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Persistent Na^+ and K^+ channel dysfunctions after chronic exposure to insecticides and pyridostigmine bromide

T.J. Nutter, N. Jiang, Brian Y. Cooper *

Division of Neuroscience, Dept. of Oral and Maxillofacial Surgery, JHMHC, University of Florida College of Dentistry, Box 100416, Gainesville, FL 32610, United States

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ABSTRACT

Many soldiers that served in the 1991 Gulf War developed widespread chronic pain. Exposure to insecticides and the nerve gas prophylactic pyridostigmine bromide (PB) was identified as risk factors by the Research Advisory Committee on Gulf War Veterans' Illnesses (GWI). We examined whether a 60 day exposure to neurotoxicants/PB (NTPB) produced behavioral, molecular and cellular indices of chronic pain in the rat. Male rats were exposed to chlorpyrifos (120 mg/kg; SC), permethrin (2.6 mg/kg; topical), and PB (13.0 mg/kg; oral) or their respective vehicles (corn oil, ethanol, and water). Permethrin can exert profound influences on voltage activated Na^+ channel proteins; while chlorpyrifos and PB can increase absorption and/or retard metabolism of permethrin as well as inhibit cholinesterases. During and after exposure to these agents, we assessed muscle pressure pain thresholds and activity (distance and rest time). Eight and 12 weeks after treatments ceased, we used whole cell patch electrophysiology to examine the physiology of tissue specific DRG nociceptor channel proteins expressed in muscle and putative vascular nociceptors (voltage dependent, activation, inactivation, and deactivation). Behavioral indices were unchanged after treatment with NTPB. Eight weeks after treatments ended, the peak and average conductance of K_v7 mediated K^+ currents were significantly increased in vascular nociceptors. When a specific K_v7 inhibitor was applied (linopirdine, 10 μM) NTPB treated vascular nociceptors emitted significantly more spontaneous APs than vehicle treated neurons. Changes to K_v7 channel physiology were resolved 12 weeks after treatment. The molecular alterations to K_v7 channel proteins and the specific susceptibility of the vascular nociceptor population could be important for the etiology of GWI pain.

1. Introduction

Although lingering musculoskeletal pain is not uncommon among veterans, there are distinct multi-symptom manifestations of a widespread chronic pain associated with soldiers of the 1991 Gulf War. Despite the relative brevity of both the conflict and the deployment to the Persian Gulf region, there have been many published reports of unusual complexes of headache, joint pain, muscle pain, and abdominal pain in Gulf War veterans (GWV; Escalante and Fischbach, 1998; Fukuda et al., 1998; Proctor et al., 1998; Gray et al., 1999; Kang et al., 2000; Steele, 2000; Gray et al., 2002; Voelker et al., 2002; Ang et al., 2006). When these, and other reports, were subjected to meta-analysis, it was confirmed that those who served in the Persian Gulf theater were more likely to suffer from a variety chronic pain conditions. In particular, the relative likelihood of joint, muscle, and/or abdominal pain was found to be 3 fold greater in GWV than in those warfighters deployed elsewhere (Thomas et al., 2006; see also Kang et al., 2000; Kelsall et al., 2004; Stimpson et al., 2006). These chronic pain conditions and other sensory, motor and cognitive disturbances are collectively recognized as Gulf War Illness (GWI).

A myriad of factors, including depleted uranium, sarin gas, oil fires, vaccination adjuvants, organophosphates, carbamates, stress and many others have been proposed and/or examined as potentially determinant or contributory factors in the development of GWI syndromes (Binns et al., 2008; see also Shoenfeld and Agmon-Levin, 2010). Substantial challenges remain before a specific etiology and effective treatment can be identified for this condition. To date, there has been relatively little examination of the instigating factors underlying the chronic pain component of GWI. The causal factors must be dissimilar to other chronic pain conditions. Although joint pain is a frequent complaint, there is no evidence of histological changes in 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.,

* Corresponding author. Tel.: +1 352 273 6763; fax: +1 352 392 7609.
E-mail addresses: tnutter@dental.ufl.edu (T.J. Nutter), nan.jiang@neurology.ufl.edu (N. Jiang), bcooper@dental.ufl.edu (B.Y. Cooper).

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2007; Pessler et al., 2008). Although sensory disturbances may be present, there is no evidence of significant physical nerve impairment. Changes in conduction velocity, that can be indicative of segmental deymelination, have not been observed in GWV (Bourdette et al., 2001; Sharief et al., 2002; Blanchard et al., 2006; but see Rivera-Zayas et al., 2001).

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). Pesticides and repellants were used liberally in theater. These included a variety of organophosphate, organochlorine, dialkylamide, carbamate and pyrethroid neurotoxicants. Some of these pesticides were prescribed for use at specific concentrations and with specific application methods and procedures. These application procedures were not always carefully followed and warfighters often supplemented prescribed agents with others they acquired on their own (US, DOD Environmental Exposure Report: Pesticides, 2003).

While stationed in the Gulf region, warfighters made ample use of the pyrethroid insecticides permethrin and allethrin (Binns et al., 2008). Burning, stinging and other skin sensations have been reported following either ingestion (Gotoh et al., 1998) or topical contact with permethrin (Kolmodin-Hedman et al., 1982; Tucker and Flannigan, 1983; Flannigan and Tucker, 1985) or other pyrethroid formulations (Knox et al., 1984; Wilks, 2000; see Wolansky and Harrill, 2008). The influence of type I and type II pyrethroids (allethrin, tetramethrin, and deltamethrin) on TTXs and TTXr Na_v voltage sensitive proteins of the DRG (dorsal root ganglion; $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$) suggests a relatively direct pathway to nociceptor activation and pain (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996; Tabarean and Narahashi, 1998, 2001). Our recent investigations have documented a wide range of potent acute interactions of the type 1 pyrethroid, permethrin, with specifically identified skin, muscle and putative vascular nociceptor $Na_v1.8$ protein (Jiang et al., 2013). The findings included the generation of spontaneous activity in muscle, but not skin or vascular nociceptors. It is not clear that the acute influences of permethrin on nociceptor function will be manifested after chronic exposure to this neurotoxicant.

It is possible that synergisms between pyrethroid neurotoxicants and other agents that either promote their activity of otherwise interact with the pain system could result in significant structural or molecular disruptions with potential clinical import. Animal studies have revealed that various combinations of permethrin, chlorpyrifos, the nerve gas prophylactic pyridostigmine bromide and other anti-cholinesterases can produce a variety of motor and cognitive signs (Abou-Donia et al., 2001, 2004; Abdel-Rahman et al., 2004a), suppress enzyme activity (Abdel-Rahman et al., 2002, 2004b) and degrade the blood brain barrier (Abdel-Rahman et al., 2002, 2004b) at levels greater than can be produced by individual agents (but see Jortner, 2006; Wille et al., 2011); but the involvement of any pain system components are unreported. Permethrin, in particular, has close linkages to the pain system. The capacity of pyrethroids to directly interact with pain system ion channel proteins that are critical to excitability provides a natural focus for investigations of the deleterious effects of chronic neurotoxicant exposure.

In the studies described below, we examined how a 60 day exposure to permethrin, chlorpyrifos, and pyridostigmine bromide affected behavioral, cellular and molecular indices of pain in rodents. Molecular and cellular studies were targeted on discrete populations of muscle and vascular nociceptors and those voltage sensitive ion channel proteins with substantial influence over nociceptor excitability ($Na_v1.8$; $K_v7.3$).

2. Methods

2.1. Preparation of cells

Young male Sprague-Dawley rats ($n = 60$; Harlan) were used in the described experiments. Before and during treatment and delay conditions, rats were allowed ad libitum food and water with a 12 h on/off light cycle. At the end of the treatment and delay intervals rats were anesthetized (Isoflurane) and euthanized by decapitation. The spinal cord 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 titruration 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, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO (U.S. Army Medical Research and Materiel Command).

2.2. Recording and characterization of muscle and vascular nociceptors

Conventional whole cell patch techniques were used. Recordings were made with an Axopatch 200B or Multiclamp700 (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 was 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 (2–4 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 on Na_v and K_v were conducted at room temperature. Some action potential studies were conducted at 35 °C.

Immediately after achieving the whole cell mode, neurons were classified as type 5 or type 8 using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2000, 2002; Rau et al., 2007; Xu et al., 2010; Ono et al., 2010). Cells not fitting these classifications were discarded. Classifications were assigned according to patterns of voltage activated currents (current signatures) that were revealed by three classification protocols (Fig. 1). Due to the considerable functional diversity that is present in the protein subunits that combine to form voltage activated Na^+ and K^+ and other cation channels, current signature patterns can be generated in order to classify neurons into functionally distinct groupings. Classification Protocol 1 (CP1) was used to examine the signature pattern of hyperpolarization activated currents (I_H). Among DRG neurons, H-currents can differ substantially in both amplitude and kinetics. With CP1, currents were evoked by a series of hyperpolarizing pulses presented from a V_H of −60 mV (10 mV per step to a final potential of −110 mV; 500 ms, 4 s interstimulus interval). Classification Protocol 2 (CP2) was used to produce outward current patterns formed by voltage activated K^+ channel proteins (K_v). From a V_H of −60 mV, a 500 ms conditioning pulse to −100 mV was followed by 200 ms depolarizing command steps (20 mV) to a final potential of +40 mV. Most DRG neurons express the fast activating K_v known as 'A-current' (I_A). The kinetics and