evoked was significantly higher in muscle nociceptors harvested from GW exposed rats (Fig. 8D). The amount of depolarization did not differ between the two groups (29.9 ± 3.6 and 22.8 ± 4.3 mV; GW exposed and vehicle treated respectively).

3.2.4. Acute studies with permethrin

Pyrethroids, like permethrin, produce powerful changes in the physiology of TTXs and TTXr Na_v proteins of DRG neurons (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996; Song and Narahashi, 1996; Tabarean and Narahashi, 1998, 2001; Jiang et al., 2013). There have been few published reports documenting the influence of permethrin on K_v channels (Jiang et al., 2013). In order to determine if the chronic changes in K_{DR} current properties might be initiated by the direct influence of permethrin during the time of exposure, we exposed muscle or vascular nociceptors to this pyrethroid. Using a custom-made glass pipette application system (see Section 2), we superfused vascular or muscle nociceptors with 10 μM permethrin or .001% ETOH vehicle for 2 min. Using a tail current analysis, we did not observe any changes in the voltage dependence (not shown), amplitude or deactivation kinetics of type 5 muscle or type 8 vascular nociceptor K_{DR} (Fig. 9).

4. Discussion

In our previous studies, we reported on molecular adaptations that appeared in deep tissue nociceptors after chronic exposure to 3 GW chemicals (Nutter et al., 2013; Nutter and Cooper, 2014). The molecular perturbations were not accompanied by measurable pain behaviors. In the present report, we examined whether a modified

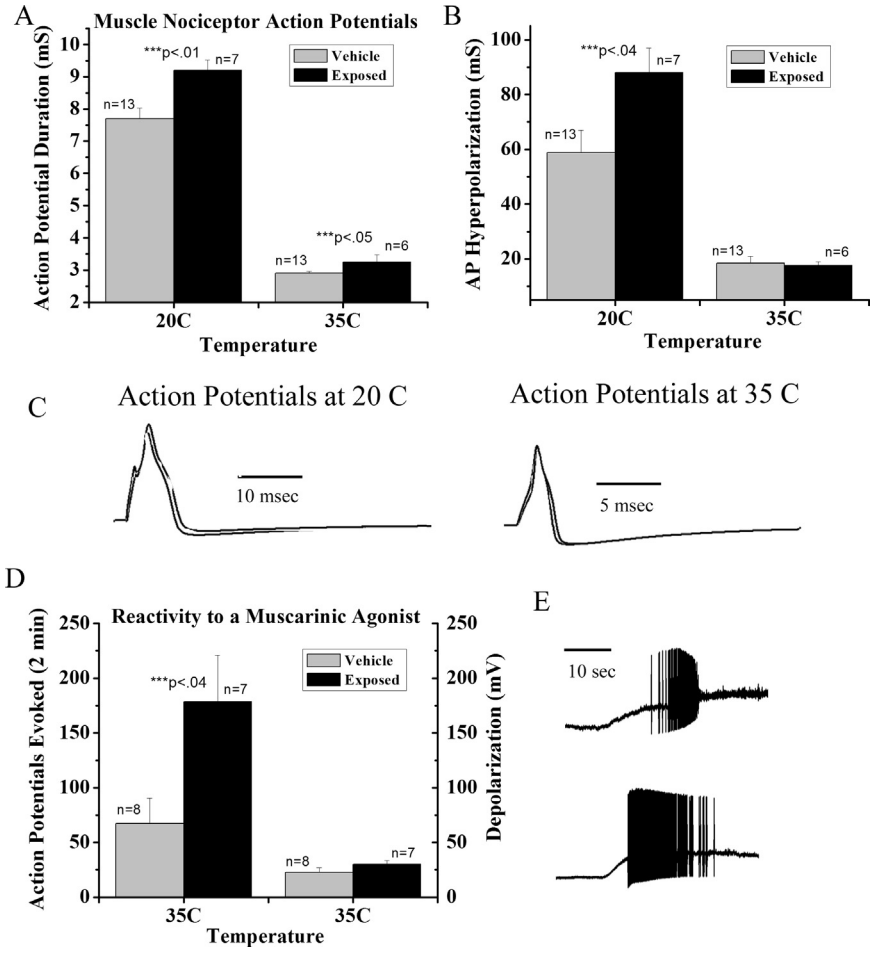

Fig. 8. Action potential characteristics and reactivity to oxotremorine-M. (A) Action potential duration in vehicle and GW chemical exposed muscle nociceptors. The AP duration was increased at both test temperatures. (B) Action potential hyperpolarization in vehicle and GW chemical exposed muscle nociceptors. The AHP duration was increased only at 20 °C. (C) Overlapping AP traces in vehicle and GW chemical exposed rats at each test temperature. The AP durations of GW exposed rat muscle nociceptors were significantly longer than those of vehicle exposed rats. (D) Total action potentials evoked (left axis) and rapid depolarization (right axis) following exposure to the broad muscarinic agonist oxotremorine-M. (E) Representative AP burst in vehicle (upper trace) and exposed (lower trace) muscle nociceptors.

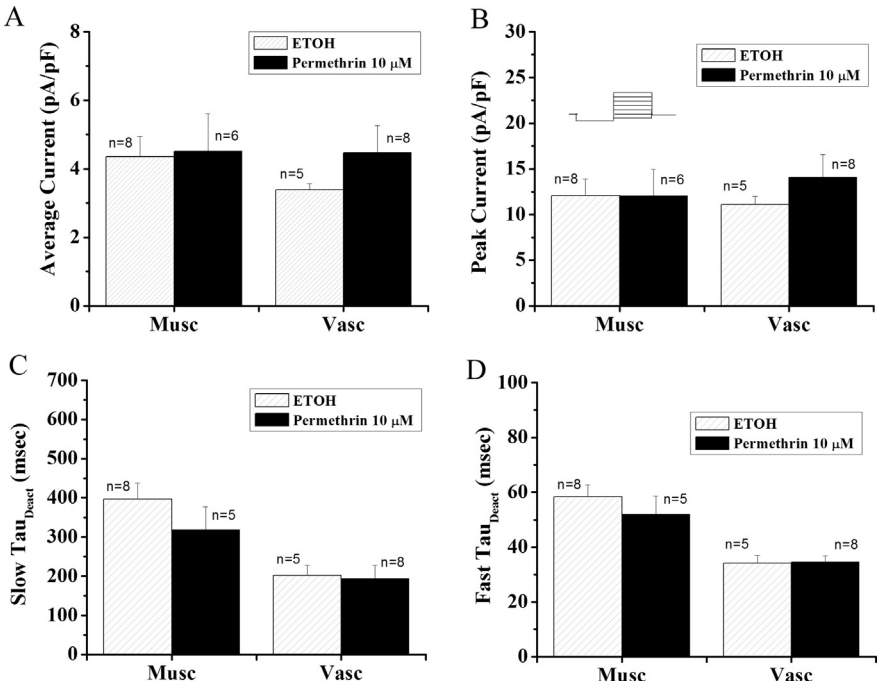

Fig. 9. Acute exposure to permethrin did not modify K_{DR} current amplitude or kinetics. The K_{DR} current was evoked in the presence of ETOH or 10 μM permethrin. (A, B) There were no significant shifts in the peak or average current in either muscle or vascular nociceptors. (C, D) Permethrin did not induce any significant shifts in the slow or fast component of the tau_{deact} in either muscle or vascular nociceptors. Evoked currents were averaged (−60 to 10 mV) and normalized for cell size. Comparisons were made between permethrin (10 μM) and ETOH (.001%) treated cases. The voltage protocol is shown as an insert in panel (B). From a V_H of −60 mV, cells were hyperpolarized to −100 mV for 2 s then stepped for 500 ms from −80 to 20 mV in 10 mV steps.

chemical exposure protocol would produce signs of enduring pain behaviors and molecular maladaptations in rats exposed to chemical agents that were used during the 1991 Persian Gulf War. In a series of behavioral studies, we demonstrated that increasing the duty cycle exposure to chlorpyrifos and PB produced signs consistent with a delayed myalgia and arthralgia that mimicked the emergence of GW illness pain reported by many veterans (Haley and Kurt, 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006). Parallel studies in these same GW chemical exposed rats, revealed a pattern of molecular maladaptations in deep tissue nociceptors that were consistent with increased nociceptor excitability and pain 12 weeks after exposure to GW agents had ended.

4.1.1. Behavior studies

The Persian Gulf War exposed troops to a wide variety of chemicals with diverse potential for neurotoxicity. Many of these chemicals were anticholinesterases. These anticholinesterases included lethal nerve gas agents (Sarin), nerve gas prophylactics (PB), and many pesticides (*e.g.*, chlorpyrifos) that were issued to suppress local insect populations (Binns et al., 2008). For the deployed warfighter, exposures to these agents would vary considerably dependent upon a variety of factors that included: (1) chemical weapon attack; (2) chemical weapon destruction; (3) use of nerve gas prophylactics; (4) authorized insecticide and repellant application; and (5) unauthorized insecticide and repellant application. The incidence, duration and concentration of these exposures were highly variable and largely undocumented. While exposure to any one agent was likely to be without risk to the health of the warfighter (with the exception of Sarin), the exposure to multiple agents, especially those converging on the same physiological targets, had the potential for significant neurotoxicity.

In our studies, we were able to show that increasing the duty cycle of two anticholinesterases (PB and chlorpyrifos) produced behavioral signs consistent with myalgia/arthralgia. Pain behaviors were manifested as increased resting periods in rats exposed for 30 or 60 days to the 3 GW agents. Shifts in resting times were replicated in the 30 and 60-day exposure groups. More importantly, the 60-day exposure plan increased resting periods for 12 weeks post-exposure and added decreased movement distance to the pain behavior complex. Paradoxical increases in movement rate, which were observed with 30-day exposures, faded in the 60-day exposure group. Most (∼75%) servicemen and women that developed symptoms of GWI pain experienced these symptoms only after they returned from overseas deployments (Kroenke et al., 1998). In our rat model, the full pattern of increased resting and decreased movement, with normalized rate, did not appear until 12 weeks after chemical exposures had ended. Therefore, the final pattern of rat activity measures, that might represent a myalgia and/or arthralgia, emerged in a delayed manner, much like that reported by most, but not all, Gulf War veterans (Kroenke et al., 1998). Because increasing the exposures to chlorpyrifos and PB produced a behavioral outcome consistent with a delayed chronic pain, the role of GW anticholinesterases as a key factor in the development of this pain syndrome was strengthened. While these outcomes are encouraging, it must be recognized that pain measurement is always an indirect assessment. The moderate elevation of rest periods and decline in movement distance, that emerged 12 weeks after GW chemical exposure, might reflect something other than myalgia and/or arthralgia.

Tests on membrane excitability tended to generally support the interpretation that the behavioral data represented a myalgia. When excitability tests were carried out at 35 °C, muscle nociceptors exhibited increased AP discharges in current injection and muscarinic agonist testing. However, it should be recognized that excitability testing, *in vitro*, cannot fully substitute for *in vivo* recordings from intact muscle nociceptors. Action potentials are normally generated at the initial segment. The mixture of Na_v and K_v ion channels, in the soma, might not accurately reflect

the composition of these channels at the initial segment. The divergence between the molecular composition at these distinct neuronal compartments must temper any conclusions regarding neuronal hyperexcitability. Alternately, we cannot rule out that ectopic discharges, originating from DRG soma, contribute to the chronic pain of GWI (Fan et al., 2011; Ren et al., 2012; Vaso et al., 2014; Xie et al., 2015). Until direct testing is made on isolated muscle nociceptors, *in vivo*, we cannot determine the site of hyperexcitability or fully evaluate its functional importance.

4.1.2. Molecular studies on K_v7

In prior reports, we found that 30–60 day exposures to permethrin, chlorpyrifos and PB significantly increased the activity of K_v7 and $Na_v1.9$ ion channels expressed in defined populations of vascular and muscle nociceptors (Rau et al., 2007; Cooper et al., 2014). These shifts were manifested as increased current amplitude and/or conductance and persisted for at least 8 weeks after exposure (Nutter et al., 2013; Nutter and Cooper, 2014). Although the persistence of the molecular adaptations was compelling, they did not occur during periods of measureable chronic pain-like behaviors; nor were the changes in channel activity obviously consistent with increased nociceptor excitability. While we have not observed shifts in $Na_v1.8$ amplitude (Nutter et al., 2013), the increased current amplitude in both $Na_v1.9$ and K_v7 channels after GW chemical exposure suggests a rebalancing of some of the depolarizing and hyperpolarizing forces that govern nociceptor excitability. Whether they achieved an appropriate rebalancing is difficult to determine in a cell soma preparation. Regardless of the limitations of the preparation, the lack of any measureable behavioral pain component, in our previous reports, indicated that whatever molecular adaptations occurred, they were insufficient to mimic a GW pain syndrome.

We hypothesized that increasing the anticholinesterase component of the GW chemical exposure might further derange the relationship between depolarizing and hyperpolarizing forces that govern neural excitability in muscle and vascular nociceptors. Increasing the duty cycle of PB exposure from 50 to 100% and that of chlorpyrifos from 7 to 14% produced a pattern of rat behavior could be interpreted as a myalgia/arthralgia. During time periods in which open field activity measures suggested pain, tests on muscle nociceptor physiology revealed a significant decline in K_v7 (and K_{DR}) activity. This decline in K_v channel activity, in muscle nociceptors, was clearly consistent with the presence of a myalgia (Du and Gamper, 2013; Tsantoulas and McMahon, 2014). Nevertheless, it is assumed that depolarizing forces ultimately drive nociceptor activity. As we have documented that there are increases in $Na_v1.9$ amplitude, inadequate opposition to this strong depolarizing force, by K_v7, could maintain a chronic pain state in our model. Still, we have yet to determine if maladaptations to $Na_v1.9$ also occur following exposure to the protocol used in this report.

Intracellular pathways that produce long term changes in the expression of K_v7 are not thoroughly understood, but have been linked to the development of neuropathic-like pain behaviors exhibited in some animal models of chronic pain (Rose et al., 2011; Cai et al., 2013). Neural activity can promote the expression of K_v7, through the Ca^{2+}/calcineurin dependent induction and nuclear translocation of members of the NFAT family of transcription factors (nuclear factor of activated T cell; Rao et al., 1997; Graef et al., 1999; Smith, 2009; Zhang and Shapiro, 2012). There is evidence that this pathway functions as an excitation driven negative feedback loop through an 'activity reporter' that is sensitive to action potential enabled entry of Ca^{2+} *via* the L-type voltage activated Ca^{2+} channel ($Ca_v1.3$; Mucha et al., 2010; Zhang and Shapiro, 2012). Exposure to pyrethroids, such as permethrin, could contribute substantially to drive this regulatory pathway due to their influence on Na_v inactivation and deactivation (Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006; Jiang et al., 2013). The permethrin induced broadening of the Na_v component of the nociceptor action potential would enhance Ca^{2+} entry through all voltage dependent Ca^{2+} channels to produce a false report of excessive activity. Pyrethroid dependent enhancement of Ca^{2+} entry has been shown to promote the expression of BDNF (brain derived neurotrophic factor) and other neurotrophins (Imamura et al., 2006; Cao et al., 2011; Ihara et al., 2012; Matsuya et al., 2012; but see Imamura et al., 2000). BDNF is one of several neurotrophins that drive expression of NFAT transcription factors (Groth and Mermelstein, 2003; Groth et al., 2007; Kim et al., 2014).

Calcium enters neurons during action potential depolarization. Ca^{2+} entry into nociceptors tends to be greater due to their broad action potentials; this is due, in part, to the slow inactivation kinetics of $Na_v1.8$. If exposure to permethrin induced a disproportionate entry of Ca^{2+} into DRG nociceptive neurons, relatively minor activity of nociceptors could result in gene transcriptions that are normally initiated by the intense activity associated with inflammatory or post-traumatic discharge. Muscle nociceptors would be a prime candidate for such a mechanism as, unlike other nociceptor populations that are mainly inactive, and would not provide a path for Ca^{2+} entry, muscle nociceptors are activated by ischemic pain related to muscle exertion. Ischemic muscle pain would be a frequent experience for the deployed warfighter. Accordingly, we can hypothesize a scenario where exaggerated Ca^{2+} entry, under the influence of permethrin exposure, could drive the expression of NFAT transcription factors to substantially elevate expression of K_v7. There are also indications from our studies that the maladaptive entry of excessive calcium might persist into the post-exposure period. Following a 60-day exposure to permethrin, chlorpyrifos and PB, we have documented a slowing of $Na_v1.8$ inactivation kinetics that persisted for 8 weeks and coincided with the upregulation of K_v7 (Nutter et al., 2014). The slowing of $Na_v1.8$ kinetics implies a continuing dysregulation of Ca^{2+} entry that could falsely report excessive discharge rates and spur BDNF/NFAT expression long after exposures had ended (Cao et al., 2010; Cao et al., 2011). In the present study, we found that both action potential duration and after hyperpolarization were significantly longer in muscle nociceptors 12 weeks after GW chemical exposure. Because the family of Ca^{2+} activated K^+ channels govern the AHP, the longer AHP in muscle nociceptors implies that enhanced, Ca^{2+} entry persists 12 weeks after exposure. The known acute and possible chronic impact of pyrethroids on voltage activated Ca^{2+} channels could further magnify AP dependent Ca^{2+} entry (Shafer and Meyer, 2004; Neal et al., 2010).

Ultimately, it is the down regulation of K_v7, in muscle nociceptors, that we detected 12 weeks post-exposure, which is likely to be a key event in the delayed emergence of the pain-like behavior syndrome. Due to its unique capacity to actively oppose membrane depolarization, inhibition of K_v7 leads to increased excitability and spontaneous activity in neurons (Marrion, 1997; Robbins, 2001; Brown and Passmore, 2009). Pathways that lead to acute inhibition of K_v7 have been characterized in multiple preparations, including DRG, where they contribute to the development of inflammatory pain (Passmore et al., 2012). Those factors that decrease expression of K_v7 are not well understood.

NRSF/REST (neuron-restrictive silencer factor; repressor element 1 – silencing transcription factor) has been identified as a transcription factor that suppresses the expression of several K_v channels, including K_v7 ($K_v4.3$, $K_v3.4$, $K_v7.2$, $K_v7.3$; Chien et al., 2007; Uchida et al., 2010; Mucha et al., 2010; Rose et al., 2011). We have demonstrated the expression of $K_v7.3$ in both type 5 muscle

and type 8 vascular nociceptors (Nutter et al., 2013). In a nerve injury model of neuropathic pain, the appearance of chronic pain-like behaviors was accompanied by the elevated expression of NRSF/REST (Uchida et al., 2010; Rose et al., 2011). Interestingly, significant REST expression was delayed 15–30 days following neural insult and well past the onset of pain behaviors (Rose et al., 2011). Delayed expression of REST could play a key role in the delayed decline of K_v7 activity and the appearance of pain-like activity patterns in our model. Factors contributing to the time dependent expression of REST have not been determined.

4.1.3. Molecular studies on K_{DR}

While decreased expression of K_v7 is likely to be an important event leading to increased neural excitability, we also observed down regulation of total K_{DR} channel activity, in muscle nociceptors, during those periods when rats exhibited pain-like behaviors. It is possible that the reduction of K_v7 activity may have only been part of a broader decline of K_v channels expressed in muscle nociceptors. Recent studies have identified an antisense mRNA that induces broad reductions of total K_v current in DRG neurons. Expression of the long non-coding antisense mRNA *kcna2* (AS mRNA *kcna2*) has been shown to decrease total K_v current in DRG neurons, increase neuronal excitability and produce neuropathic pain-like behaviors (Zhao et al., 2013; Lutz et al., 2014; Li et al., 2015;). Long antisense mRNA *kcna2* expression is very low in normal DRG, but rises sharply after nerve injury (Zhao et al., 2013). It is expressed only in select populations of DRG neurons that make up the medium and large diameter pools (Zhao et al., 2013; Fan et al., 2014). Both the type 5 muscle and type 8 vascular nociceptors come from the medium diameter pool of DRG neurons (Petruska et al., 2002). While the decline in the activity of K_{DR} and K_v7 ion channel proteins might be due to increased expression of long antisense mRNA *kcna2*, there is no demonstrated linkage between permethrin, chlorpyrifos or PB and the expression of *kcna2.*

5. Conclusion

Our studies have demonstrated that increasing chronic exposure to certain anticholinesterases (PB, chlorpyrifos), in the presence of permethrin, can produce delayed pain-like behaviors and long lasting maladaptations in muscle and vascular nociceptor ion channel function. In muscle nociceptors, the up and down regulation of K_v7 and K_{DR} ion channel proteins covaried with the appearance of pain-like behaviors that appeared 12 weeks after toxin exposures had ended. The diverse nature of the chemicals the warfighters were actually exposed to in the Gulf War, and the ultimate distribution of these chemicals into blood, muscle, synovial fluid and fat depots would expose tissue specific populations of nociceptive neurons to similar maladaptive perturbations that could ultimately produce the highly varied pain symptoms exhibited by GW veterans.

Conflict of interest

The authors declare that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Muscarinic Receptor Maladaptations following Exposure to Gulf War Illness Chemicals

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Abstract

Chronic pain is a frequent 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. Following an 8 week exposure of rats to a mixture of anticholinesterases (pyridostigmine bromide, chlorpyrifos) and a Na_v deactivation inhibiting pyrethroid, permethrin, a behavior pattern emerged that was consistent with a delayed myalgia (Nutter et al., 2015). This myalgia-like behavior was accompanied by persistent changes to K_v 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). Contrasts between muscle nociceptors harvested from vehicle and GW chemical exposed rats revealed that mAChr suppression of K_v7 activity was enhanced in exposed rats, but simultaneously diminished against a Stromatoxin insensitive component of the K_{DR}. We also provide further evidence that both muscle and vascular nociceptors exhibited a unique mAChr induced depolarization and burst discharge (MDBD). Examination of the molecular basis of the MDBD revealed that it was present in both vascular and muscle nociceptor but was not dependent upon inhibition of K_v7. 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.

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). A large portion of those suffering from GWI complained of chronic pan 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 in 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 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 as series of studies that were designed to reproduce the myalgia and arthralgia of GWI in a rat model. Certain GW chemicals (pyrethroids, anticholinesterases) have properties that could directly (pyrethroids) or indirectly (anticholinesterases) interact with important membrane ion channel and receptor proteins expressed in peripheral nociceptors (e.g., $Na_v1.8$: Narahashi et al., 1998; Narahashi, 2000; Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006; Soderlund, 2010; Jiang et al., 2013; Muscarinic receptors: Abou-Donia et al., 2003; Abou-Donia et al., 2004; 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 for 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 support this interpretation, as K_v7 and other K_{DR} 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 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.

Methods

Exposure Protocol. Juvenile male rats weighing between 90 and 110 g were used in all studies (chronic Studies, n= 33; acute studies: n= 21; Sprague-Dawley; Harlan/Envigo). In chronic experiments, 13 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). 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 (20) additional rats received only vehicle exposures using an identical administration schedule. Rats were sacrificed for electrophysiological studies 8 and 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; n=20, Vehicle; and 430.8 +/- 8.6 g. n=16, Exposed; $p<.28$). Rats were weighed once per week throughout the studies and doses were adjusted accordingly.

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.

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 (right 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 chemically exposed (permethrin, chlorpyrifos, PB) and vehicle treated (ETOH, corn oil, water) animals over an identical time course. Rats were tested once per week on the behavioral tasks. Most tests were conducted in 'blinded' conditions. Only 'blinded' testing scores were used in the analysis.

Electrophysiological Studies

Preparation of Cells. Dorsal root ganglion neurons (DRG) were harvested from chemically and vehicle exposed rats 8 and 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 10 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 1000 Hz (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 were conducted at room temperature (21 ° 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 studies on K^+ 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. 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 Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 65-70% 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.

Current Clamp Experiments. Type 5 muscle, type 8 and type 19 vascular neurons were identified and brought into current clamp mode (see also Nutter et al., 2015). The recorded cell was exposed to 10 uM oxotremorine-M for 30 seconds (OXO-M). In some tests, linopirdine (10 uM, ethanol), HC-030031 (2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamid; 10 uM), or TTX (1 uM) were presented for 2-3 minutes prior to application of OXO-M. All compounds were purchased from Sigma-Aldrich. When an antagonist preceded OXO-M, the antagonist (or its vehicle) was included in the OXO-M test. Tests were conducted at 35 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.

Isolation and Characterization of K_{DR} Currents.

K^+ currents were characterized in a 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.

K_{DR} Currents. For the purpose of this study, the K_{DR} current was defined as the residual total K^+ current following removal of the K_v7 component. Attempts to remove other components using K_v1 inhibitors Maurotoxin, Margatoxin or Dendrotoxin-K were unsuccessful. The voltage dependent activation of the residual total K_{DR} current, was assessed, as a tail current, after a 3 minute application of the K_v7 inhibitor linopirdine (10 uM). From a holding potential of -60 mV, a 2000 msec conditioning pulse (-100 mV) was followed by 11 consecutive command steps to 20 mV (10 mV increments; returning to -60 mV). The amplitude of the tail current at -60 mV was measured from the peak relative to the baseline current recorded 2500 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 $V_{1/2}$ determined for each individual cell. Student's T-test was used to compare the pooled $V_{1/2}$ values for vehicle and GW chemical treated cells. To assess average and peak 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 (-50 to 0 mV) to obtain a mean current amplitude. The normalized peak current was determined by inspection. Student's T-tests were performed on peak and average amplitude as described above. The alpha level was set at .05.

Stromatoxin Sensitive K_{DR} Currents. Tests were conducted to isolate those portions of the K_{DR} that were modified by GW chemicals. The K^+ isolation (Kiso) solution was used to isolate all K_{DR} current as described above. Ten micromolar linopirdine was added to Kiso to block K_v7 components of the current. Bovine serum albumin (BSA, 0.1%) was also added to all solutions as a carrier for the toxins and for vehicle test contrasts against toxin testing. 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 ($K_v2.1$, $K_v2.2$; Escoubas et al., 2002) or Maurotoxin ($K_v1.1$, $K_v1.2$; Kharrat et al., 1996; Castle et al., 2003) 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. The evoked current at 20 mV was leak corrected, on line, using the P/N procedure utility of Clampex 9.0. 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. Toxins were applied continuously by close superfusion. 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, and to a lesser extent, Maurotoxin was effective in tests on muscle and vascular nociceptors. The Maurotoxin or Stromatoxin sensitive current was determined as the amount of current that decreased after 5 minutes of toxin application. Leak corrected 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 for cell capacitance. The Stromatoxin insensitive current was determined from the currents remaining after the 5 minute application 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 (itox) was the average of the last 3 tests evoked after the 5 minute toxin presentation. The percentage of toxin insensitive current in the K_{DR} was then determined as a ratio: 1-(itox/base).

Muscarinic Influences on K_{DR}. Tests were conducted to determine if the K_{DR} currents were sensitive to a muscarinic agonist (OXO-M) and if that sensitivity was altered by GW chemicals. The K^+ isolation (Kiso) solution was used to isolate K_{DR} current as described above. Ten micromolar (10 uM) 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 uM oxotemorine-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 from leak corrected data (P/N). 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). There was little indication that the influence of OXO-M changed over the 4 minute application period or after a 3 minute washout test that followed.

Statistics. A repeated measures ANOVA was used to assess the influence of chemical exposure and weeks on dependent measures of muscle pain threshold (grams), 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 (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 contrast amplitude measures of K_{DR} and K_v7 activity in GW exposed and Oxotremorine 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.

Results

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).

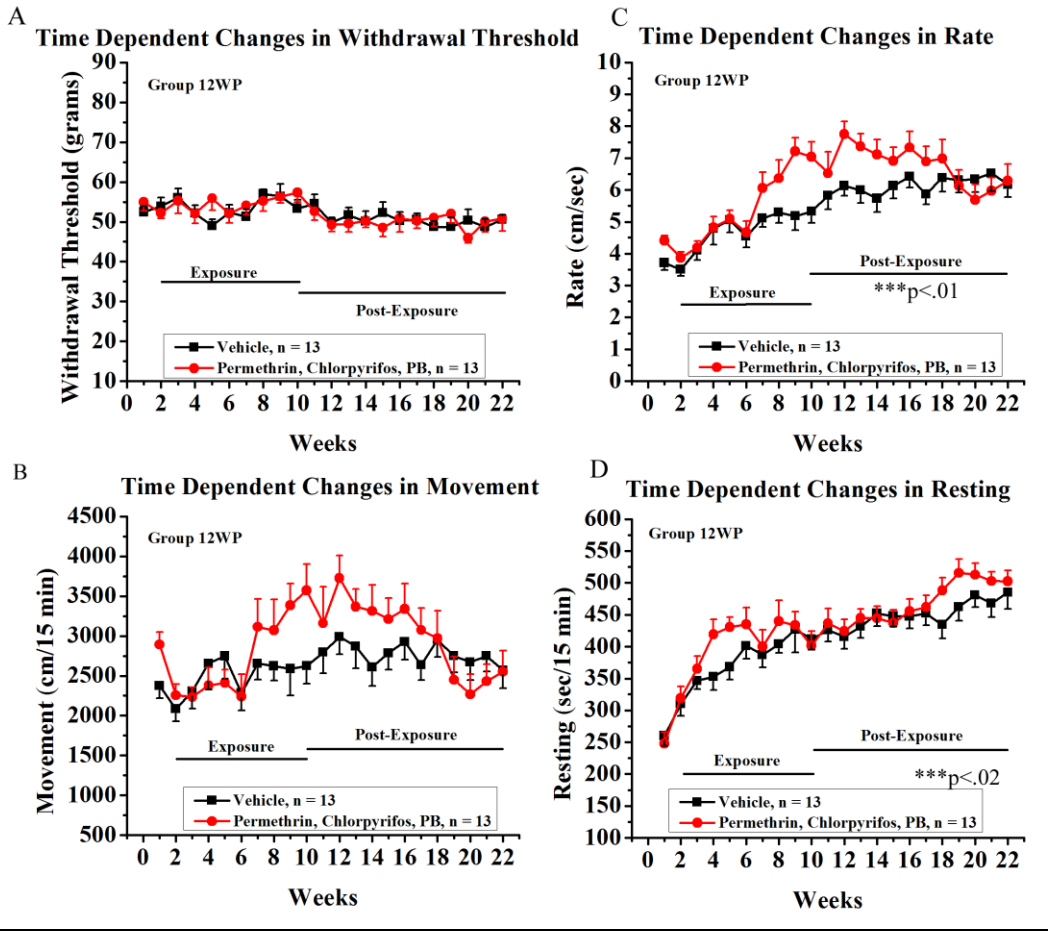

Figure 1. The Intensified Exposure Protocol Produced a Delayed Pain-Like Behavior Pattern in the 12 Week post Exposure Group. A)-B) Muscle pain pressure threshold, and movement distance were unchanged following the 60 day exposure to GW chemicals. **C)** Movement rate significantly increased during weeks 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 ($F=5.82$; $p<.02$). Tests were not conducted on any measure 1-4 weeks post exposure or during exposure to GW chemicals.

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

Rats from GW chemical and vehicle treated groups were sacrificed 12 weeks following exposures. DRG neurons were plated on 35 mm Petri dishes and physiology studies were carried out within 10 hours after plating. 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. K_{DR} currents were isolated in a K-iso solution (see Method). Consistent with our previous approach, linopirdine (10 uM) was added to the isolation solution to block the K_v7 component of the K_{DR}, (3 minute application; Nutter et al., 2015). Following a pre-pulse to -100 mV, a 500 msec voltage pulse to +20 mV was applied at 30 second intervals. Evoked currents were leak corrected on line using the P/N procedure. Following collection of 6-9 baseline samples, current amplitude stabilized sufficiently to present a toxin.

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=11) failed to produce significant reduction of the K_{DR} current. 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. 4AP was included in the K_{iso} solution to inhibit A-currents expressed by these nociceptors (I_A; 2 mM; Castle et al., 1994; Grissmer et al., 1994; Russel et al., 1994). 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; Bocksteins et al., 2009). 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 3).

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} current 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, 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 $K_v1.3$.

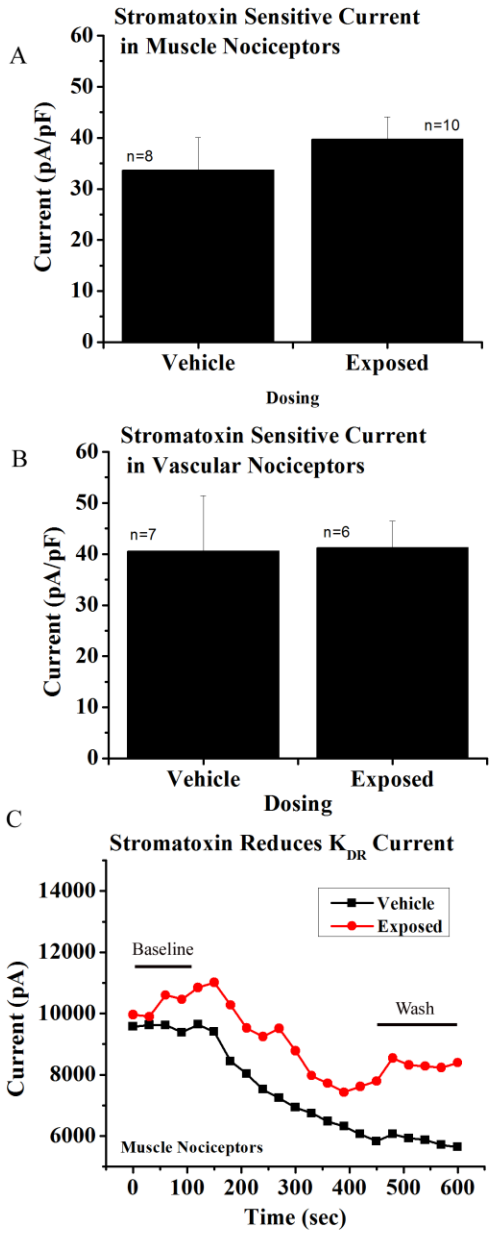

Figure 2. Stromatoxin Sensitive Currents (K_v2.1, K_v2.2) in GW Exposed Nociceptors. The total K_v2.1 and K_v2.2 currents were not modified by chronic exposure to GW chemicals. **(A)** Muscle nociceptors **(B)** Vascular nociceptors. **C)** A representative case showing the time dependent change in peak K_{DR} current after exposure to StTX. Cells were harvested 12 weeks after exposures had ceased.

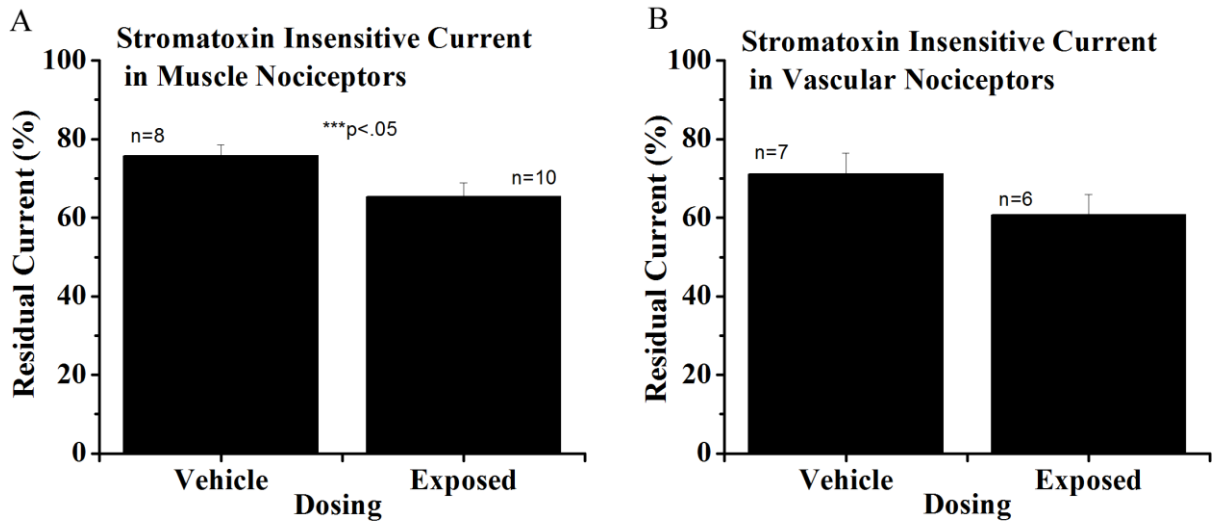

Figure 3. Stromatoxin Insensitive Currents in GW Exposed Nociceptors. StTX sensitive currents ($K_v2.1$ and $K_v2.2$) normally comprised about 25 to 30% of the total K_{DR} current in muscle and vascular nociceptors, respectively (vehicle tested rats). The StTX insensitive current, the proportion of 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.

Modification of mAChr Reactivity after Exposure to GW Chemicals

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 uM 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 (not shown; 1.17 +/- 0.03 and 1.13 +/- 0.03; n=8 vehicle and 6 exposed, respectively).

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 7B, 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.

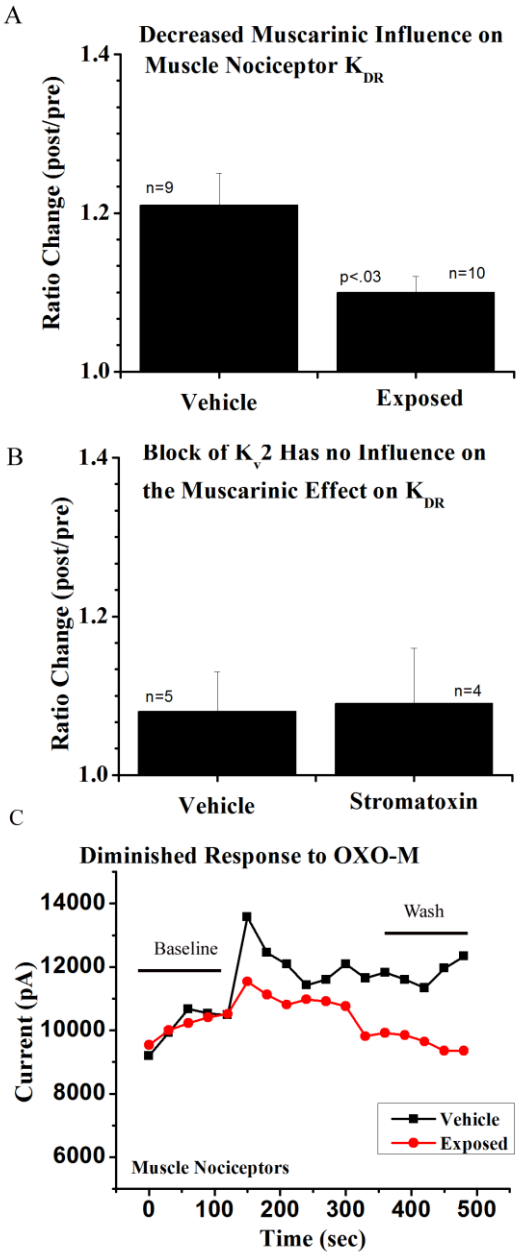

Figure 4. GW Chemicals Decreased the Muscarinic Sensitivity of K_{DR} Currents. A) A broadly active muscarinic agonist (OXO-M) increased K_{DR} currents of muscle nociceptors harvested from both vehicle exposed and GW chemical exposed muscle nociceptors. Muscle nociceptors from exposed rats were significantly less reactive to the muscarinic agonist. **B)** 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. **C)** 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.

Both muscle and vascular nociceptors express K_v7 channel proteins (Nutter et al., 2013). Cholinergic modulation (inhibition), via the muscarinic receptor activated pathway, is one of the ways by which K_v7 enhances neuronal activity (Brown and Passmore, 2009). We have shown that the activity of K_v7 currents was modified by exposure to GW toxicants (Nutter et al., 2013; Nutter et al., 2015). One level of modification could occur via activation of muscarinic receptors.

K_v7 channel proteins are open at the resting membrane potential. This allows K^+ to flow out of the cell and hyperpolarizes the membrane. Closure of K_v7 depolarizes the membrane and increases neuronal excitability. We examined how exposure to a broadly active muscarinic agonist, OXO-M, altered the resting currents in both muscle and vascular nociceptors (OXO-M, 10 uM; 30 sec). 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).

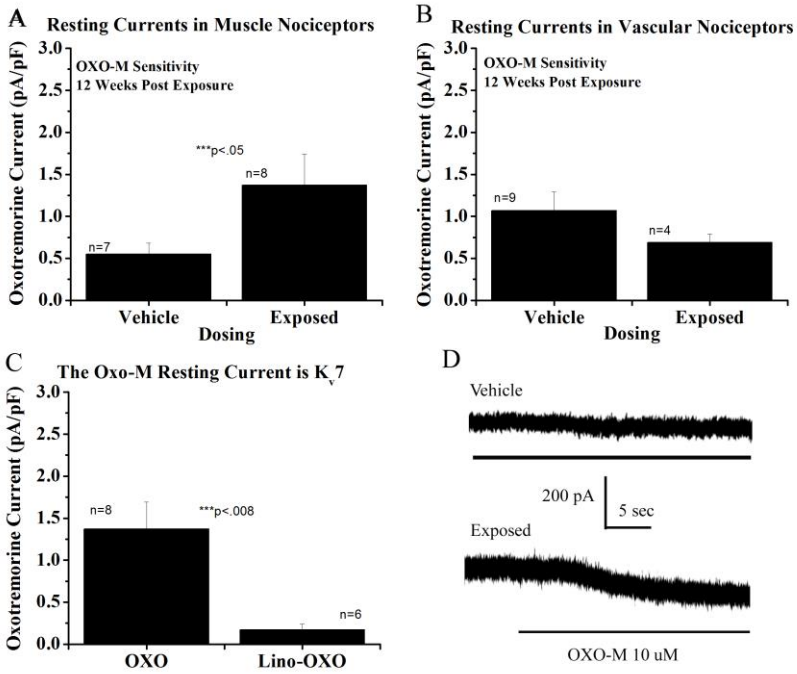

Figure 5. Muscle Nociceptors Exhibit Enhanced Sensitivity to a Muscarinic Agonist. A) Shifts in the holding current following a 30 second exposure to OXO-M (10 uM) suggested enhance suppression of K_v7 currents in muscle nociceptors exposed to GW chemicals. **B)** Resting currents in vascular nociceptors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

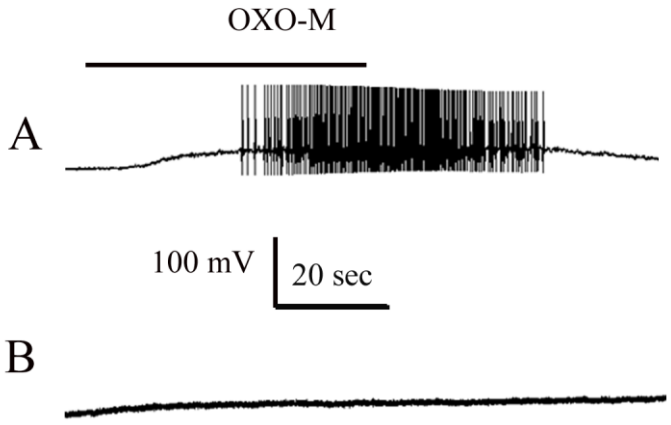

Figure 6. A Muscarinic Burst Discharge in a Muscle Nociceptor from Rats Exposed to GW Chemicals. A) MDBD in a muscle nociceptor following the application of oxotremorine-M (10 uM). **B)** Depolarization without action potential discharge in a vascular nociceptor from vehicle exposed rats (OXO-M, 10 uM). Studies were conducted in Tyrode's solution.

In a recent publication, we demonstrated that a muscarinic agonist evoked a burst discharge (MDBD; muscarinic depolarization and burst discharge) 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 pathophysiology of GWI, we further developed this line of investigation.

Experiments were carried out in current clamp mode in a Tyrode's bath solution. Using muscle and vascular nociceptors that were harvested from GW chemical and vehicle exposed rats, we applied 10 uM OXO-M by close superfusion. Depolarization with and without action potential bursts were observed in both groups of cells (figure 6). Two of 3 muscle nociceptors from chemically treated rats rapidly depolarized (20.7 +/- 3.1 mV; n=3). Depolarization was accompanied by a burst discharge (255.5 +/- 251.5 APs; 13.1 +/- 8.1 sec burst; n=2). Weaker depolarization and discharge was observed in vehicle exposed groups (12.3 +/- 9.3 mV; n=3 and 155 APs; n=1; 11.9 sec burst). These results were similar to our previous report. We also performed tests on vascular nociceptors that had not been previously studied. Following application of 10 uM OXO-M, an MDBD was evoked in 2 of 4 cases. Tests were limited to vehicle exposed rats. Depolarization was substantial (9.7 +/- 8.1 mV; n=4) and 2 cells exhibited a burst discharge (70.5 +/- 34.5 APs; 2.3 +/- 2.5 sec; n=2; figure 6).

Molecular Basis of the MDBD

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).

The molecular basis of the hippocampal MDBD involved muscarinic receptor activation coupling to a persistent, TTXs, Na^+ current (Chiange et al., 2010; Yamada-Hanff and Bean, 2013). We examined whether the molecular basis of the MDBD in DRG was similar to that described in hippocampus. These studies were carried out in young adult 'naïve' rats.

Experiments were conducted in a Tyrode's bath solution in current clamp mode (n=8 rats). Muscle and vascular afferents were identified in the usual manner (Method). 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 uM) 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 35 C by a servo-controlled probe during the conditioning phase (see Method).

In vehicle treatment conditions, type 5 muscle and type 8 vascular nociceptors both manifested MDBD (figure 7A). Instances of burst discharge were less frequent in naïve rats than in GW chemical exposed rats. While all nociceptors exhibited rapid depolarization in excess of 10 mV (11.9 +/- 2.8 and 10.8 +/- 5.1 mV; muscle and vascular nociceptors respectively), burst discharges were only observed in one muscle nociceptor (213 Aps, 9.2 msec burst; n=1). Additional tests were conducted on type 19 vascular afferents (Cooper et al., 2014; Henao et al., 2015). Type 19, non-nociceptive vascular afferents did not exhibit significant depolarization or any burst discharge to OXO-M (n=5; not shown).

MDBD could be dependent on depolarization consequent to inhibition of K_v7 subsequent to activation of mAChr (Linely et al., 2008; Brown and Passmore, 2009). To test this hypothesis, we applied linopirdine for 3 minutes prior to presentation of OXO-M (10 uM). During the

application of linopirdine, only weak depolarizations were observed. When OXO-M was presented in the presence of linopirdine, both type 5 muscle (n=5) and type 8 vascular (n=4) nociceptors strongly depolarized (14.4 +/- 2.1 mV and 8.3 +/- 1.4 mV), but only muscle nociceptors emitted AP bursts (63.0 +/- 20.5 APs; 14.4 +/- 5.2 sec; n=3; figure 7B). Pre-treatment with the K_v7 blocker, linopirdine, did not block depolarization but may have increased the likelihood of AP discharges evoked by OXO-M. Nor was there any evidence that pretreatment with TTX and linopirdine could block depolarization or AP burst discharge (n=1).

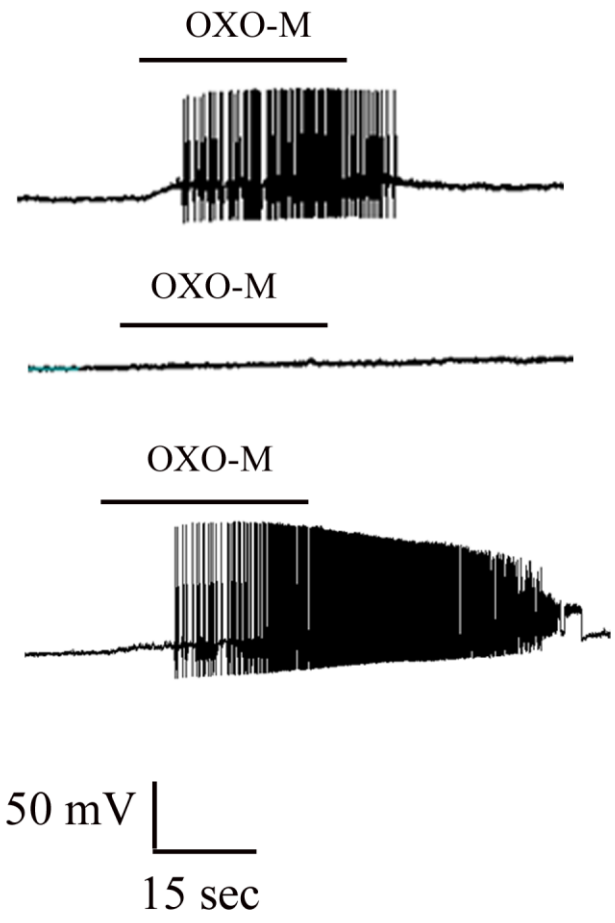

Figure 7. Block of TRPA1 and/or Kv7 Modifies MDBD. A and B) Muscle nociceptor depolarization is unaffectred by pre-treatment with K_v7 antagonist linopirdine, but significantly reduced by TRPA1 antagonist HC-

030031. The probability of discharge was significantly increased in the presence of linopiridine only. **C)** Representative trace of a high frequency burst discharge in a muscle nociceptor following OXO-M (10 uM). **D)** Inclusion of HC-030031 reduced depolarization to OXO-M. E) When HC-030031 was washed-out for 5 minutes, OXO-M produced powerful bursts (same cell as 'D'. All tests conducted at 35 C.

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.

Behavior 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 suppressed 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). 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 (Rau et al., 2013; 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.

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 et al., 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 et al., 2015).

For our purposes, the action of of ACh at mAChr is particularly relevant. Muscarinic M2 and M4 receptors are expressed in 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; Padilla et al., 2005 Pung et al., 2006; Abou-Donia et al., 2004; see also Abou-Donia et al., 2003; Abdel Rahman et al., 2004a; Abdel Rahman et al., 2004b; Udarbe et al., 2008; 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.

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 K. Krnjević et al., 1971). Mechanistically, it also closely resembles the phenominon 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 may be accompanied by a burst discharge (Klink and Alonso, 1997; Ergov 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.

ADP is a consequence mAChr activation, has been shown to require coupling to Gq/11 (Araneda and Andrade, 1991; Greene et al., 1994; Sidiropoulou et al., 2009), 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 a non-selective cation current (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; Yue and Yaari, 2006; Brown and Randall, 2009; and may require and activation of LVA Ca^{++} currents, the nature 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 MDBD in DRG was dependent on the inhibition of K_v7, nor was it sensitive to the blockade of TRPA1 or a TTX sensitive Na_v. As the principle Na_v in DRG nociceptors is a TTXinsensitive Nav1.8, it is not surprising the block of TTXsensitive channel proteins did not modify MDBD. It remains unclear whether these powerful mAChr dependent burst discharges in DRG nociceptors are mechanistically related to CNS ADP. Nor is it clear that ADP itself is a mechanistically uniform phenomenon. The prevalence of ADP in hippocampus, at times including muscarinic bursting discharges similar to those that we observed in DRG, could have important implications for GWI. In the CNS, ADP is believed to contribute to learning and memory (Rahman and Berger, 2011). We have shown that MDBD is potentiated by chronic exposure to GW chemicals (Nutter et al., 2015). If such effects were exhibited in hippocampus, it could be a compelling link to cognitive deficits associated with this disorder.

In DRG, the MDBD was widespread in muscle nociceptors (Nutter et al., 2015). Presently, we report that vascular nociceptors also exhibited an MDBD, but it was present only in about half of

the vascular nociceptor population. The latter observation was consistent with our studies in which vascular nociceptors from rat tail vein were directly traced and characterized (Cooper et al., 2014; Henao et al., 2015). About half of these traced type 8 nociceptors exhibited slow, small inward persistent currents shifts, when challenged with ACh. Those currents were consistent with activation of mAChr. The other half exhibited large nicotinic responses we have previously characterized as $\alpha_3\beta_4\alpha_5$ mediated in type 8 cells (Rau et al., 2005). In the absence of a muscarinic response component, no MDBD would occur in that subclass of type 8 cells. We have identified at least 3 functional variants of the type 8 cell signature in tracings from skin, muscle, colon and vessels (Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). Other significant functional variants may exist.

Muscarinic receptor dependent burst discharges could play a key role in symptoms of GWI pain. In sensory neurons, MDBD could contribute to postsynaptic spinal cord neuroplasticity (i.e., central sensitization) that is associated with some chronic pain syndromes (Willis, 2001; Latremoliere and Woolf, 2009). Central sensitization, can be initiated by high frequency discharge dependent release of the paracrine vasoactive neuropeptide SP from primary afferent nociceptors. In the spinal cord, afferent derived SP can produce prolonged depolarizations of post-synaptic relay neurons that relieve Mg^{++} blockade of the NMDA receptor. The activation of the NMDA receptor is essential for central sensitization (Dougherty et al., 1993; Willis, 2001). Moreover, central release of the coexpressed neuropeptide CGRP activates resident microglia that synthesize and/or release of a number of cytokines (IL-1β, IL-6) chemokines (CCL2), nitric oxide and other pro-inflammatory agents (Li et al., 2008; De Corato et al., 2011; Malon et al., 2011) in the CNS. In DRG, SP, CGRP, $K_v7.3$ and $Na_v1.9$ are known to be expressed in type 5 and type 8 nociceptors (Petruska et al., 2002; Nutter et al., 2013; Nutter and Cooper, 2014).

An eight week exposure to permethrin, chlorpyrifos and PB, suppresses K_v7 activity, increases $Na_v1.9$ amplitude, enhances muscarinic coupling to K_v7, K_{DR} and potentiates an MDBD burst to a broadly active muscarinic agonist (Nutter and Cooper, 2014; Nutter et al., 2015). It is likely the SP and CGRP would be released by the high frequency discharges observed in MDBD (Holz et al., 1988). By shifting the balance of muscarinic modulation of K_v7, a GW chemical induced maladaptation functionally expressed as an exaggerated MDBD could contribute to the development and chronicity of Gulf War Illness pain syndromes through central sensitization. We have shown that increasing the exposure to anticholinesterases played an important role in the development of a delayed myalgia. The consequence of heightened exposure to anticholinesterases is consistent with the pattern of a modified mAChr dependent phenomena (K_v7, K_{DR}) and its functional expression through amplification of MDBDs.

Acknowledgements

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