**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 $\text{Na}_v1.9$ 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 were 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

49 K_v7 channel proteins was consistent with increased nociceptor excitability and a delayed
50 myalgia in rats exposed to GW chemicals.

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69 **Introduction**

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71 Many soldiers returning from the Persian Gulf War suffered from unusual complexes of
72 headache, joint, muscle and abdominal pains associated with Gulf War illness (GWI;
73 Haley Syndrome 3; Haley et al., 1997; Blanchard et al., 2006; Stimpson et al., 2006;
74 Thomas et al., 2006; Haley et al., 2013; see also Gopinath et al., 2012). While it is well
75 recognized that veterans returning from service overseas often report lingering
76 musculoskeletal pain, the relative likelihood of joint, muscle, and/or abdominal pain
77 were 3 fold greater in GW veterans than in those that were deployed elsewhere (Thomas
78 et al., 2006; Kelsall et al., 2004; Stimpson et al 2006; see also Kang et al., 2000). The
79 appearance of these symptoms was typically delayed, but up to 25% of those stationed
80 in the Persian Gulf exhibited GWI related pain prior to their return to the States
81 (Kroenke et al., 1998). In the years that followed, the symptoms of GWI tended to
82 remain the same or worsen over time (Hotopf et al., 2003).

83 The Research Advisory Committee on Gulf War Illness (GWI) concluded that pesticides
84 could have contributed to the development of the symptoms of GWI (Binns et al., 2008;
85 RAC2014). During the brief course of the Gulf War, soldiers were potentially exposed
86 to 67 insecticides and repellants containing 37 distinct active ingredients (DoD
87 Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008). These included a
88 variety of organophosphate, organochlorine, dialkylamide, carbamate and pyrethroid
89 pesticides and repellants. Our laboratory has identified molecular adaptations in pain
90 system neurons that result from exposure to GWI suspected pesticide neurotoxicants
91 (permethrin, chlorpyrifos) and the nerve gas prophylactic, pyridostigmine bromide (PB;
92 Jiang et al., 2013; Nutter et al., 2013; Nutter and Cooper, 2014). These chemicals have

93 direct interactions with important proteins expressed in the peripheral pain (nociceptor)
94 system. Permethrin acutely modifies the physiology of central and peripheral nervous
95 system Na^+ channels that are essential for pain coding ($\text{Na}_v1.6$, $\text{Na}_v1.7$, $\text{Na}_v1.8$, $\text{Na}_v1.9$;
96 Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006; Nutter et al., 2013;
97 Nutter and Cooper, 2014). Chronic exposure to anticholinesterases chlorpyrifos and PB
98 upregulate expression of muscarinic acetylcholine receptor proteins that couple to
99 important voltage sensitive K^+ channels that control neuronal excitability (K_v7 ; Abou-
100 Donia et al., 2003; Abou-Donia et al., 2004). Following an 8 week exposure to
101 permethrin, chlorpyrifos and PB (rats), we were unable to demonstrate a pattern of
102 behavior consistent with a chronic pain condition. Nevertheless, we observed
103 upregulation of ion channel proteins $\text{Na}_v1.9$ and K_v7 (Nutter et al., 2013; Nutter and
104 Cooper, 2014). These molecular adaptations persisted 8 weeks after neurotoxicant
105 exposure had ceased.

106 The K_v7 family of ion channel proteins belong to the large family of K_v (voltage
107 sensitive K^+) proteins that contribute to neural excitability and conduction in multiple
108 ways (Du and Gamper, 2013; Tsantoulous and McMahon, 2014). The adaptations
109 exhibited by K_v7 channels, following GW chemical exposure, could represent a general
110 adaptation affecting K_v channels or a more specific adaptation of the K_v7 family through
111 their linkages to muscarinic receptors whose expression may have been perturbed by
112 anticholinesterases (Abou-Donia et al., 2003; Abou-Donia et al., 2004; Passmore et al.,
113 2012). Because a variety of K_v channels have been implicated in the development of
114 chronic pain, determining the scope of this defect could aid in the interpretations of
115 determinant factors contributing to the development and treatment of GWI pain

syndromes (Du and Gamper, 2013; Tsantoulous 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.

Methods

Behavioral Studies

Subjects. Young adult male rats weighing 229.4 +/- 2.6 grams (~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.

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. Chlorpyrifos 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; $p < .35$).

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.

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 (2 mg/ml) and Dispase II (5 mg/ml; Roche Chemical). A 15 ml centrifuge tube containing the dissected ganglia was placed in a heated, shaking water bath for 90 minutes at 35° C (EDVOTEK Digital Shaking Water Bath). Gentle trituration was then used to break up visible strips of ganglia. The dispersed neurons were then digested for an additional 45 minutes, and then spun at 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 were conducted at room temperature (21 ° C) within 10 hours of plating. Only one cell was used per Petri dish. Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). 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.

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., 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 °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 μ M 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. 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 MgCl₂, 0.2 CaCl₂, 1 CsCl₂, 2 4-amino pyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with HCl. The pipette solution contained (in mM): 120 KCl, 5 Na₂-ATP, 0.4 Na₂-GTP, 5 EGTA, 2.25 CaCl₂, 5 MgCl₂, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

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 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 from -80 to 20 mV (10 mV increments; 500 msec duration). The amplitude of the tail current at -60 mV was measured from the peak relative to the baseline current recorded 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_{.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 (τ_{deact}) was determined by fit of a function of the form: $A_1 \exp(-(t-k)/\tau_1) A_2 \exp(-(t-k)/\tau_2) \dots + C$, 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 (τ_{slow}) and a fast time constant (τ_{fast}).

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 ($10 \mu M$ 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.

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 (10) 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 minutes 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 minute application of a K-ISO solution containing 1 mM CsCl, 2 mM 4-AP and 10 uM 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 msec duration). To remove inactivation, each command step was preceded by 2000 msec step to -100 mV. Permethrin 10 uM (or .001% ethanol) in the K-ISO solution was applied for 2 minutes 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.

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 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.

Results

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 (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). None of these animals were sacrificed for physiological studies.

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 ($F= 7.23$; $p<.009$). 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 (8 weeks post) were animals that were sacrificed for physiology experiments 8 weeks post-exposure (figure 2). Group 12WP (12 weeks post) 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). 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 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.

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365 **Figure 2. The Intensified Exposure Protocol Produced Some Signs of Lasting Pain Behaviors in the 8**
 366 **Week Post Exposure Group.** **A and B)** Muscle pain pressure thresholds and distance moved were
 367 unchanged following the 60 day exposure to GW chemicals. **C and D)** Movement rate and resting were
 368 both increased during the post exposure period. Resting was significantly increased 5-8 weeks post-
 369 exposure ($F=7.43$; $p<.008$). Movement rate was significantly increased at the same time delay ($F= 9.23$;
 370 $p<.003$). Tests were not conducted on any measure 1-4 weeks post exposure.

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

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387 **Molecular Studies on Chronically Exposed Rats**

388 Eight and 12 weeks after chemical exposures ended, muscle and vascular nociceptors
389 (type 5 and type 8, respectively) were identified in DRG harvested from rats exposed for
390 60 days to GW chemicals or their vehicles (Petruska et al., 2002; Rau et al., 2007; Cooper
391 et al., 2014). Using a tail current analysis, we examined the voltage dependence and
392 amplitude of the K_{DR} and the conductance of the K_v7 current component (isolated as the
393 linopirdine sensitive deactivation tail current). These currents were isolated from other
394 voltage dependent currents using conventional procedures (see Methods; also see Nutter
395 et al., 2013). We observed persistent changes in both K_v7 and the K_{DR} currents following
396 the intensified exposure protocol. Some of these changes predicted increased neural
397 excitability and pain.

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

407 At 12 weeks post-exposure, K_v7 currents were now significantly decreased in muscle
408 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 -60 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 μ M), 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 figure 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; figure 5A and B) and vascular nociceptors (12 weeks; figure 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 (figure 6A and C) paralleled changes in voltage dependence (figure 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 (figure 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.

449 **Figure 5. The Voltage Dependence of Delayed Rectifier Channels (K_{DR}) 8 and 12 Weeks Following**
450 **Exposure to Permethrin, Chlorpyrifos and PB.** **A and B)** In muscle nociceptors, the computed
451 activation voltage constants (V₅₀) were hyperpolarized (leftward shift) at both the 8 and 12 weeks post
452 exposure periods. **C and D)** Vascular nociceptors were shifted only at the 12 week delay. Voltage
453 constants (V₅₀) were derived from Boltzmann fits to individual cells while the curves above reflect the
454 average of all cells. Curves were formed from tail currents evoked by the voltage protocol illustrated in
455 'D'. From a V_H of -60 mV, cells were hyperpolarized to -100 mV for 2 sec then stepped for 500 msec from
456 -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 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 sec then stepped for 500 msec from -80 to 20 mV in 10 mV steps.

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 (τ_{deact}), exponential fits were made to the K_{DR} deactivation tails (at -30 mV; see Method). 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 τ_{deact} of exposed and vehicle treated rats revealed significant influences of GW chemicals on type 5 muscle nociceptor K_{DR} channel

deactivation (figure 7). Trends toward slowed deactivation occurred in both nociceptor groups at both delays. Significant changes in the slow $\text{Tau}_{\text{deact}}$ were observed at both the 8 and 12 week delays in muscle nociceptors (figure 7C and D). These shifts paralleled significantly increased tail current amplitude at 8 weeks (figure 6C), but diverged from those at 12 weeks (figure 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 (figure 7D) could not account for a significant decrease in the K_{DR} at this time delay (figure 6D). A significant slowing of the fast component of deactivation was only observed at the 12 week delay (figure 7B).

Figure 7. Deactivation Time Constants (Tau) were Increased by Exposure to GW Chemicals. A and B) Eight (8) 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 and D)** Significant changes in the slow decay constants were present at 8 and 12 weeks. All significant increases were in muscle nociceptors

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 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 two minutes for signs of spontaneous activity (20 °C). Subsequently, the RMP was held, by current injection, at -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 a series of depolarizing current injections (0.1 nA/step; 10 steps; 250 msec). 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 Method).

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 (figure 8A 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 nonspecific muscarinic agonist Oxotremorine-M (10 μ M; 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 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).

Figure 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.

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

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Figure 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 μ M 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 τ_{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 μ M) 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 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 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 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. 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 repellent application, and 5) unauthorized insecticide and repellent 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.

Molecular Studies on K_v7. In prior reports, we found that 30 to 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 were 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/arthritis. 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 ($\text{Ca}_v1.3$; 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 $\text{Na}_v1.8$. 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 $\text{Na}_v1.8$ inactivation kinetics that persisted for 8 weeks and coincided with the upregulation of K_v7 (Nutter et al., 2014). The slowing of $\text{Na}_v1.8$ 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. Due to its unique capacity to actively oppose membrane depolarization, inhibition of K_v7 leads to increased excitability and spontaneous activity in neurons (Marion 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.

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*.

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.

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784 **References**

785 Aquilonius, S. M., S. A. Eckernas, P. Hartvig, B. Lindstrom and P. O. Osterman (1980).
786 "Pharmacokinetics and oral bioavailability of pyridostigmine in man." Eur J Clin
787 Pharmacol **18**(5): 423-428.

788

789 Abou-Donia, M. B., A. Abdel-Rahman, L. B. Goldstein, A. M. Dechkovskaia, D. U.
790 Shah, S. L. Bullman and W. A. Khan (2003). "Sensorimotor deficits and
791 increased brain nicotinic acetylcholine receptors following exposure to
792 chlorpyrifos and/or nicotine in rats." Arch Toxicol **77**(8): 452-458.

793 Abou-Donia, M. B., A. M. Dechkovskaia, L. B. Goldstein, A. Abdel-Rahman, S. L.
794 Bullman and W. A. Khan (2004). "Co-exposure to pyridostigmine bromide,
795 DEET, and/or permethrin causes sensorimotor deficit and alterations in brain
796 acetylcholinesterase activity." Pharmacol Biochem Behav **77**(2): 253-262.

797 Binns JH, Barlow C, Bloom FE, et al (2008) Research Advisory Committee on Gulf War
798 Veterans' Illnesses. Gulf War Illness and the Health of Gulf War Veterans.
799 Washington, DC: Department of Veterans Affairs.
800

801 Birtley, R. D., J. B. Roberts, B. H. Thomas and A. Wilson (1966). "Excretion and
802 metabolism of [¹⁴C]-pyridostigmine in the rat." Br J Pharmacol Chemother **26**(2):
803 393-402.
804

805 Blanchard, M. S., S. A. Eisen, R. Alpern, J. Karlinsky, R. Toomey, D. J. Reda, F. M.
806 Murphy, L. W. Jackson and H. K. Kang (2006). "Chronic multisymptom illness
807 complex in Gulf War I veterans 10 years later." Am J Epidemiol **163**(1): 66-75.

808 Bradberry, S. M., S. A. Cage, A. T. Proudfoot and J. A. Vale (2005). "Poisoning due to
809 pyrethroids." Toxicol Rev **24**(2): 93-106.

810 Breyer-Pfaff, U., U. Maier, A. M. Brinkmann and F. Schumm (1985). "Pyridostigmine
811 kinetics in healthy subjects and patients with myasthenia gravis." Clin Pharmacol
812 Ther **37**(5): 495-501.
813

814 Brown, D. A. and G. M. Passmore (2009). "Neural KCNQ (Kv7) channels." Br J
815 Pharmacol **156**(8): 1185-1195.

816 Cai, Y. Q., S. R. Chen and H. L. Pan (2013). "Upregulation of nuclear factor of activated
817 T-cells by nerve injury contributes to development of neuropathic pain." J
818 Pharmacol Exp Ther **345**(1): 161-168.

819 Cao, X. H., H. S. Byun, S. R. Chen, Y. Q. Cai and H. L. Pan (2010). "Reduction in
820 voltage-gated K⁺ channel activity in primary sensory neurons in painful diabetic
821 neuropathy: role of brain-derived neurotrophic factor." J Neurochem **114**(5):
822 1460-1475.

823 Cao, Z., T. J. Shafer and T. F. Murray (2011). "Mechanisms of pyrethroid insecticide-
824 induced stimulation of calcium influx in neocortical neurons." J Pharmacol Exp
825 Ther **336**(1): 197-205.

826 Cardenas, C. G., L. P. Del Mar and R. S. Scroggs (1995). "Variation in serotonergic
827 inhibition of calcium channel currents in four types of rat sensory neurons
828 differentiated by membrane properties." J Neurophysiol **74**(5): 1870-1879.

- 829 Chien, L. Y., J. K. Cheng, D. Chu, C. F. Cheng and M. L. Tsaor (2007). "Reduced
830 expression of A-type potassium channels in primary sensory neurons induces
831 mechanical hypersensitivity." J Neurosci **27**(37): 9855-9865.
- 832 Cook, D. B., A. J. Stegner and L. D. Ellingson (2010). "Exercise alters pain sensitivity in
833 Gulf War veterans with chronic musculoskeletal pain." J Pain **11**(8): 764-772.
- 834 Cooper, B.Y., Nutter, T.J., Dugan, V.P., Johnson, R.D (2014). Classification and
835 characterization of vascular afferents in the rat. Society for Neuroscience
- 836 Du, X. and N. Gamper (2013). "Potassium channels in peripheral pain pathways:
837 expression, function and therapeutic potential." Curr Neuropharmacol **11**(6): 621-
838 640.
- 839 Fan, N., P. Sikand, D. F. Donnelly, C. Ma and R. H. Lamotte (2011). "Increased Na⁺ and
840 K⁺ currents in small mouse dorsal root ganglion neurons after ganglion
841 compression." J Neurophysiol **106**(1): 211-218.
842
- 843 Fan, L., X. Guan, W. Wang, J. Y. Zhao, H. Zhang, V. Tiwari, P. N. Hoffman, M. Li and
844 Y. X. Tao (2014). "Impaired neuropathic pain and preserved acute pain in rats
845 overexpressing voltage-gated potassium channel subunit Kv1.2 in primary
846 afferent neurons." Mol Pain **10**: 8.
- 847 Ginsburg, K. S. and T. Narahashi (1993). "Differential sensitivity of tetrodotoxin-
848 sensitive and tetrodotoxin-resistant sodium channels to the insecticide allethrin in
849 rat dorsal root ganglion neurons." Brain Res **627**(2): 239-248.
- 850 Gopinath, K., P. Gandhi, A. Goyal, L. Jiang, Y. Fang, L. Ouyang, S. Ganji, D. Buhner,
851 W. Ringe, J. Spence, M. Biggs, R. Briggs and R. Haley (2012). "fMRI reveals
852 abnormal central processing of sensory and pain stimuli in ill Gulf War veterans."
853 Neurotoxicology **33**(3): 261-271.
- 854 Groth, R. D., L. G. Coicou, P. G. Mermelstein and V. S. Seybold (2007). "Neurotrophin
855 activation of NFAT-dependent transcription contributes to the regulation of pro-
856 nociceptive genes." J Neurochem **102**(4): 1162-1174.
- 857 Groth, R. D. and P. G. Mermelstein (2003). "Brain-derived neurotrophic factor activation
858 of NFAT (nuclear factor of activated T-cells)-dependent transcription: a role for
859 the transcription factor NFATc4 in neurotrophin-mediated gene expression." J
860 Neurosci **23**(22): 8125-8134.
- 861 Haley, R. W., E. Charuvastra, W. E. Shell, D. M. Buhner, W. W. Marshall, M. M. Biggs,
862 S. C. Hopkins, G. I. Wolfe and S. Vernino (2013). "Cholinergic autonomic

dysfunction in veterans with Gulf War illness: confirmation in a population-based sample." JAMA Neurol **70**(2): 191-200.

Haley, R. W. and T. L. Kurt (1997). "Self-reported exposure to neurotoxic chemical combinations in the Gulf War. A cross-sectional epidemiologic study." JAMA **277**(3): 231-237.

Hotopf, M., A. S. David, L. Hull, V. Nikalaou, C. Unwin and S. Wessely (2003). "Gulf war illness--better, worse, or just the same? A cohort study." BMJ **327**(7428): 1370.

Husain, M. A., J. B. Roberts, B. H. Thomas and A. Wilson (1968). "The excretion and metabolism of oral ¹⁴C-pyridostigmine in the rat." Br J Pharmacol **34**(2): 445-450.

Ihara, D., M. Fukuchi, D. Honma, I. Takasaki, M. Ishikawa, A. Tabuchi and M. Tsuda (2012). "Deltamethrin, a type II pyrethroid insecticide, has neurotrophic effects on neurons with continuous activation of the Bdnf promoter." Neuropharmacology **62**(2): 1091-1098.

Imamura, L., H. Hasegawa, K. Kurashina, A. Hamanishi, A. Tabuchi and M. Tsuda (2000). "Repression of activity-dependent c-fos and brain-derived neurotrophic factor mRNA expression by pyrethroid insecticides accompanying a decrease in Ca(2+) influx into neurons." J Pharmacol Exp Ther **295**(3): 1175-1182.

Imamura, L., M. Yasuda, K. Kuramitsu, D. Hara, A. Tabuchi and M. Tsuda (2006). "Deltamethrin, a pyrethroid insecticide, is a potent inducer for the activity-dependent gene expression of brain-derived neurotrophic factor in neurons." J Pharmacol Exp Ther **316**(1): 136-143.

Jiang, N., T. J. Nutter and B. Y. Cooper (2013). "Molecular and cellular influences of permethrin on mammalian nociceptors at physiological temperatures." Neurotoxicology **37**: 207-219.

Kang, H. K., C. M. Mahan, K. Y. Lee, C. A. Magee and F. M. Murphy (2000). "Illnesses among United States veterans of the Gulf War: a population-based survey of 30,000 veterans." J Occup Environ Med **42**(5): 491-501.

Kelsall, H. L., M. R. Sim, A. B. Forbes, D. C. Glass, D. P. McKenzie, J. F. Ikin, M. J. Abramson, L. Blizzard and P. Ittak (2004). "Symptoms and medical conditions in Australian veterans of the 1991 Gulf War: relation to immunisations and other Gulf War exposures." Occup Environ Med **61**(12): 1006-1013.

897 Kim, M. S., L. P. Shutov, A. Gnanasekaran, Z. Lin, J. E. Rysted, J. D. Ulrich and Y. M.
898 Usachev (2014). "Nerve growth factor (NGF) regulates activity of nuclear factor
899 of activated T-cells (NFAT) in neurons via the phosphatidylinositol 3-kinase
900 (PI3K)-Akt-glycogen synthase kinase 3beta (GSK3beta) pathway." J Biol Chem
901 **289**(45): 31349-31360.

902 Kroenke, K., P. Koslowe and M. Roy (1998). "Symptoms in 18,495 Persian Gulf War
903 veterans. Latency of onset and lack of association with self-reported exposures." J
904 Occup Environ Med **40**(6): 520-528.

905 Li, Z., X. Gu, L. Sun, S. Wu, L. Liang, J. Cao, B. M. Lutz, A. Bekker, W. Zhang and Y.
906 X. Tao (2015). "Dorsal root ganglion myeloid zinc finger protein 1 contributes to
907 neuropathic pain after peripheral nerve trauma." Pain **156**(4): 711-721.

908 Lutz, B. M., A. Bekker and Y. X. Tao (2014). "Noncoding RNAs: new players in chronic
909 pain." Anesthesiology **121**(2): 409-417.
910

911 Marrion, N. V. (1997). "Control of M-current." Annu Rev Physiol **59**: 483-504.

912 Matsuya, Y., D. Ihara, M. Fukuchi, D. Honma, K. Itoh, A. Tabuchi, H. Nemoto and M.
913 Tsuda (2012). "Synthesis and biological evaluation of pyrethroid insecticide-
914 derivatives as a chemical inducer for Bdnf mRNA expression in neurons." Bioorg
915 Med Chem **20**(8): 2564-2571.

916 Mucha, M., L. Ooi, J. E. Linley, P. Mordaka, C. Dalle, B. Robertson, N. Gamper and I.
917 C. Wood (2010). "Transcriptional control of KCNQ channel genes and the
918 regulation of neuronal excitability." J Neurosci **30**(40): 13235-13245.

919 Neal, A. P., Y. Yuan and W. D. Atchison (2010). "Allethrin differentially modulates
920 voltage-gated calcium channel subtypes in rat PC12 cells." Toxicol Sci **116**(2):
921 604-613.

922 Nutter, T. J., N. Jiang and B. Y. Cooper (2013). "Persistent Na⁺ and K⁺ channel
923 dysfunctions after chronic exposure to insecticides and pyridostigmine bromide."
924 Neurotoxicology **39**: 72-83.
925

926 Nutter, T. J. and B. Y. Cooper (2014). "Persistent modification of Nav1.9 following
927 chronic exposure to insecticides and pyridostigmine bromide." Toxicol Appl
928 Pharmacol **277**(3): 298-309.

929 Ono, K., S. Xu and K. Inenaga (2010). "Isolectin B(4)binding in populations of rat
930 trigeminal ganglion cells." Neurosci Lett **486**(3): 127-131.

931 Passmore, G. M., J. M. Reilly, M. Thakur, V. N. Keasberry, S. J. Marsh, A. H. Dickenson
 932 and D. A. Brown (2012). "Functional significance of M-type potassium channels
 933 in nociceptive cutaneous sensory endings." Front Mol Neurosci **5**: 63.

934 Petruska, J. C., J. Napaporn, R. D. Johnson and B. Y. Cooper (2002). "Chemical
 935 responsiveness and histochemical phenotype of electrophysiologically classified
 936 cells of the adult rat dorsal root ganglion." Neuroscience **115**(1): 15-30.

937 Petruska, J. C., J. Napaporn, R. D. Johnson, J. G. Gu and B. Y. Cooper (2000).
 938 "Subclassified acutely dissociated cells of rat DRG: histochemistry and patterns
 939 of capsaicin-, proton-, and ATP-activated currents." J Neurophysiol **84**(5): 2365-
 940 2379.

941 Research Advisory Committee on Gulf War Veterans' Illnesses *Gulf War Illness and the*
 942 *Health of Gulf War Veterans: Research Update and Recommendations, 2009-*
 943 *2013* Boston, MA: U.S. Government Printing Office, April 2014
 944

945 Rau, K. K., N. Jiang, R. D. Johnson and B. Y. Cooper (2007). "Heat sensitization in skin
 946 and muscle nociceptors expressing distinct combinations of TRPV1 and TRPV2
 947 protein." J Neurophysiol **97**(4): 2651-2662.

948 Rau, K. K., R. D. Johnson and B. Y. Cooper (2005). "Nicotinic AChR in subclassified
 949 capsaicin-sensitive and -insensitive nociceptors of the rat DRG." J Neurophysiol
 950 **93**(3): 1358-1371.

951 Rau, K.K., Jiang, N., Johnson, R.D. and Cooper, B.Y. Tissue Specific Distribution of
 952 Nociceptors with nAChR. In: Arias, HR (Ed.) *Pharmacology of Nicotinic*
 953 *Acetylcholine Receptors from the Basic and Therapeutic Perspectives*, Research
 954 *Signpost*, 2012, pp 223-245.

955 Rau, K. K., J. C. Petruska, B. Y. Cooper and R. D. Johnson (2014). "Distinct
 956 subclassification of DRG neurons innervating the distal colon and glans
 957 penis/distal urethra based on the electrophysiological current signature." J
 958 Neurophysiol **112**(6): 1392-1408.
 959

960 Ray, D. E. and J. R. Fry (2006). "A reassessment of the neurotoxicity of pyrethroid
 961 insecticides." Pharmacol Ther **111**(1): 174-193.

962 Ren, Y. S., N. S. Qian, Y. Tang, Y. H. Liao, Y. L. Yang, K. F. Dou and M. Toi (2012).
 963 "Sodium channel Nav1.6 is up-regulated in the dorsal root ganglia in a mouse
 964 model of type 2 diabetes." Brain Res Bull **87**(2-3): 244-249.
 965

- 966 Robbins, J. (2001). "KCNQ potassium channels: physiology, pathophysiology, and
967 pharmacology." Pharmacol Ther **90**(1): 1-19.
- 968 Rose, K., L. Ooi, C. Dalle, B. Robertson, I. C. Wood and N. Gamper (2011).
969 "Transcriptional repression of the M channel subunit Kv7.2 in chronic nerve
970 injury." Pain **152**(4): 742-754.
- 971 Shafer, T. J. and M. F. Hughes (2010). "Accumulation of pyrethroid compounds in
972 primary cultures from rat cortex." Toxicol In Vitro **24**(7): 2053-2057.
- 973 Shafer, T. J. and D. A. Meyer (2004). "Effects of pyrethroids on voltage-sensitive
974 calcium channels: a critical evaluation of strengths, weaknesses, data needs, and
975 relationship to assessment of cumulative neurotoxicity." Toxicol Appl Pharmacol
976 **196**(2): 303-318.
- 977 Smith, H. S. (2009). "Calcineurin as a nociceptor modulator." Pain Physician **12**(4):
978 E309-318.
- 979 Smith, J. N., J. A. Campbell, A. L. Busby-Hjerpe, S. Lee, T. S. Poet, D. B. Barr and C.
980 Timchalk (2009). "Comparative chlorpyrifos pharmacokinetics via multiple
981 routes of exposure and vehicles of administration in the adult rat." Toxicology
982 **261**(1-2): 47-58.
983
- 984 Soderlund, D. M., J. M. Clark, L. P. Sheets, L. S. Mullin, V. J. Piccirillo, D. Sargent, J. T.
985 Stevens and M. L. Weiner (2002). "Mechanisms of pyrethroid neurotoxicity:
986 implications for cumulative risk assessment." Toxicology **171**(1): 3-59.
- 987 Song, J. H., K. Nagata, H. Tatebayashi and T. Narahashi (1996a). "Interactions of
988 tetramethrin, fenvalerate and DDT at the sodium channel in rat dorsal root
989 ganglion neurons." Brain Res **708**(1-2): 29-37.
- 990 Song, J. H. and T. Narahashi (1996b). "Modulation of sodium channels of rat cerebellar
991 Purkinje neurons by the pyrethroid tetramethrin." J Pharmacol Exp Ther **277**(1):
992 445-453.
- 993 Stimpson, N. J., C. Unwin, L. Hull, T. David, S. Wessely and G. Lewis (2006).
994 "Prevalence of reported pain, widespread pain, and pain symmetry in veterans of
995 the Persian Gulf War (1990-1991): the use of pain manikins in Persian Gulf War
996 health research." Mil Med **171**(12): 1181-1186.
- 997 Tabarean, I. V. and T. Narahashi (1998). "Potent modulation of tetrodotoxin-sensitive
998 and tetrodotoxin-resistant sodium channels by the type II pyrethroid
999 deltamethrin." J Pharmacol Exp Ther **284**(3): 958-965.

- 1000 Tabarean, I. V. and T. Narahashi (2001). "Kinetics of modulation of tetrodotoxin-
1001 sensitive and tetrodotoxin-resistant sodium channels by tetramethrin and
1002 deltamethrin." J Pharmacol Exp Ther **299**(3): 988-997.
- 1003 Tatebayashi, H. and T. Narahashi (1994). "Differential mechanism of action of the
1004 pyrethroid tetramethrin on tetrodotoxin-sensitive and tetrodotoxin-resistant
1005 sodium channels." J Pharmacol Exp Ther **270**(2): 595-603.
- 1006 Tsantoulas, C. and S. B. McMahon (2014). "Opening paths to novel analgesics: the role
1007 of potassium channels in chronic pain." Trends Neurosci **37**(3): 146-158.
- 1008 Uchida, H., K. Sasaki, L. Ma and H. Ueda (2010). "Neuron-restrictive silencer factor
1009 causes epigenetic silencing of Kv4.3 gene after peripheral nerve injury."
1010 Neuroscience **166**(1): 1-4.
- 1011 U.S. Department of Defense, Office of the Special Assistant to the Undersecretary of
1012 Defense (Personnel and Readiness) for Gulf War Illnesses Medical Readiness and
1013 Military Deployments. *Environmental Exposure Report: Pesticides Final Report*.
1014 Washington, D.C. April 17, 2003.
- 1015 Vaso, A., H. M. Adahan, A. Gjika, S. Zahaj, T. Zhurda, G. Vyshka and M. Devor (2014).
1016 "Peripheral nervous system origin of phantom limb pain." Pain **155**(7): 1384-
1017 1391.
- 1018
1019 Xie, W., J. A. Strong and J. M. Zhang (2015). "Local knockdown of the NaV1.6 sodium
1020 channel reduces pain behaviors, sensory neuron excitability, and sympathetic
1021 sprouting in rat models of neuropathic pain." Neuroscience **291**: 317-330.
- 1022
1023
1024
- 1025 Xu, S., K. Ono and K. Inenaga (2010). "Electrophysiological and chemical properties in
1026 subclassified acutely dissociated cells of rat trigeminal ganglion by current
1027 signatures." J Neurophysiol **104**(6): 3451-3461.
- 1028 Zhao, X., Z. Tang, H. Zhang, F. E. Atianjoh, J. Y. Zhao, L. Liang, W. Wang, X. Guan, S.
1029 C. Kao, V. Tiwari, Y. J. Gao, P. N. Hoffman, H. Cui, M. Li, X. Dong and Y. X.
1030 Tao (2013). "A long noncoding RNA contributes to neuropathic pain by silencing
1031 Kcna2 in primary afferent neurons." Nat Neurosci **16**(8): 1024-1031.

1032