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2 **Exposure to Gulf War Illness Chemicals Induces Functional**
3 **Muscarinic Receptor Maladaptations in Muscle Nociceptors**

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25 **Key Words: Gulf War Syndrome; Chlorpyrifos; K_v7; Nociceptor; Muscarinic; ADP**

Abstract

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

1. Introduction

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

Diverse risk factors, including exposure to Sarin gas, depleted uranium, oil fires, vaccination adjuvants, organophosphates and combat stress have been proposed as factors contributing to the development of GWI. However, no single factor has been able to account for the wide ranging symptoms of this complex multisymptom disease. The Research Advisory Committee on Gulf War Illness concluded that pesticides could have contributed to the development of the symptoms of GWI (Binns et al., 2008; RAC2014). While deployed to the Persian Gulf, soldiers were potentially exposed to 67 insecticides and repellants that contained 37 distinct ingredients (DoD Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008). Organophosphate, organochlorine, dialkylamide, carbamate and pyrethroid pesticides and repellants were used

liberally in the Gulf theater. Coincident with exposure to these agents, warfighters were self-administering a nerve gas prophylactic, pyridostigmine bromide (PB), which shares the anticholinesterase properties of many insecticidal chemicals. Pesticides were to be used at specific concentrations and with specific application methods and procedures. These application procedures were not always carefully followed and warfighters often supplemented designated agents with others they acquired on their own (US, DoD Environmental Exposure Report: Pesticides, 2003). While the levels of exposure to any one of these pesticides may not have posed a significant detriment to the health of the troops, synergisms arising from multiple chemical exposures could have converged on one or more molecular targets to produce long lasting physiological maladaptations and/or physical damage to nervous system components.

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

Several years ago we began a series of studies that were designed to reproduce the myalgia and arthralgia of GWI in a rat model. Certain GW chemicals have properties that could directly (pyrethroids) or indirectly (anticholinesterases) interact with important membrane ion channel and receptor proteins expressed in peripheral nociceptors (e.g., voltage activated Na^+ channel 1.8 ($\text{Na}_v1.8$): Narahashi et al., 1998; Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006; Jiang et al., 2013; Muscarinic receptors: Abou-Donia et al., 2003; Abou-Donia et al., 2004; voltage activated K^+ channel 7 (K_v7): Marion 1997; Robbins, 2001; Brown and Passmore, 2009). Following a 60 day exposure to the anticholinesterases pyridostigmine bromide and chlorpyrifos and the pyrethroid insecticide permethrin, we discovered that the physiology of vascular and/or muscle nociceptor ion channel proteins $\text{Na}_v1.9$ and K_v7 were altered 8 to 12 weeks after exposures had ceased (Nutter et al., 2013; Nutter and Cooper, 2014). In contrast, the physiology of $\text{Na}_v1.8$ was unaffected in these nociceptor pools (Nutter et al., 2013). Despite a relatively consistent pattern of perturbed ion channel physiology, we could not demonstrate behavioral changes consistent with the development of a chronic pain syndrome.

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

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

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

2. Methods

2.1. Behavioral Studies

2.1.1. Exposure Protocol

All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO. Juvenile male rats, initially weighing between 90 and 110 g, were used in all studies (chronic studies: n=35; acute studies: n=17; Sprague-Dawley; Harlan/Envigo). In chronic experiments, 15 rats were exposed to permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), and pyridostigmine bromide (PB; 13 mg/kg), for 60 days. Permethrin, in ETOH, was

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

2.1.2. Assessment of Pain Behaviors

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

PB) and vehicle treated (ETOH, corn oil, water) animals over an identical time course. From baseline testing (figure 1) until they were sacrificed, the rats were tested once per week on the 4 behavioral tasks. In order to avoid potential experimenter test bias, all the manual PAM tests were conducted under ‘blinded’ conditions (with the exception of two days). Automated activity testing did not require experimenter blinding. None of the ‘non-blinded’ PAM data fell within the periods of statistical testing.

2.2. Electrophysiological Studies

2.2.1. Preparation of Cells

Dorsal root ganglion neurons (DRG) were harvested from GW chemical and vehicle exposed rats 12 weeks after termination of chemical exposures. Rats were anesthetized (Isoflurane) and rapidly euthanized by decapitation (Harvard Instruments). The spinal column was removed, bisected and the DRG were dissected free from T11 to S1. Ganglia were trimmed, cut into strips and digested in Tyrode’s solution containing collagenase A (4 mg/ml) and Dispase II (10 mg/ml; Roche Chemical). A 15 ml centrifuge tube containing the dissected ganglia was placed in a heated, shaking water bath for 90 minutes at 35° C (EDVOTEK Digital Shaking Water Bath). Gentle trituration was then used to break up visible strips of ganglia. The dispersed neurons were then digested for an additional 45 minutes, and subsequently spun at 1000 RPM (30 sec). The supernatant was discarded. The remaining pellet was dispersed into 2 ml of Tyrode’s, triturated and plated on 8-10, 35 mm, polylysine coated, Petri dishes (Fluorodish). Plated neurons were bathed continuously in a Tyrode’s solution, containing (in mM) 140 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. All electrophysiological studies on specific ion channels (voltage clamp tests on K_{v7} and K_{DR}) were

conducted at room temperature (20 °C) within 10 hours of plating. Studies on membrane excitability were conducted at 34 °C (current clamp testing). Only one cell was used per Petri dish (9 Petri dishes per daily experiment). Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). Unless otherwise noted, the pipette solution contained (in mM): 120 KCl, 5 Na₂-ATP, 0.4 Na₂-GTP, 5 EGTA, 2.25 CaCl₂, 5 MgCl₂, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

2.2.2. Recording and Characterization of Muscle and Vascular Nociceptors

Whole cell patch clamp recordings were made with an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 60-70% with Clampex compensation circuitry. Whole cell resistance and capacitance were determined by the Clampex 9.0 software utility. Recorded currents were sampled at 10-20 kHz and filtered at 2 kHz (Bessel filter).

Once the whole cell mode was achieved, neurons were classified as type 5 (muscle), type 8 (vascular) nociceptors or type 19 (vascular) using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2000; 2002; Cooper et al., 2014; see also Xu et al., 2010; Ono et al., 2010). Anatomical targets of type 5, type 8 and type 19 neurons were determined by a series of anatomic tracing experiments (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). Cells not fitting the classification criteria were discarded.

2.2.3. Current Clamp Experiments

Type 5 muscle, type 8 and type 19 vascular neurons were identified in voltage clamp. Studies were then conducted in current clamp mode (see also Nutter et al., 2015). The recorded cell was exposed to 10 μ M oxotremorine-M for 30 seconds (OXO-M; Sigma-Aldrich). In some tests, linopirdine (10 μ M in ethanol; Sigma Aldrich) or HC-030031 (10 μ M in DMSO; Sigma Aldrich), were presented for 2-3 minutes prior to the application of OXO-M. Linopirdine is a specific antagonist of K_v7 channels. It was applied when it was necessary to remove resting and evoked K_v7 channel activity (aka M-current; Brown and Passmore, 2009) from a given recording. OXO-M is a broad activator of mAChRs that is used to inhibit resting and evoked K_v7 activity that follows muscarinic receptor activation in many neurons. K_v7 inhibition can produce a depolarization due to the presence of a resting or voltage activated outward K^+ current (Brown and Passmore, 2009). HC-030031 is a specific antagonist of TRPA1 (McNamara et al., 2007). It was used to determine whether TRPA1 contributed to a depolarization evoked by OXO-M. When an antagonist (linopirdine or HC-030031) preceded OXO-M presentation, the antagonist (or its vehicle) was included in the OXO-M application.

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

2.2.4. Isolation and Characterization of K_v Currents

K^+ currents (K_{DR} , K_v7) were characterized in a 'Kiso' (potassium current isolation) solution containing (in mM): 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 10 glucose, 10 HEPES, adjusted to pH 7.4 with HCL. On the day of the experiment, 1mM $CsCl_2$ and 2 mM 4-amino pyridine were added to block H -currents (I_H) and A -currents (I_a), respectively. The pipette solution contained (in mM): 120 KCl, 5 Na_2 -ATP, 0.4 Na_2 -GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

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

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

toxins and for vehicle test contrasts against toxin testing. Toxins were applied continuously by close superfusion (~1 mm). Following toxin presentation, the Kiso solution (BSA and linopirdine added) was applied for 3 minutes as a washout test. There was little indication that the effect of a toxin could be washed out in 3 minutes.

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

2.2.5. Muscarinic Influences on K_{DR}

K_{DR} currents, expressed by type 5 and type 8 nociceptors harvested from GW chemical or vehicle treated rats, were exposed to a muscarinic agonist (OXO-M). The K^+ isolation (Kiso) solution was used to isolate K_{DR} current as described above. Ten micromolar (10 μM)

linopirdine was added to block K_v7 components of the current. Following a 3 minute application of the Kiso solution, a series of strong depolarization were used to evoke the K_{DR} . From a holding potential of -60 mV, a 2,000 msec conditioning pulse (-100 mV) was followed by a 500 msec step to +20 mV. The evoked current was leak corrected using the P/N procedure utility of pClamp 9.0. A pre-EXO-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 μ M oxotremorine-M (EXO-M; Sigma Aldrich) was applied for 4 minutes by close superfusion.

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

Statistics. The influence of chemical exposure on dependent measures of muscle pain threshold (grams), movement distance (cm/15 min), movement rate (cm/sec) and rest time (sec/15 min) were assessed by ANOVA for repeated measures. Follow-up tests were not conducted on significant main effects or interactions. Separate analyses of pain behaviors were performed on periods in which rats were sacrificed for molecular studies (5-8 weeks and 9-12 weeks post chemical exposure). We did not perform analyses on other time periods either during or after chemical exposures. Student's t-test was used to compare amplitude measures of K_{DR} and K_v7

activity in GW exposed and oxotremorine-M exposed nociceptors with vehicle treated cell groups. The alpha level was set at .05. Multiple tests within the same experiment were corrected for Type 1 error using the Bonferroni procedure.

3. Results

3.1. Behavioral Studies

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

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

3.2. Molecular Studies

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

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

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

After exposure to StTX, a substantial portion of the K_{DR} current remained. It was apparent that the $K_{v2.1}$ and $K_{v2.2}$ currents comprised about 25 and 35% of the 4AP and linopirdine insensitive K_{DR} currents present in muscle and vascular nociceptors, respectively (figure 3). As the precise composition of the residual current is unclear, we will refer to this as the StTX insensitive current. In contrast to the StTX sensitive current, the 8 week exposure to GW chemicals significantly decreased the StTX insensitive K_{DR} current in muscle nociceptors (figure 3A). Therefore, those specific muscle nociceptor K_{DR} component currents that were down regulated by exposure to GW chemicals were not $K_{v2.1}$ or $K_{v2.2}$, nor were they likely to be $K_{v1.1}$, $K_{v1.2}$ or $K_{v1.3}$.

Figure 2. Stromatoxin-Sensitive Currents ($K_{v2.1}$, $K_{v2.2}$) in GW Exposed Nociceptors. The total $K_{v2.1}$ and $K_{v2.2}$ currents were not modified by chronic exposure to GW chemicals. (A) Muscle nociceptors (B) Vascular nociceptors. (C) A representative case showing the time dependent change in peak K_{DR} current after exposure to StTX. Attempts to washout StTX and restore the K_{DR} currents to baseline levels generally failed. Cells were harvested 12 weeks after exposures had ceased. Experiments were conducted on cells collected from 13 rats.

Figure 3. Stromatoxin-Insensitive Currents in GW Exposed Nociceptors. StTX sensitive currents ($K_{v2.1}$ and $K_{v2.2}$) normally comprised about 25 to 30% of the total K_{DR} current in muscle and vascular nociceptors, respectively (vehicle tested rats). The StTX insensitive current, the proportion of K_{DR} currents, in muscle nociceptors that remained after pre-treatment with linopirdine and StTX, were modified in muscle nociceptors by chronic exposure to GW chemicals. (A) Muscle nociceptors (B) Vascular nociceptors. Cells were harvested 12 weeks after exposures had ceased. Experiments were conducted on cells collected from 13 rats.

3.2.2. Modification of mAChR Reactivity after Exposure to GW Chemicals

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

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

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

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

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

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Figure 4. GW Chemicals Decreased the Muscarinic Sensitivity of K_{DR} Currents. **A)** A broadly active muscarinic agonist (OXO-M) increased K_{DR} currents of muscle nociceptors harvested from both vehicle exposed and GW chemical-exposed muscle nociceptors. Muscle nociceptors from exposed rats were significantly less reactive to the muscarinic agonist. **B)** Vascular nociceptor K_{DR} reactivity to OXO-M was not influenced by GW chemicals. **C)** Block of the $K_{v2.1}$ and $K_{v2.2}$ current by StTX did not prevent the increase in K_{DR} current by OXO-M in muscle nociceptors. **D)** A representative case showing the time dependent change in peak K_{DR} current after exposure to OXO-M. Values in (A) and (B) represent amplitudes measured 1 minute after the superfusion of OXO-M. Experiments were conducted on cells collected from 13 rats.

K_{v7} is a member of the broader K_{DR} family. We have shown previously that the activity of K_{v7} currents was modified by an 8 week exposure to GW toxicants (Nutter et al., 2013; Nutter et al., 2015). One level of modification could occur via activation of muscarinic receptors. Cholinergic modulation, via the muscarinic receptor activated pathway, is one of the ways by which inhibition of K_{v7} potentiates neuronal activity (Brown and Passmore, 2009). We examined how exposure to a broadly active muscarinic agonist, OXO-M, altered the resting

currents in both muscle and vascular nociceptors harvested from rats exposed to GW agents (OXO-M, 10 μ M; 30 sec).

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

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

3.2.3. Molecular Basis of the MDBD

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

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

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

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

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

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

498 MDBD could be the result of inhibition of K_v7 currents that are active at the resting membrane
499 potential. Activation of mAChR is the classic pathway to K_v7 channel inhibition (Linely et al.,
500 2008; Brown and Passmore, 2009). To test this hypothesis, we applied, by close superfusion, the
501 specific K_v7 inhibitor, linopirdine ($10 \mu\text{M}$), to muscle and vascular nociceptors for 3 minutes
502 prior to the presentation of OXO-M ($10 \mu\text{M}$). Despite pretreatment with linopirdine, muscle and
503 vascular nociceptors were strongly depolarized by OXO-M (figure 6A, 6B). Seven of 14 cases
504 also emitted AP bursts (73.0 ± 17.9 APs, $n=6$ of 8; 115 APs, $n=1$ of 6, muscle and vascular
505 nociceptors, respectively). Although depolarization amplitude was essentially unchanged in
506 those cases pretreated with linopirdine, the duration of the AP burst discharges was significantly
507 increased ($p<.04$; 6.73 ± 1.98 vs 17.83 ± 4.01 sec, OXO-M, $n=6$, and linopirdine treated, $n=7$,
508 respectively; not shown). Therefore, pre-treatment with the K_v7 blocker, linopirdine, did not
509 block the mAChR dependent depolarizations, but did modify the AP bursts that often
510 accompanied depolarization.

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

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

520 TRPA1 is a Ca^{++} activated non-selective cation channel that is expressed in about half of the
521 capsaicin sensitive nociceptor population (Julius, 2013). We examined whether TRPA1 was an
522 I_{can} mediating MDBD in capsaicin sensitive muscle and vascular nociceptors (Petruska et al.,
523 2002; Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). Using the methods described
524 above, nociceptors were pretreated with linopirdine and the specific TRPA1 antagonist HC-
525 030031 (10 μM) for 3 minutes prior to presentation of OXO-M.

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

532
533

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

4. Discussion

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

4.1. Behavioral Studies

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

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

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

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

4.2. Molecular Studies

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

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

Exposure to chlorpyrifos or PB alters the expression of muscarinic receptors in the CNS (Nostrandt et al., 1997; Liu et al., 1999; Huff et al., 2001; Zhang et al., 2002; Abou-Donia et al., 2004; Padilla et al., 2005; Pung et al., 2006; see also Abou-Donia et al., 2003; Abdel Rahman et al., 2004a; Abdel Rahman et al., 2004b; Zou et al., 2006; Proskocil et al., 2010). We have now demonstrated that chronic exposure to GW agents disturbed the normal relationship between ACh muscarinic receptors and certain K_v proteins in the PNS. Twelve weeks after exposure to GW toxicants, the muscarinic up-regulation of an inhibitory StTX insensitive K_{DR} in muscle nociceptors was diminished; simultaneously the muscarinic down regulation of inhibitory K_v7 channel proteins in muscle nociceptors was enhanced. This is a combination that is highly suggestive of increased nociceptor excitability that could contribute to a chronic myalgia.

4.2.1. Role of MDBD in GW Pain

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

645 Following mAChR activation, ADP has been shown to depend upon rapid development of a
646 persistent, TTX sensitive Na^+ current (Azouz et al., 1996; Yue et al., 2005; Chiang, et al., 2010;
647 Yamada-Hanff and Bean, 2013; but see Hofman and Frazier, 2010) and/or activation of a non-
648 selective cation current (I_{can} ; Haj-Dahmane and Andrade, 1998; Hofman and Frazier, 2010;
649 Zhang et al., 2011; Dasari et al., 2013). ADP is modulated by K_v7 activity (Yue and Yaari,
650 2004, 2006; Brown and Randall, 2009); and may require activation of LVA Ca^{++} currents, the
651 phenotype of which may differ in distinct functional cell classes (Dreyfus et al., 2010; Geier et
652 al., 2011; Park and Spruston, 2012).

653 In our studies, we found little evidence that mAChR induced depolarization in DRG muscle and
654 vascular nociceptors was dependent on the inhibition of K_v7 . Nevertheless, K_v7 played a
655 significant role in MDBD by modulating the duration of AP discharges when such activity
656 occurred. ADP has been shown to depend upon a flufenamic acid sensitive I_{can} , the identity of
657 which remains unknown (Hoffman and Frazier, 2010; Tsuruyama et al., 2013; Alvares et al.,
658 2014). We were able to demonstrate that inhibition of TRPA1, an I_{can} widely expressed in DRG
659 nociceptors (Julius, 2013), reduced, by half, the mAChR evoked depolarization. The remaining
660 current could represent activity in a distinct channel and/or residual activity in TRPA1.

661 It remains unclear whether these powerful mAChR dependent burst discharges in PNS
662 nociceptors are mechanistically related to CNS ADP, or whether TRPA1 contributes to ADP in
663 the CNS. Nor is it clear that ADP is a mechanistically uniform phenomenon throughout the
664 nervous system. The prevalence of ADP in hippocampus, at times including muscarinic bursting
665 discharges similar to those that we observed in DRG (Klink and Alonso, 1997; Egorov et al.,
666 2002; Zhang et al., 2011), could have broad implications for the pathophysiology of GWI. In the
667 CNS, ADP is believed to contribute to learning and memory formation (Rahman and Berger,

2011). We have shown that MDBD is potentiated by chronic exposure to GW chemicals (Nutter et al., 2015). Those affected DRG ion channels and receptors are also expressed in hippocampus (Na_v1.9, K_v7, mAChR; see Nutter and Cooper, 2014; Nutter et al., 2015). If such GWI chemical maladaptations were also manifested in hippocampus, it could be a compelling functional link to cognitive deficits associated with this disorder (Haley et al., 1997).

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

691

692 **5. Conclusion**

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

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711 **Acknowledgements**

712 Studies were funded by DoD W81XWH-13-1-0355/CDMRP/GWIRP, GW120039 to BC and
713 RDJ and by the University of Florida Research Foundation (BC). We would like to thank Linda
714 Flunker for her contributions to the behavioral studies.

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