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# Persistent modification of $Na_v1.9$ following chronic exposure to insecticides and pyridostigmine bromide

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ABSTRACT

Many veterans of the 1991 Gulf War (GW) returned from that conflict with a widespread chronic pain affecting deep tissues. Recently, we have shown that a 60 day exposure to the insecticides permethrin, chlorpyrifos, and pyridostigmine bromide (NTPB) had little influence on nociceptor action potential forming $Na_v1.8$, but increased $K_v7$ mediated inhibitory currents 8 weeks after treatment. Using the same exposure regimen, we used whole cell patch methods to examine whether the influences of NTPB could be observed on $Na_v1.9$ expressed in muscle and vascular nociceptors. During a 60 day exposure to NTPB, rats exhibited lowered muscle pain thresholds and increased rest periods, but these measures subsequently returned to normal levels. Eight and 12 weeks after treatments ceased, DRG neurons were excised from the sensory ganglia. Whole cell patch studies revealed little change in voltage dependent activation and deactivation of $Na_v1.9$, but significant increases in the amplitude of $Na_v1.9$ were observed 8 weeks after exposure. Cellular studies, at the 8 week delay, revealed that NTPB also significantly prolonged action potential duration and afterhyperpolarization (22 °C). Acute application of permethrin (10 μM) also increased the amplitude of $Na_v1.9$ in skin, muscle and vascular nociceptors. In conclusion, chronic exposure to Gulf War agents produced long term changes in the amplitude of $Na_v1.9$ expressed in muscle and vascular nociceptors. The reported increases in $K_v7$ amplitude may have been an adaptive response to increased $Na_v1.9$, and effectively suppressed behavioral pain measures in the post treatment period. Factors that alter the balance between $Na_v1.9$ and $K_v7$ could release spontaneous discharge and produce chronic deep tissue pain.



## Introduction

Widespread joint and muscle pain is a common complaint associated with GWI[1] (Gulf War Illness; Blanchard et al., 2006; Haley and Kurt, 1997; Stimpson et al., 2006; Thomas et al., 2006). Despite the years that have passed, the etiology of this disorder has remained obscure, and there is little evidence of spontaneous recovery (Kang et al., 2009; Ozakinci et al., 2006). The sources of GWI pain symptoms appear to be dissimilar from other chronic pain conditions. Although joint pain is a frequent complaint, there is no evidence of histological changes in the joints, nor is there the presence of inflammatory markers that are associated with classic joint diseases such as osteo- or rheumatoid arthritis (Diaz-Torne et al., 2007; Pessler et al., 2008). Chronic sensory disturbances are reported from a variety of deep tissue sources, but there is no evidence of significant physical nerve impairment (e.g., segmental demyelination; Blanchard et al., 2006; Bourdette et al., 2001; Sharief et al., 2002; but see Rivera-Zayas et al., 2001). Although commonly recognized as a post-deployment syndrome, about 25% of warfighters that ultimately suffered from chronic GWI pain, reported symptoms while still in theatre (Kroenke et al., 1998).

The Research Advisory Committee on Gulf War Illness determined that pesticides may have contributed to the development of the symptoms of GWI (Binns et al., 2008). During the brief course of the Gulf War, veterans were potentially exposed to 67 insecticides and repellants containing 37 distinct active ingredients (DoD Environmental Exposure Report: Pesticides, 2003; see Binns et al., 2008). These included a variety of organophosphate, organochlorine, dialkylamide, carbamate and pyrethroid neurotoxicants; some of which have known relationships to the development of chronic sensory disorders (OPIDN; Organophosphate-Induced Delayed Neuropathy; Abdollahi and Karami-Mohajeri, 2012).

The most significant contributor to GWI pain may have been the type 1 pyrethroid, permethrin. Due to its interaction with critical sensory system proteins ($Na_v1.6$, $Na_v1.7$, $Na_v1.8$), this agent could play a key role in the development of sensory maladaptations. While deployed,

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[1] AP: action potential; APD: action potential duration; AHP: action potential hyperpolarization; BDNF: brain derived neurotrophic factor; DoD: Dept. of Defense; GWI: Gulf War Illness; GNDF: Glia cell line derived neurotrophic factor; NTPB: neurotoxicants and pyridostigmine bromide; PB: pyridostigmine bromide; VGSC: voltage-gated $Na^+$ Channel;

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soldiers were provided permethrin to apply to their uniforms. Reports suggested that the prescribed application procedures may not have been followed closely. As a result, the daily exposure levels could have been high, and the physiological impacts of this contact could have been compounded by simultaneous exposures to other insecticides, repellants, and nerve gas prophylactics that were used over the course of the conflict (Binns et al., 2008; Steele et al., 2011). Some of these agents (i.e., chlorpyrifos and PB) increase dermal absorption and/or retard catabolism of permethrin (Baynes et al., 2002; Choi et al., 2004; Rose et al., 2005).

It is well established that both type 1 and type 2 pyrethroid insecticides alter the physiology of voltage-gated sodium channels (VGSC, or $Na_v$). While there have been few studies on the influence of the type 1 pyrethroid, permethrin, on mammalian channel protein function (Jiang et al., 2013), the influence of structurally similar type 1 and type 2 pyrethroid neurotoxicants has been studied extensively. These studies confirmed that several pyrethroids (allethrin, tetramethrin, deltamethrin) dramatically alter the physiology of $Na_v$ expressed in mammalian DRG (dorsal root ganglion; Ginsburg and Narahashi, 1993; Song and Narahashi, 1996; Song et al., 1996; Tabarean and Narahashi, 1998, 2001; Tatebayashi and Narahashi, 1994). When applied at room temperature, pyrethroids alter activation, steady state inactivation and deactivation across the diverse family of mammalian TTXs (tetrodotoxin sensitive) and TTXr (tetrodotoxin resistant) $Na_v$ (e.g., $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, $Na_v1.8$; Breckenridge et al., 2009; Choi and Soderlund, 2006; Dekker et al., 2005; Meacham et al., 2008; Motomura and Narahashi, 2001; Smith and Soderlund, 1998; Song et al., 1996; Tan and Soderlund, 2009, 2010). We have recently shown that, in skin, muscle and vascular nociceptors, permethrin alters the voltage dependence of activation, deactivation and kinetics of $Na_v1.8$; the main action potential forming $Na_v$ in mammalian nociceptors (Jiang et al., 2013).

Pyrethroids are considered relatively safe for humans, in part, because lethal influences on the physiology of TTXs $Na_v$ are greatly diminished at mammalian body temperatures (tetramethrin; Song and Narahashi, 1996). TTXr $Na_v$ also exhibits decreased sensitivity to acute permethrin at 35 °C; however, significant changes to activation, deactivation and current decay are retained (Jiang et al., 2013). Given that warfighters were chronically exposed to permethrin and other neurotoxicants over a period of months, a persistent perturbation to a key pain system $Na_v$ may have induced direct and indirect maladaptive changes in the physiological makeup of nociceptor proteins. Consistent with this notion, chronic exposure to permethrin and other neuroactive agents (chlorpyrifos, pyridostigmine bromide (PB)), produced persistent changes to $Na_v1.8$ decay kinetics lasting at least 8 weeks after the neurotoxicant treatments had ceased. In addition, a key protein controlling spontaneous activity was up-regulated in vascular nociceptors ($K_v7$; Nutter et al., 2013). Although no changes in neuronal excitability or pain behaviors were observed in parallel with molecular adaptations, the specific $K_v7$ inhibitor linopirdine, released significantly more spontaneous discharge in neurotoxicant exposed rats (Nutter et al., 2013). We hypothesized that the increase in $K_v7$ was an adaptive response to suppress an increased tendency for spontaneous discharge. Although changes to $Na_v1.8$ decay kinetics were suggestive, they did not seem sufficient qualitatively or quantitatively, to drive spontaneous discharge.

In the experiments described below, we examined whether a 60 day exposure to the neurotoxicants (permethrin, chlorpyrifos) and pyridostigmine bromide (NTPB) could induce long term maladaptations to another important nociceptor $Na_v$ ($Na_v1.9$; Liu and Wood, 2011; Rogers et al., 2006; Rush et al., 2007), and whether these changes were accompanied by shifts in nociceptor excitability and action potential features that would persist 8 to 12 weeks after NTPB exposure. Studies were conducted on rat muscle and vascular nociceptors. Behavioral tests of muscle pain threshold and activity accompanied molecular and functional studies.

## Methods

### *Preparation of cells*

Male Sprague–Dawley rats (n = 73; Harlan) were anesthetized (Isoflurane) and euthanized by decapitation. The spinal column was dissected free and split longitudinally. The dorsal root ganglia were excised, trimmed, cut into several sections and digested at 35 °C in a Tyrode's solution containing 2 mg/ml type 1 collagenase and 5 mg/ml Dispase II (90 min; Sigma Aldrich; Roche Chemicals). Digested ganglia were dispersed by trituration and digested for an additional 45 min. Isolated neurons were spun down (1000 rpm), re-suspended and plated on 10, 35 mm Petri dishes. Plated neurons were bathed continuously in Tyrode's solution, containing (in mM): 140 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters. Rats were fed ad lib and maintained on a 12 hour light/dark cycle. 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).

### *Recording and characterization of muscle and vascular nociceptors*

Conventional whole cell patch techniques were used. Recordings were made with an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and a Digidata 1322A (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 from a capacitive trace. Recorded currents were sampled at 10–20 kHz and filtered at 2 kHz (Bessel filter). Electrodes (2–5 MΩ) were formed from borosilicate glass stock that was pulled to a suitable tip size by a Sutter P1000 (Sutter Instruments, Novato, CA). Recordings were completed within 2–10 h after plating. Only one cell was used per Petri dish. Studies were conducted at room temperature unless otherwise indicated.

Cells were classified as muscle or vascular nociceptors according to the pattern of voltage activated currents evoked by three protocols (Cardenas et al., 1995; Petruska et al., 2002; Rau et al., 2007). Type 5 and type 8 neurons were identified as muscle and vascular nociceptors, respectively in prior tracing studies (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2012). Type 8 nociceptors were traced from all tissue sites (skin, muscle, colon, mucosa; Rau et al., 2007, 2012) and are presumed to be vascular in origin. More detailed descriptions of the cell classification methods have been presented elsewhere (Nutter et al., 2013; Petruska et al., 2000).

### *Exposure protocol*

#### *Chronic exposure to NTPB*

Juvenile male rats (90–110 g) were used in all studies. By the end of the NTPB treatment studies, the average weights were 440 +/− 8 (vehicle) and 428 +/− 8 g (non-significant by Student's *t*-test). On arrival, rats were acclimated to the behavioral procedures for 2 weeks before dosing began (baseline testing). Neurotoxicants and PB were administered over a 60 day period. During exposure, permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), in ETOH, was applied every day to a shaved area of the back. Chlorpyrifos (120 mg/kg; Sigma Aldrich) was administered by subcutaneous injection (corn oil) once every 14 days. PB was given by oral gavage (13 mg/kg; water) for 14 consecutive days, beginning on day 1 and again on day 30. Animals were euthanized for electrophysiological experiments 8 or 12 weeks after the dosing period had ended. Groups of rats receiving equal volumes of ETOH, corn oil and water over the identical time period served as controls.