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14. ABSTRACT

We examined whether chronic exposure to combinations of two neurotoxicants (chlorpyrifos and permethrin) with pyridostigmine bromide (PB) could produce a delayed neuropathic pain condition in rats; and whether corresponding molecular changes would occur in nociceptive neurons coding for pain in skin, muscle or vasculature. Following a 30 day exposure to neurotoxicants/PB, we observed molecular dysfunctions in membrane Kv proteins (Kv7, KDR) that persisted at least 4 weeks after exposure had ended. K+ channel protein malfunctions were limited to neurons we have attributed to the vascular system. Neurons that innervate skin and muscle were not affected. The physical location of vascular nociceptive neurons renders them most exposed to concentrations of circulating neurotoxicants/PB as well as to any blood borne secondary influences (endocrine, immune) these agents might induce. As a result, vascular nociceptors could be the first.

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Introduction

Chronic, widespread joint and muscle pain is a frequent and debilitating component of GWI syndrome (Gulf War Illness; Stimpson et al., 2006; Thomas et al., 2006). The events leading to the development of GWI pain are unknown, but it has been reported that the use of pyridostigmine bromide (PB) and a variety of insecticides/repellants co-varied with the development of this condition (Research Advisory Committee on Gulf War Veterans' Illnesses, 2008; Steele et al., 2011). In the SOW (statement of work), we outlined a series of behavioral and electrophysiological (molecular) studies to determine the dose combinations of pyridostigmine bromide (PB), permethrin and chlorpyrifos that produced a condition of persistent muscle pain in a rodents. These behavioral studies were supported by neurophysiological experiments to determine the molecular basis of the chronic pain and provide a specific direction for the exploration of treatments to ameliorate the condition. The hypotheses were guided, in part, by the known acute effects of permethrin on the structurally and functionally similar insect and mammalian protein, $Na_{v1.8}$ (TTX resistant VGSC; Choi and Soderlund, 2006), and the high degree of association of this protein with mammalian nociceptors. Behavioral studies assessed concurrent and delayed manifestation of muscle pressure pain that followed 30-60 days exposure to combinations of the neurotoxicants with PB. Molecular studies were conducted, 4 weeks after dosing, in specific sub-populations of nociceptors that innervated skin, muscle and vasculature.

Body

Synergisms that might occur between insecticides, repellants and nerve agent prophylactics used during the GW could modify or damage the machinery of protein expression in ways that ultimately result in chronic pain and other symptoms of GWI (Bradberry et al., 2005). The Research Advisory Committee on Gulf War Veterans' Illnesses (Binns et al., 2008) emphasized a number of agents with the potential to produce such synergisms. An animal model that reproduces the pain symptoms of GW veterans would be a valuable asset to the discovery of a molecular defect in the pain system. This discovered molecular defect might be generalized to other neuronal populations in the CNS with wider implications for GWI syndromes. We used a

rat model of muscle pain to provide a means to assess the influence of insecticides and PB on sensory processing.

Chronic Influences of Neurotoxicants and PB on Pain Behavior

Task 1: We will examine the development, time course and persistence of a myalgia in rats exposed to combinations of neurotoxicants pyridostigmine bromide, permethrin and chlorpyrifos.

Two neurotoxicant/PB dosing regimens (NR1 and NR2) were completed during the reporting period. A third NR is currently active (NR3). These specific dose regimens are presented in Table 1. In both NR1 and NR2, treatments continued for 30 days, after which a 4 week delay passed before animals were sacrificed for electrophysiological experiments. Delays were instituted to ensure that any pain that developed from the synergism of neurotoxicants would persist, like GWI pain, after the exposure had ended.

Young adult male rats were treated with permethrin and chlorpyrifos along with a standard military dose of pyridostigmine bromide (assuming 70 kg body weight). Treatments continued for 30 days. Pressure pain measures were taken using a computer regulated, hand held test device by which pressure was applied via an 8 mm diameter transducer to the semitendinosus and biceps femoris muscles (right hind limb; PAM, Ugo Basile). To complement pressure pain testing, activity levels (movement distance, rest time) were recorded automatically in an activity box (15 min test period; AccuScan). It was hypothesized that pain threshold and movement distance indices would decrease in animals experiencing widespread pain while rest periods would increase. Behavior tests were assessed in both treated (permethrin, chlorpyrifos, PB) and vehicle (ETOH, corn oil, water) exposed animals over an identical time course. Behavior testing occurred in blinded conditions.

Table I

	PB mg/kg, Oral	Permethrin mg/kg, Topical	Chlorpyrifos mg/kg, SC	Exposure days
NR1	13	1.3	60	30
NR2	13	2.6	120	30
NR3	13	2.6	120	60

Permethrin is applied every day
Chlorpyrifos is injected once every 2 weeks
PB is given by gavage every day for 2 weeks every 4 weeks

When combinations of neurotoxicants and PB were administered in two neurotoxicant regimens, transient pain appeared only in NR2 and was found to be associated with molecular maladaptations in a specific nociceptor sub-population (vascular nociceptors). The changes we observed were highly consistent, persisted at least 4 weeks past the period of drug administration, and were limited to putative vascular nociceptors. The lack of concurrent behavioral and molecular changes could have indicated that: 1) the molecular shifts were pre-clinical, and that longer dosing periods were required to observe simultaneous pain and molecular defects; 2) the molecular shifts were compensatory to the pain previously manifested but that had since subsided; 3) the muscle pain sensitivity test was not appropriate for what might have been a vascular pain condition. Sixty day treatments are currently underway in the expectation that persistent pain reactions may occur. Detailed findings are presented below.

The 30 day treatment with NR1 did produce any changes in behavioral pain measures (figure 1A). However, after a 30 day exposure to NR2, we did observe significant decreases in muscle pain threshold during the NR2 dosing period. The decreases in muscle pain subsided and actually reversed during the weeks that followed dosing of the animals. Other pain correlates, such as rest times, also shifted, consistent with a pain condition, during dosing with neurotoxicants, but eventually reversed polarity 4 weeks after treatments had terminated (figure 1B). In contrast, movement distance measures mainly increased relative to controls in all treatment conditions and periods. The increase in movement distance coupled to an increase in rest times indicated more rapid movement during periods of activity, as the overall time of observation was fixed at 15 minutes. Overall, the behavior data indicated, at best, that the higher doses of neurotoxicants and PB could produce a transient increase in the muscle pressure pain, but only during the 4 weeks of dose application. Studies are currently underway to determine whether muscle pain will persist after dosing if we increase the dosing period to 60 days as specified in the SOW.

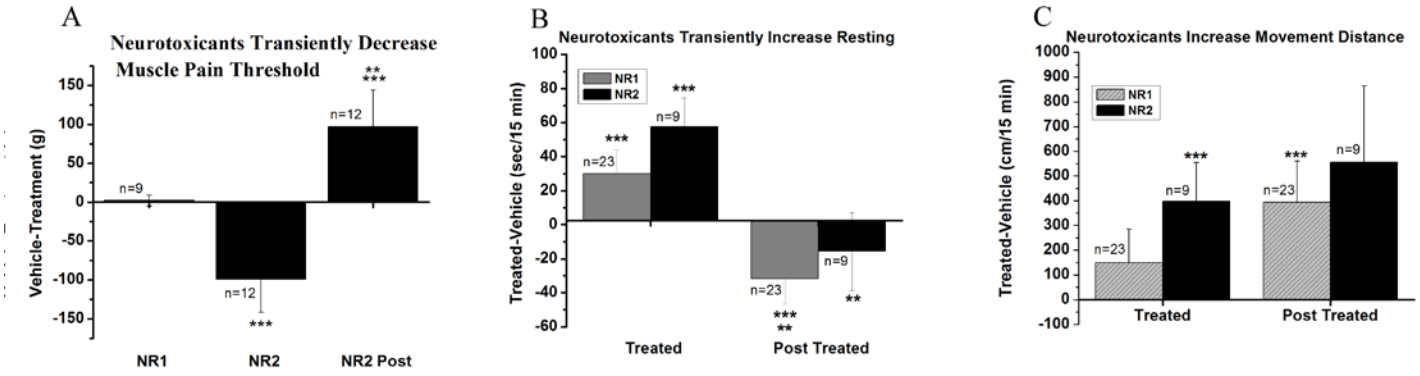

Figure 1. Neurotoxicants Transiently Decrease Pain Threshold and Increases Resting Time. A) Muscle pain threshold drops during NR2 treatment relative to controls, but reverts after treatment ceases. **B)** Neurotoxicant-induced rest time increases paralleled pain measures in NR2 (black bars). **C)** Rat movement distance increases with both NR treatments, suggesting faster movement in less time. The data are presented as difference scores between vehicle and dosed animals (double blinded tests). Activity measured by automated collection system in 15 minute observation periods. *** significantly different from vehicle dosed; ** significantly different from rats concurrently dosed with neurotoxicants.*

Although NR1 treatment failed to produce any persistent behavioral changes, this did not rule out the possibility of pre-clinical shifts in the activity of neurotoxicant vulnerable proteins that might be present in skin, muscle and vascular nociceptors. Nor did it rule out that the animals might experience mild pain that we were unable to detect or pain from tissue sources that were not assessed by the muscle pressure apparatus (e.g., joint or vascular pain). Accordingly, as described in the SOW, we examined whether changes Na_v or Kv_7 proteins were altered in any way by neurotoxicant/PB exposure.

Persistent Influences of Neurotoxicants and PB on Nociceptor Protein Function

Task 4: We will assess nociceptors for changes in K_{v7} function that could contribute to chronic and widespread pain.

GWI pain is often described by veterans as a widespread pain affecting muscle, joints and other deep tissues (Stimpson et al., 2006; Thomas et al., 2006). Accordingly, we focused our molecular studies on nociceptive neurons that innervated deep tissues with extensive bodily representation (muscles and vessels). Electro-physiological methods were used to examine the status of membrane proteins expressed in categories of skin, muscle and vascular nociceptors harvested from treated and control animals 4 weeks after dosing had ceased (Rau et al., 2007; Rau et al., 2012).

Persistent Alteration in K^+ Channel Protein Function

K^+ currents were isolated as described in the Appendix (p. 57). The linopirdine sensitive Kv_7 'tail' current was assessed across a series of deactivating voltages. In type 8 vascular nociceptors, significant decreases in the amplitude of the Kv_7 currents were associated with treatment of rats with neurotoxicants in NR1, 30 days after the treatment had ceased ($F= 7.92$, $p<.002$; $n=6,6$; figure 2A). These decreases were significant at voltages near the resting membrane potential (-70, -50 mV; figure 3A), where activation of Kv_7 currents are engaged in opposing neural discharge. This implies heightened excitability in this nociceptor family. In contrast, no

differences were observed in muscle nociceptor (type 5) Kv_7 function after NR1. This interesting Kv_7 sensitivity to neurotoxicants was replicated following NR2 treatments. In both studies, the changes in Kv_7 amplitude were specific to vascular nociceptors (figure 2). Although the Kv_7 protein sensitivity to neurotoxicants was confirmed ($F=5.28$, $p<.03$, n=9,9), the polarity of the change *reversed* in NR2, where treated vascular nociceptive neurons manifested increased Kv_7 current amplitude. Although the overall amplitude was increased, the voltage dependent changes in Kv_7 amplitude were shifted to depolarized levels that would *not* oppose AP initiation (-30 and -40 mV; figure 3C). While the neurotoxicant action on Kv_7 voltages were, to an extent, mixed, it is more important that we did observe, and replicated, that a neural protein was sensitive to neurotoxicant treatment; and that this sensitivity was unique to one class of tissue specific nociceptors and persisted beyond the immediate dosing period.

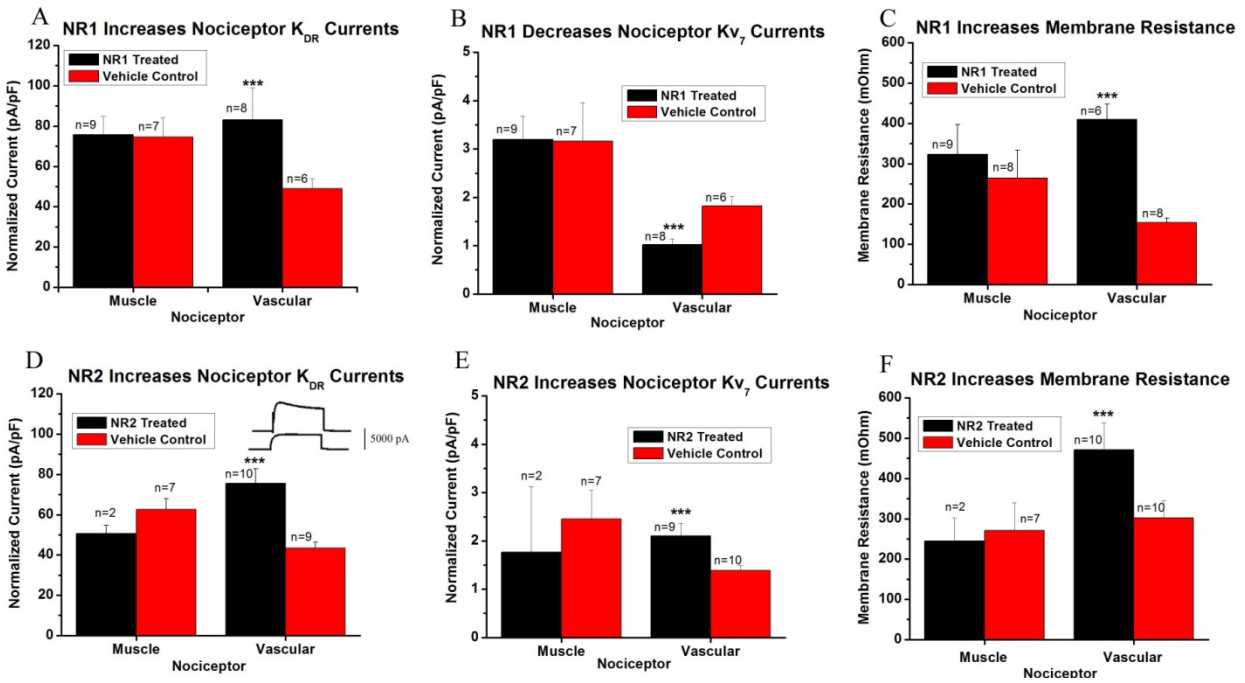

Figure 2. Neurotoxicants Altered K_v Protein Reactivity in Putative Vascular Nociceptors 4 Weeks After Treatment Ended. A-B) K^+ Channel proteins were modulated by NR1. **D-E)** The same K^+ channel proteins were modulated by NR2. Insert shows representative K_{DR} currents (upper: NR2 treated). **C and F)** Membrane resistance was increased by NR1 and NR2. Note the reversal of Kv_7 outcomes in 'B and E'. Na_v proteins were unaffected in skin, muscle or vascular nociceptor populations (not shown). *** significantly different from vehicle control.

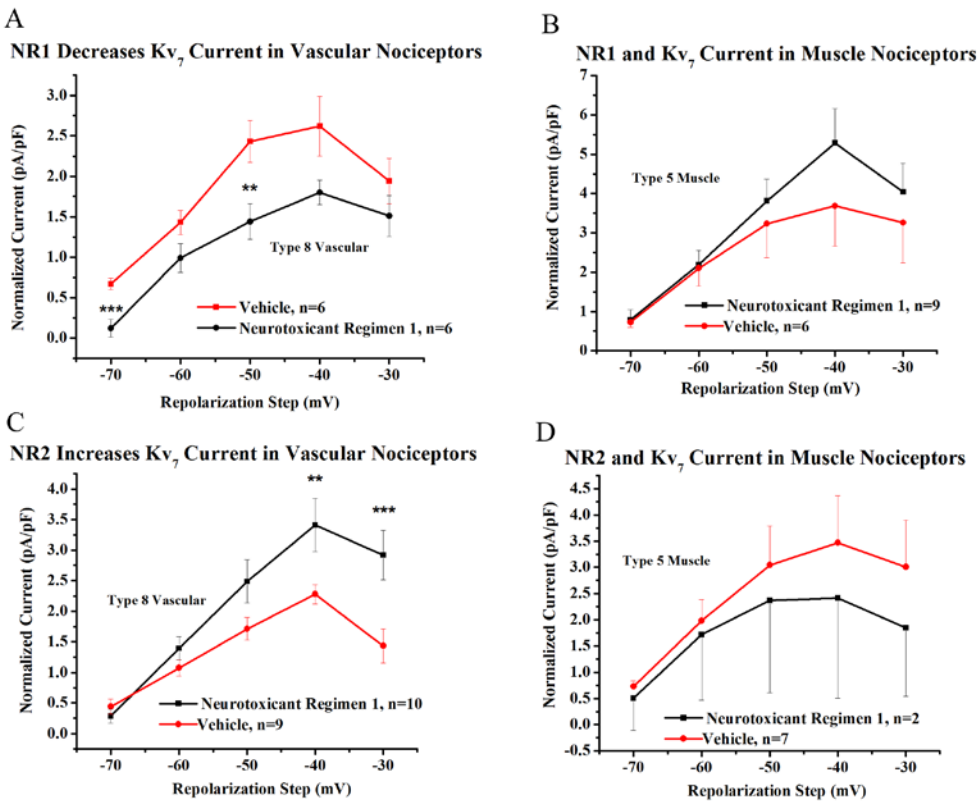

Figure 3. Neurotoxicants Alter Voltage Dependence of Kv_7 in Vascular Nociceptors. A) Kv_7 mediated currents are decreased near the resting membrane potential after NR1 (~-60 mV). **B)** Muscle nociceptor Kv_7 was not shifted by NR1 treatment. **C)** Kv_7 mediated currents are increased at depolarized voltages after NR2 (~-60 mV). **D)** There was insufficient muscle nociceptor Kv_7 data for a statistical test. NR1: *** p< .003, ** p< .02; NR2: *** p <.009, ** p< .03. Bonferroni corrected for type 1 error.

In the course of assessing Kv_7 currents we were able to simultaneously measure the total delayed rectifier currents (K_{DR}) present in muscle and vascular nociceptors. Although not part of the SOW, capture of this data was a natural consequence of the Kv_7 experiments. K_{DR} currents are critical for directional stabilization of axonal conduction in axons. To our surprise, K_{DR} currents were substantially and significantly increased in vascular, but not muscle nociceptors, following both NR1 and NR2 treatments (figure 2B, E). Parallel increases in whole cell membrane resistance complicated the interpretation of this finding, as other K^+ current proteins that govern membrane resistance (K2p) could indirectly influence K_{DR} voltage reactivity (figure 2C,F). Distinct experiments are needed to unravel the relative contributions K2p and K_{DR} proteins. Regardless, it is clear that vascular nociceptors, and multiple K^+ currents expressed by these

nociceptors, exhibit unique sensitivity to the influence of neurotoxicants chlorpyrifos, permethrin and PB. It is unclear at this point, whether these changes represent a pre-clinical shift, as no behavioral changes occurred simultaneously with K^+ protein maladaptations. The shifts may also represent compensatory response to neurotoxicant influences on other membrane proteins. Potentially, longer duration exposures to NR2 would clarify the relationship between pain behavior and molecular K^+ protein reactivity in vascular nociceptors. The 60 day exposure experiments are currently underway.

Chronic Neurotoxicant/PB Administration and Na_v Protein Function in Skin, Muscle and Vascular Nociceptors

Due to the demonstrated interactions between type I pyrethroids and Na_v proteins (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi 2001) we hypothesized that long term exposure to permethrin and other neurotoxicants would produce persistent derangements in Na_v activity. Alterations of K^+ channel activity observed after NR1 and NR2 could reflect compensation for increased Nav function. Accordingly, we examined $Nav_{1.8}$ deactivation and steady-state inactivation in rats treated with neurotoxicants/PB following a 30 day exposure to NR1 or NR2.

Task 2: We will assess nociceptors for changes in Na_v function that could contribute to chronic and widespread pain following treatment with 3 neurotoxicants

Nociceptors express the TTXr (tetrodotoxin resistant) $Nav_{1.8}$ protein (Djouhri et al., 2003a,b; Amir et al., 2006; Jiang et al., 2011). Neurons were harvested from chronically treated rats (Table 1). Following isolation of $Nav_{1.8}$ in skin, muscle or vascular nociceptors (see Appendix p. 58), we applied protocols to characterize fundamental properties of voltage sensitive proteins (activation, inactivation and deactivation) Studies were conducted 4 weeks after dosing with neurotoxicants and PB had ended. Activation reflects the tendency for $Nav_{1.8}$ protein to form action potentials and is an important gauge of excitability. These data were assessed by formation of classic sigmoidal activation curves (Boltzman functions) where a $V_{.50}$ activation statistic reflects the voltage at which half of the proteins are activated. Inactivation was also

assessed by classic methods. A $V_{.50}$ was derived that reflected the voltage at which half of the $Nav_{1.8}$ proteins were available to be activated. A depolarizing (more positive) shift of the inactivation $V_{.50}$ indicates that a larger portion of the total pool of $Nav_{1.8}$ proteins are capable of being activated. Deactivation refers to the movement of proteins from an open to a closed state due to a hyperpolarizing ion flux. The slowing of deactivation leads to greater excitability. Deactivation was assessed by deriving time constants (tau_{deact}) that characterized the exponential decay of $Nav_{1.8}$ mediated current in proportion to a series of deactivating voltage steps. Normalized peak currents were also assessed, where the observed current were normalized by an index of cell size (membrane capacitance; pF).

Although $Nav_{1.8}$ is greatly influenced by acute permethrin administration (see figures 11 and 14), we did not observe a lasting influence on $Nav_{1.8}$ expressed in muscle or vascular nociceptors that had been *chronically* exposed to permethrin as a component of the neurotoxicant regimens. There was no indication that the tau_{deact} of $Na_{v1.8}$ was modified in either mean duration or voltage dependence 4 weeks after a 30 day exposure to permethrin, chlorpyrifos and PB (NR1, NR2; figure 4). Nor were changes in steady state inactivation apparent in any tissue specific nociceptor population examined (figure 5). Fits of exponential decay functions to the decay phase of the $Nav_{1.8}$ were also unaffected by NR1 or NR2 treatments (see figure 5 imbeds). With the exception of skin nociceptors, the normalized peak $Nav_{1.8}$ current was also unaffected (I_{max}/C_m; figure 6), as was, in limited studies, the voltage dependent activation (figure 7). Therefore, the acute influences of permethrin on $Nav_{1.8}$, demonstrated during 'Acute Studies' of Task 4 (see below, p. 18), and in some instances demonstrated with other type I pyrethroids (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi 2001) were not present 4 weeks after chronic exposure to permethrin and other suspect GWI agents. If any changes occurred in Na_v protein function occurred concurrent with dosing, resulting in compensatory shifts in K^+ currents that were observed in both NR1 and NR2, they would have to have been transient changes limited to the period of neurotoxicant exposure. Otherwise, the changes in K^+ channel activity, exhibited in vascular nociceptors, were actually direct effects of the neurotoxicant/PB treatments.

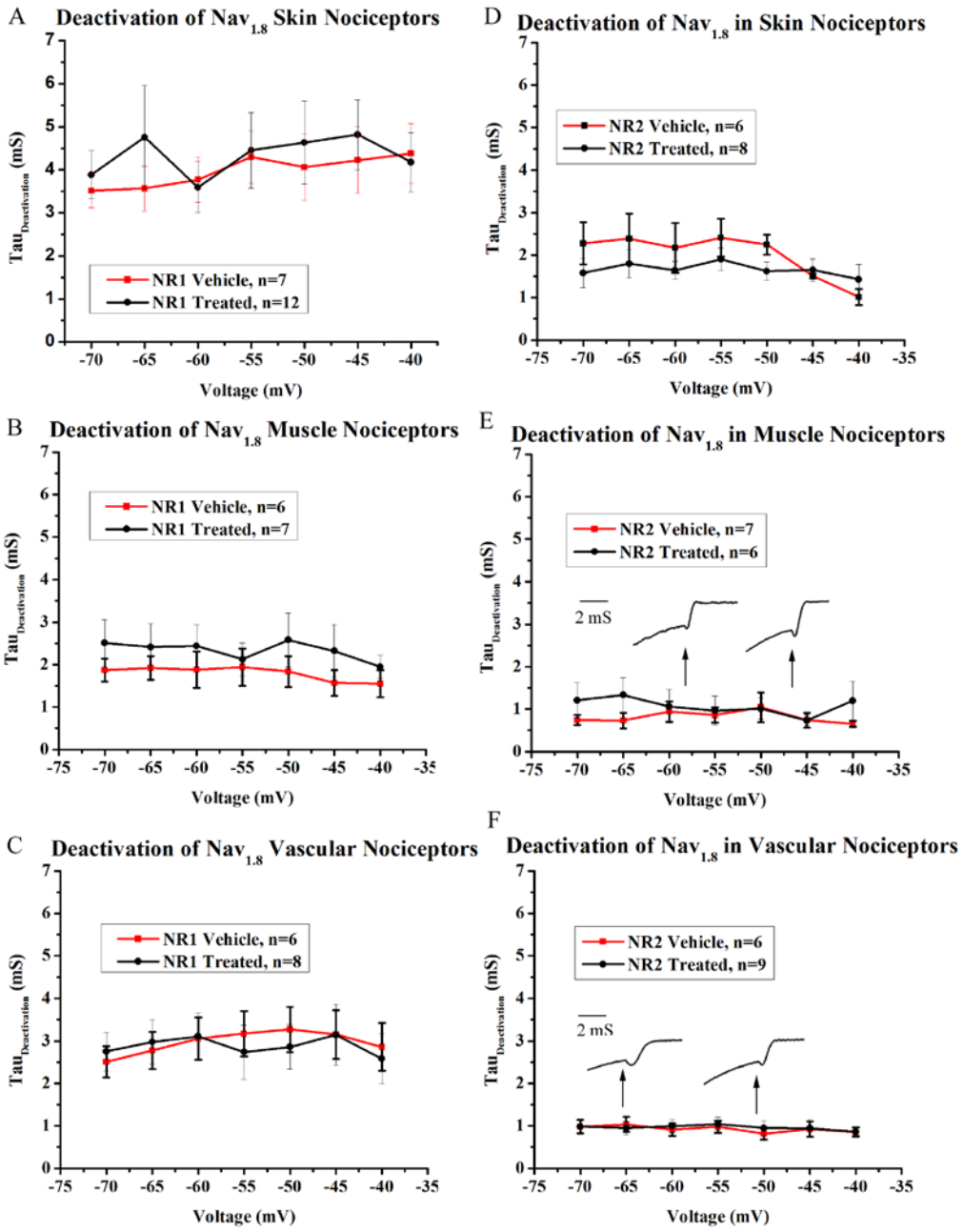

Figure 4. Dosing with NR1 or NR2 did not Modify $Nav_{1.8}$ Deactivation. A-C) Four weeks after NR1 dosing, there were no differences in the tau_{deact} in skin (A), muscle (B) or vascular nociceptors (C). **D-F)** Four weeks after NR2 dosing, there were no differences in the tau_{deact} of in skin (D), muscle (E) or vascular nociceptors (F). Inserts in E and F are truncated representative deactivation currents in muscle and vascular nociceptors (-40 mV). The arrow indicates the point of deactivation. The upper trace came from a vehicle exposed animal. The lower trace came from a neurotoxicant treated animal. The Nav current trace was greatly truncated to enable presentation of the deactivation tail current. See figure 14 for a deranged tail current typical of acute permethrin treatment.

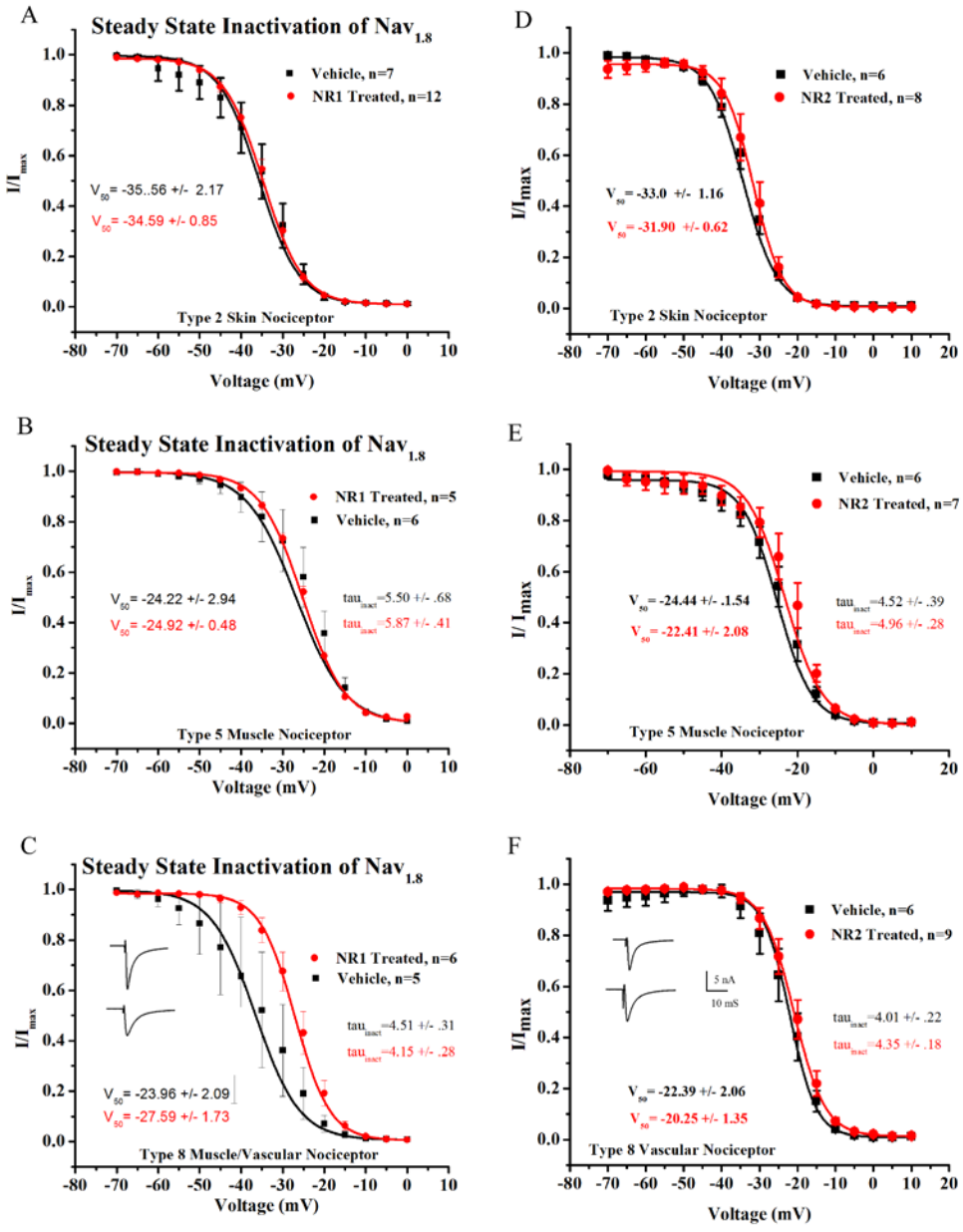

Figure 5. Dosing with NR1 or NR2 did not Modify $Nav_{1.8}$ Inactivation. A-C) Four weeks after NR1 dosing, there were no differences in the V_{50} of steady state inactivation in skin (A), muscle (B) or vascular nociceptors (C). **D-F)** Four weeks after NR2 dosing, there were no differences in the V_{50} (mV) of steady state inactivation in skin (D), muscle (E) or vascular nociceptors (F). Inserts in (C) and (F) are representative $Nav_{1.8}$ currents in vascular nociceptors (0 mV). The upper trace came from a vehicle exposed animal. The lower trace came from a neurotoxicant treated animal. Scale bars in 'F' apply to 'C'. Calculated tau_{inact} (mS) is shown as an insert in plots of muscle and vascular nociceptors. There were no significant differences in treatment conditions.

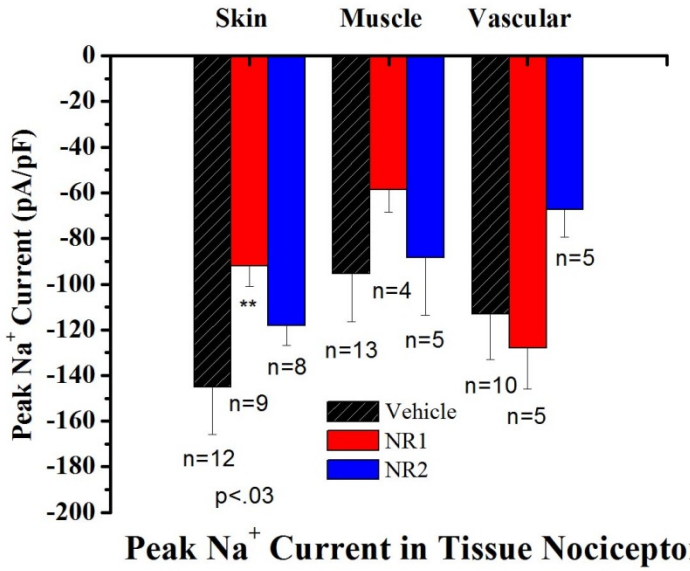

Figure 6. Peak Na^+ Currents and Neurotoxicants. Mainly decreasing trends were observed in peak Nav1.8 currents in tissue specific nociceptors. A significant decline in skin nociceptors (**) was observed following NR1 only but it was not replicated in NR2. It was non-significant with a Bonferroni correction for type 1 error.

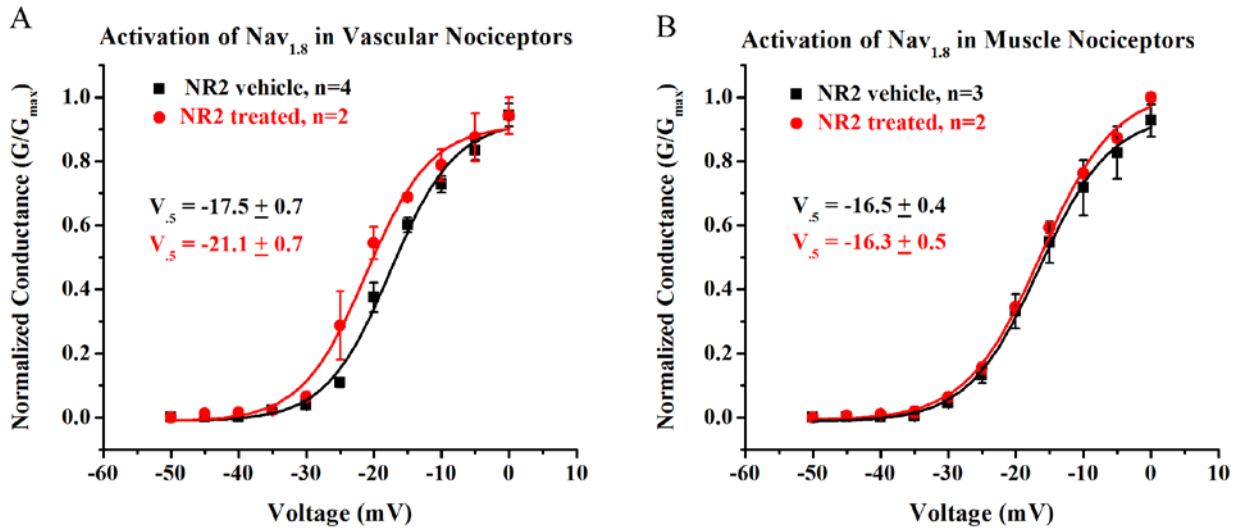

Figure 7. Dosing with NR2 did not Modify $Nav_{1.8}$ Activation. A) The $V_{.5}$ for putative vascular nociceptor activation was shifted, but insufficient data was obtained for a statistical test. **B)** The $V_{.5}$ of muscle nociceptors exhibited little neurotoxicant influence 4 weeks after NR2 treatment.

Spontaneous Activity and Nociceptor Excitability

The reduction of Kv_7 protein activity during NR1 suggested that nociceptors expressing such proteins could become spontaneously active and/or more sensitive to various physiological conditions following exposure to neurotoxicants. Accordingly we examined the excitability and spontaneous activity of muscle and vascular nociceptors harvested from rats treated with NR1.

Task 2c: Assess Excitability in Muscle Nociceptors

Task 2d: Assess Excitability in Vascular Nociceptors

Task 4c: Assess Spontaneous Activity in Nociceptors

Task 4d: Assess the Nociceptor Phenotype Exhibiting Spontaneous Activity

Action potentials represent voltage shifts resulting from the net current flow of many membrane proteins (Na_v, K_v, Ca_v, K_{ca}) that are expressed in multiple forms and differentially expressed across nociceptor phenotypes. As such, changes in the shape or excitability of the action potential represents changes in the contribution of these many proteins. We determined whether NR1 produced any shifts in action potential features 4 weeks after NR1 administration.

Neurons were harvested from rats receiving vehicle or NR1 treatment. Nociceptors were characterized as muscle or vascular nociceptors and brought into current clamp mode. The resting membrane potential was adjusted to -60 mV and action potentials (3) were evoked by current injection (3-5 nA; 1 msec). Action potential features were assessed; including action potential duration (APD) and afterhyperpolarization duration (AHD; Petruska et al, 2000; Petruska et al., 2002). Excitability was subsequently determined by stepwise current injection, and the total number of APs evoked by 15 consecutive current injection steps were tallied (see Appendix, p. 56).

Contrasts performed between vascular and muscle nociceptors harvested from NR1 treated and vehicle treated rats did not reveal any changes in action potential duration (APD; figure 8A), afterhyperpolarization (AHD; figure 8B), resting membrane potential (figure 8C) or excitability in muscle or vascular nociceptors (figure 8D and E). Although trending higher, there were no changes observed in or membrane resistance (figure 8F). The pattern of changes were similar after NR2 (figure 8). Following tests on excitability, cells from rats exposed to NR1 or NR2 ells

were observed for the presence of spontaneous activity (2.5 min). There were no significant differences in spontaneous activity between neurotoxicant and vehicle treated rats, and little spontaneous activity was observed in any nociceptor class (tests conducted at 35 C; data not shown).

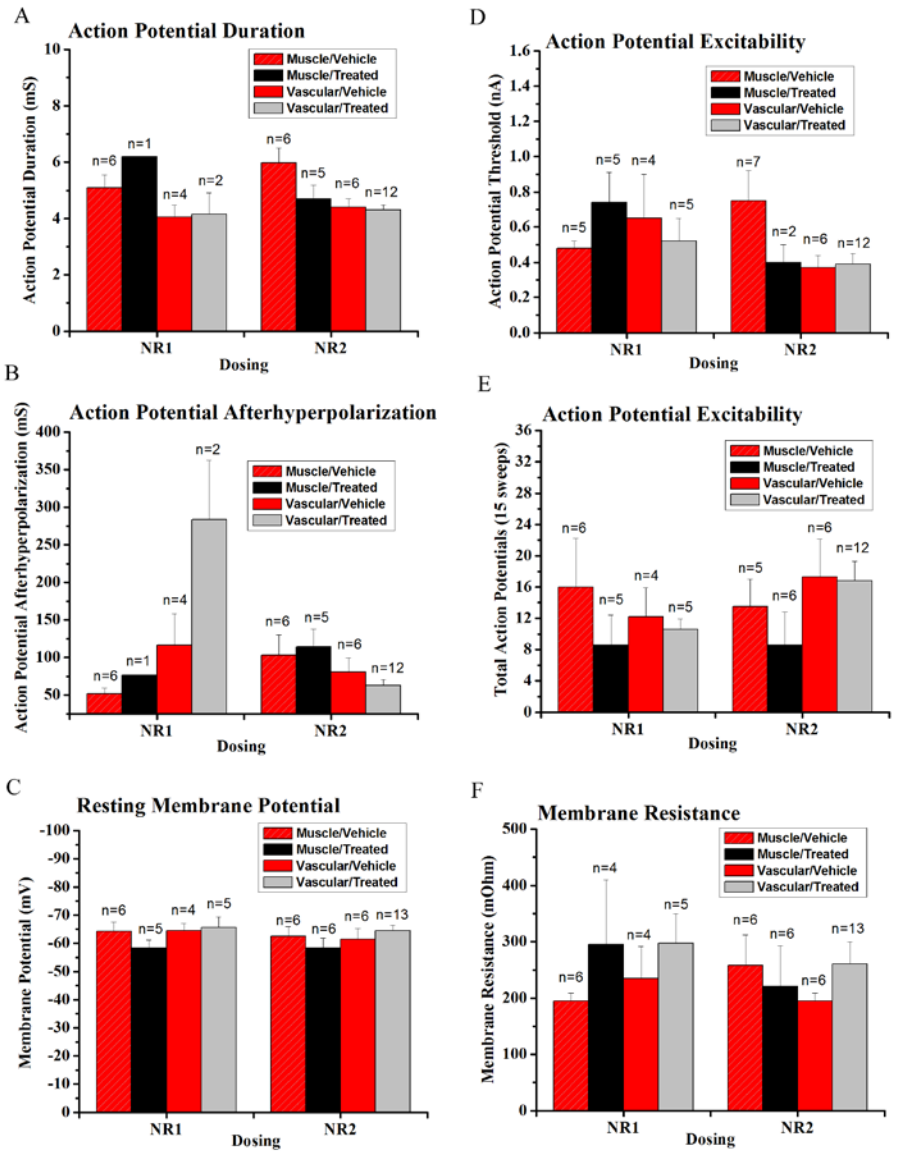

Figure 8. Neurotoxicant Treatments and Action Potential Features. A-C) Action potential features remained stable in muscle and vascular nociceptors after NR1 and NR2. **D-E)** Action potential excitability (threshold and evoked APs) were unaffected by neurotoxicant treatments. **F)** Although membrane resistance was consistently higher, there were no significant effects on membrane resistance in any nociceptor class.

Acute Influences of Permethrin on Skin, Muscle and Vascular Nociceptors

During the brief course of the Gulf war, GW veterans were exposed to 13 or more pesticides (DOD Environmental Exposure Report: Pesticides, 2003). Some of these pesticides have direct interactions with the pain system (pyrethroids: permethrin, phenothrin). The pyrethroid class of insecticides alters activity of mammalian proteins that are closely related to insect proteins by which pyrethroids exert their lethal effects (voltage gate sodium channels, VGSC, or Nav). One of these mammalian proteins (i.e., $Nav_{1.8}$) is prominent in nociceptor (pain) coding (Djouhri et al., 2003; Jiang et al., 2011). Investigations of pyrethroid interactions with mammalian sensory neurons of the DRG (dorsal root ganglion), confirmed that powerful influences between several pyrethroids (allethrin, tetramethrin, deltamethrin) and sensory perceptions were possible (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi 2001). While stationed in the Gulf region, GW veterans made liberal use of the type 1 pyrethroid insecticide permethrin. However the specific interactions between permethrin and peripheral nervous system nociceptors and/or functional $Nav_{1.8}$ channels expressed in these nociceptors is not known. Moreover, there are important functional differences between nociceptors innervating superficial (skin) and deep tissues (e.g., muscle) that could qualitatively affect their interaction with neurotoxicants. Accordingly, we initiated a series of studies to examine how permethrin interacts with specific skin, muscle and vascular nociceptors that express pyrethroid sensitive protein $Nav_{1.8}$, and other nociceptor proteins that might contribute to a chronic pain disorder.

As will be shown below, the acute influence of permethrin on nociceptor Na_v was extensive, and while affecting skin, muscle and vascular nociceptors alike, was especially potent and more pervasive in type 5 muscle nociceptors. This pattern of acute affects stands somewhat in contrast to the chronic influence of neurotoxicants, including permethrin, on pain system nociceptors. Following chronic administration, the persistent changes in protein function were clearly focused on vascular, type 8, nociceptors and their K^+ channel activity.

Task 3: We will examine the acute effects of permethrin on Na_v proteins in skin, muscle and vascular nociceptors

A series of studies on tissue specific nociceptor populations indicated that permethrin had broad effects on nociceptors, with additional influences specific to muscle nociceptors. Isolated nociceptors were characterized and identified as skin (type 2), muscle (type 5) or putative, type 8, vascular nociceptors (Appendix, p. 48; Rau et al., 2007; Rau et al., 2012). Cells were brought into current clamp mode and the influence of permethrin (10 μM) on nociceptor action potentials was assessed. As previously demonstrated in mammalian sensory neurons using the type II pyrethroid, deltamethrin (Tabarean and Narahashi, 1998), the type I pyrethroid, permethrin, significantly increased action potential duration of skin, muscle and vascular nociceptors (figure 9). The increase in duration was substantial and so extensive as to completely obscure the afterhyperpolarization period (6.96 +/- 0.8 vs 163.7 +/- 44.2; 8.8 +/- 0.7 vs. 105.9 +/- 12.2, and 6.9 +/- 0.6 vs. 82.7 +/- 12.4 ms for types 2, 5 and 8; $p<.001$, n=4-6; figure 9). The afterhyperpolarization (AHP; figure 1b) opposes repetitive discharge and plays a critical stabilizing role in axonal transmission by preventing the occurrence of ectopic discharge that is believed to contribute to certain chronic pain syndromes (Devor, 2006; Baron, 2006).

We assessed whether permethrin-induced changes in AP characteristics would be associated with altered action potential excitability. Holding the cell at -60 mV to remove resting membrane potential biases on excitability, a series of current injections were performed on ETOH and permethrin treated nociceptors (10 current injection steps of 0.1-1.0 nA; 225 ms/test). Over an observation period of 10 minutes (1 min test intervals), the number of action potentials evoked by a series of current injections were increased in type 5 muscle nociceptors (13.0 +/- 4.5 APs; n=5; figure 10) but not in skin or vascular nociceptor classes. Often excitability tests on type 5 muscle nociceptors did not last the full 10 min period due to the development of spontaneous activity. Permethrin is known to produce depolarization and spontaneous activity in mammalian neurons in various species and preparations (Carlton 1977; Parkin and Le Quesne 1982, Takahashi and Le Quesne 1982; Staatz-Benson & Hosko, 1986; Meyer et al., 2008); however the instigation of spontaneous activity is not a universal property of type I pyrethroids like permethrin (Wright et al., 1988; Ray and Fry, 2006). This is the first demonstration that direct activation of nociceptors is possible and that a specific subclass of nociceptors exhibits distinct vulnerability to this neurotoxicant.

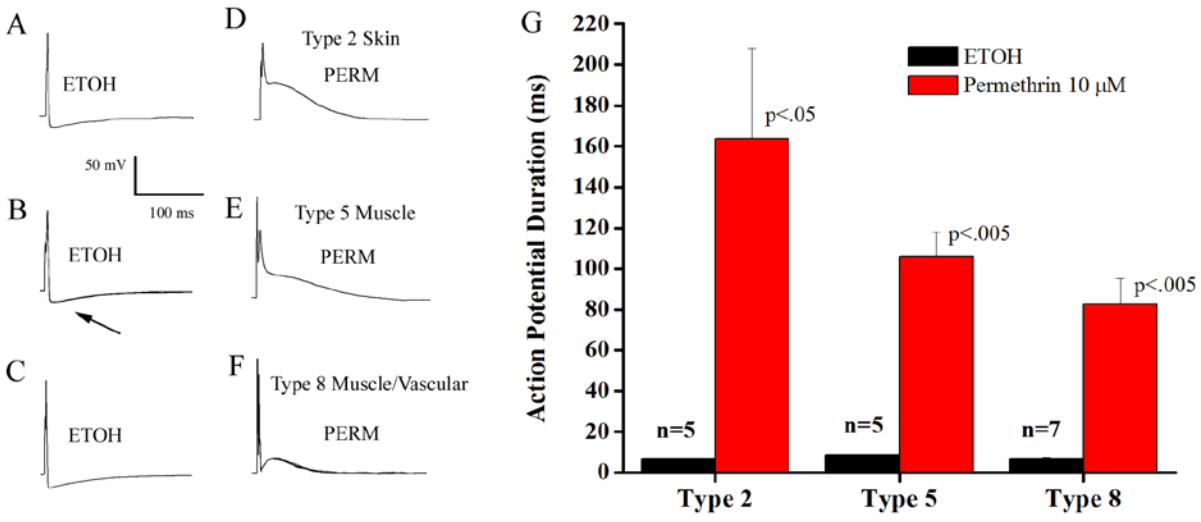

Figure 9. Permethrin Increases AP Duration in Nociceptors. A-C) Action potentials in tissue specific nociceptors following exposure to ETOH vehicle. The arrow highlights the afterhyperpolarization. **D-F)** Action potentials in the same nociceptors following exposure to 10 μM permethrin for 6-10 minutes. After-hyperpolarizations are absent in all treated neurons. **G)** Summary data for 3 nociceptive classes. Duration increases were highly significant. Action potentials were evoked by a 3 nA injection for 1 ms.

With the application of permethrin, all type 5 muscle nociceptors depolarized and exhibited spontaneous discharge (34.4 +/- 12.2 APs/5 min permethrin vs. 0.0 APs/5min ETOH; figure 10). In contrast, despite gradual depolarization of all cells in these conditions, neither vascular nor skin nociceptor populations exhibited spontaneous activity following permethrin application (n=16). ETOH vehicle did not produce spontaneous activity in any nociceptor class over equivalent application periods (n=8). It is noteworthy that depolarizations induced by permethrin exceeded those associated with ETOH vehicle alone (-6.98 +/- 2.2 vs. -17.5 +/- 2.7 mV in 5 min; n= 6 and 11 (TTX cases included)). This was consisted with a distinct contribution of permethrin to the shift in resting membrane potential to depolarized levels sufficient to initiate spontaneous activity. We initiated studies to identify the molecular basis of spontaneous activity in muscle nociceptors.

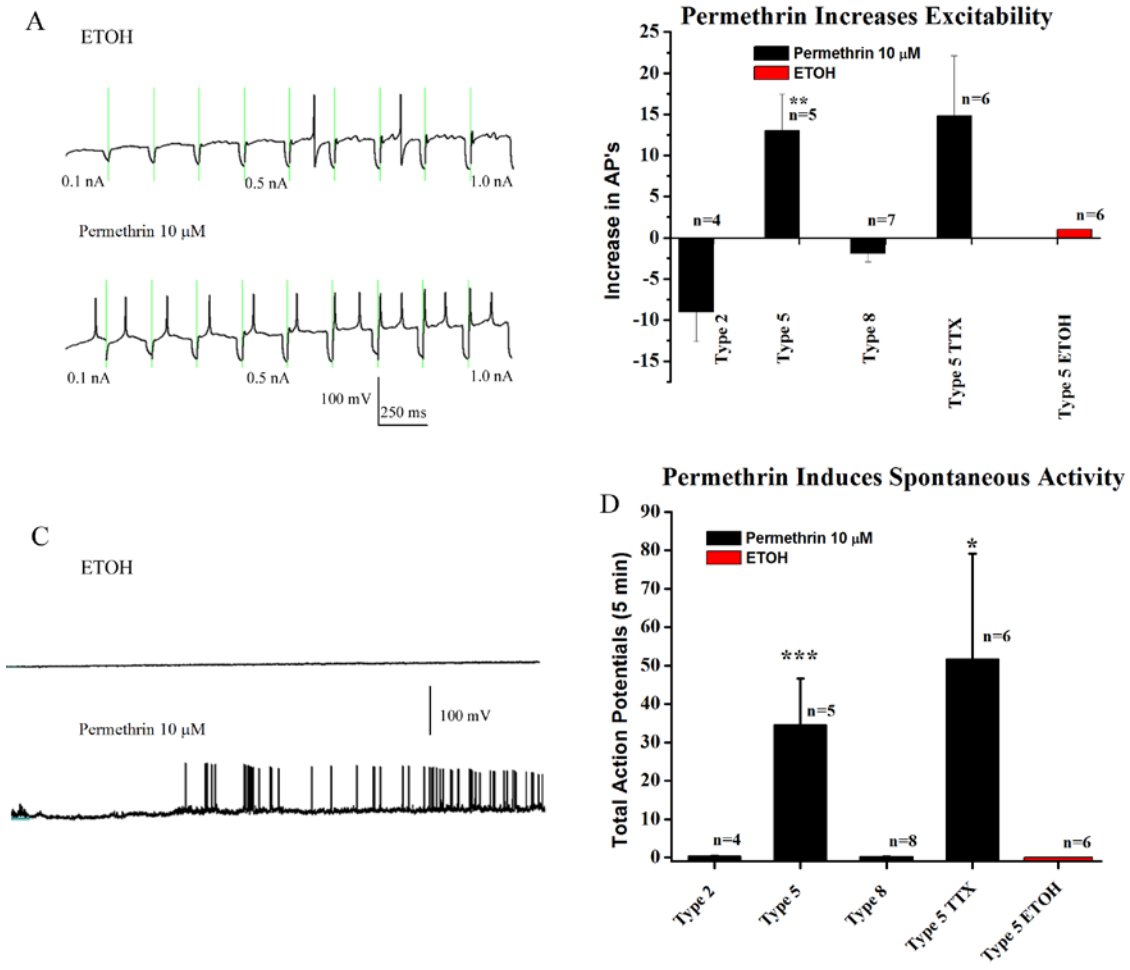

Figure 10. Permethrin Increases Excitability and Induces Spontaneous Activity in Muscle Nociceptors. A) Excitability testing in a type 5 muscle nociceptor. Ten consecutive current injection tests in an ETOH (upper panel) and Permethrin treated case (lower panel; 10 µM, 10 min). Vertical green bars separate consecutive tests. Substantial increases in action potential discharge were evident. **B)** Summary data for excitability testing indicated that only type 5 muscle nociceptors manifested excitability shifts. TTX did not block shifts in AP excitability (** p<.03 vs ETOH). **C)** Representative spontaneous activity in a type 5 muscle nociceptor after exposure to ETOH or permethrin (10 µM). Five minutes of observation are shown. Exposure to ETOH for 5 minutes (upper panel) did not result in spontaneous activity. **D)** Summary data of the influence of permethrin on spontaneous activity. Only type 5 muscle nociceptors exhibited spontaneous activity. TTX did not impair the development of spontaneous activity. * p<.03 vs ETOH (Mann-Whitney U test; ties were not corrected). *** p<.001

Voltage gated Na^+ channels are routinely divided into families of proteins that are broadly distinguished by their sensitivity to the puffer fish toxin, TTX (tetrodotoxin). To examine whether TTXs (TTX sensitive) currents were essential to the development of spontaneous activity, we treated a group of type 5 muscle nociceptors to a combination of permethrin and TTX (10 and 1 µM; n=6). Using identical procedures as above, 5 of 6 TTX treated nociceptors

depolarized and became spontaneous in the presence of permethrin+TTX (51.6 +/- 27.5 APs/5min; n=6; figure 10d). All TTX treated cases also exhibited increased excitability (figure 10b); action potential duration was not influenced co-application of TTX (105.9 +/-12.2 vs. 115.0 +/- 6.1 ms with TTX (not shown). We can conclude from these studies that TTXr (TTXresistant) Nav proteins, such as $Nav_{1.8}$, were sufficient to maintain permethrin influences on AP duration, excitability and spontaneous activity of muscle nociceptors.

The action potential represents a transient disturbance in the net flow of ions across the neural membrane. The flow of ions is governed by the opening and closing of several families of voltage gated proteins that are differentially distributed in nociceptors. The opening and closing of voltage gated proteins are described as series of 'states' that represent specific protein shapes (conformations). These open and closed states undergo activation, inactivation and deactivation as they transition from closed to open to closed states. Activation and deactivation are voltage dependent, while inactivation, although influenced by voltage, occurs in the absence of voltage changes. In an attempt to understand the basis of shifts in neural excitability and the development of spontaneous activity, we examined the influence of permethrin on a subset of voltage gated proteins that are known to influence action potential duration.

Task 3: We will examine the acute effects of permethrin on Na_v proteins in skin, muscle and vascular nociceptors

While there have been limited studies on the influence of permethrin on neural protein function, the influence of structurally similar pyrethroid neurotoxicants has been studied extensively. The permethrin/pyrethroid promotion (or obstruction) of neural activity has been associated with changes in activation, steady-state inactivation and deactivation of insect Nav proteins (Narahashi et al., 1998; Narahashi, 2000; Ray and Fry, 2006; Soderlund, 2010). This array of functional modulation of Nav has been documented in mammalian neurons across a structurally and functionally diverse family of mammalian Nav (e.g., $Nav_{1.2}$, $Nav_{1.3}$, $Nav_{1.6}$, $Nav_{1.8}$), but the specifics of the modulation differ both qualitatively and quantitatively (Song and Narahashi, 1996; Song et al., 1996a,b; Smith and Soderlund 1998; Motomura and Narahashi, 2001; Dekker, et al., 2005; Choi and Soderlund, 2006; Meacham, et al., 2008; Breckenridge et al., 2009; Tan

and Soderlund, 2009). The relationship of $Nav_{1.3}$ to neural injury and differential distribution of Nav proteins in the central ($Nav_{1.2}$) and peripheral nervous system ($Nav_{1.6}$, $Nav_{1.8}$) may be significant (Rush et al., 2007; Dib-Hajj et al., 2009).

In the studies below, we demonstrate that permethrin exhibits powerful influences on the voltage dependence of activation and deactivation of nociceptor $Nav_{1.8}$. As we observed with action potentials, these effects were manifested across tissue specific nociceptor classes that expressed $Nav_{1.8}$ (Jiang et al., 2011). Changes in deactivation can explain AP elongation that were seen across all tissue specific classes, but neither these nor activation shifts can explain the specific effects we observed on muscle nociceptor excitability and spontaneous activity that were limited to muscle nociceptors.

Muscle nociceptors, as well as many other nociceptor types, express the TTXr $Nav_{1.8}$ protein (Djouhri et al., 2003a,b; Amir et al., 2006; Jiang et al., 2011). Accordingly, we applied protocols to examine the activation, inactivation and deactivation of $Nav_{1.8}$ in the presence of permethrin. Activation reflects the tendency for $Nav_{1.8}$ protein to form action potentials. These data were assessed by formation of classic sigmoidal activation curves where a $V_{.50}$ activation statistic reflects the voltage at which half of the proteins are activated. A leftward, hyperpolarizing, shift in the activation $V_{.50}$ for $Nav_{1.8}$ (more negative) indicates a cell that is more likely to form action potentials that will be perceived as pain. Inactivation was also assessed by classic methods. A $V_{.50}$ was derived that reflected the voltage at which half of the $Nav_{1.8}$ proteins were available to be activated. A depolarizing (more positive) shift of the inactivation $V_{.50}$ indicates that a larger portion of the total pool of $Nav_{1.8}$ proteins are capable of being activated. Deactivation refers to the movement of proteins from an open to a closed state due to a hyperpolarizing ion flux. The slowing of deactivation leads to great excitability. Deactivation was assessed by deriving time constants (tau_{deact}) that characterized the exponential decay of $Nav_{1.8}$ mediated current in proportion to a series of deactivating voltage steps (see Appendix, p. 53). Recordings were obtained from skin, muscle and vascular nociceptors.

The tendency to form action potentials (excitability) is highly related to the threshold of Nav protein activation. We examined whether the activation of $Nav_{1.8}$ was influenced by the acute application of permethrin (10 μM). Skin, muscle and vascular nociceptors were identified in the usual manner. A Na-Iso solution (Appendix, p. 53) was used to separate Na^+ currents from other

voltage sensitive membrane currents. Permethrin was applied for 2 minutes. Subsequently, a $V_{.50}$ was derived from sigmoidal functions fit to the normalized voltage-conductance (G) plots. Contrasts were then made between the $V_{.50}$ of ETOH/ETOH treated cases and ETOH/Permethrin treated cases. Consistent with increased excitability, permethrin shifted the midpoint of excitation ($V_{.50}$) in the hyperpolarizing direction (figure 11). That is, Nav proteins exposed to permethrin had a far greater sensitivity to voltage changes and were much more likely to form action potentials. No changes in peak amplitude were observed (0 mV test; figure 12).

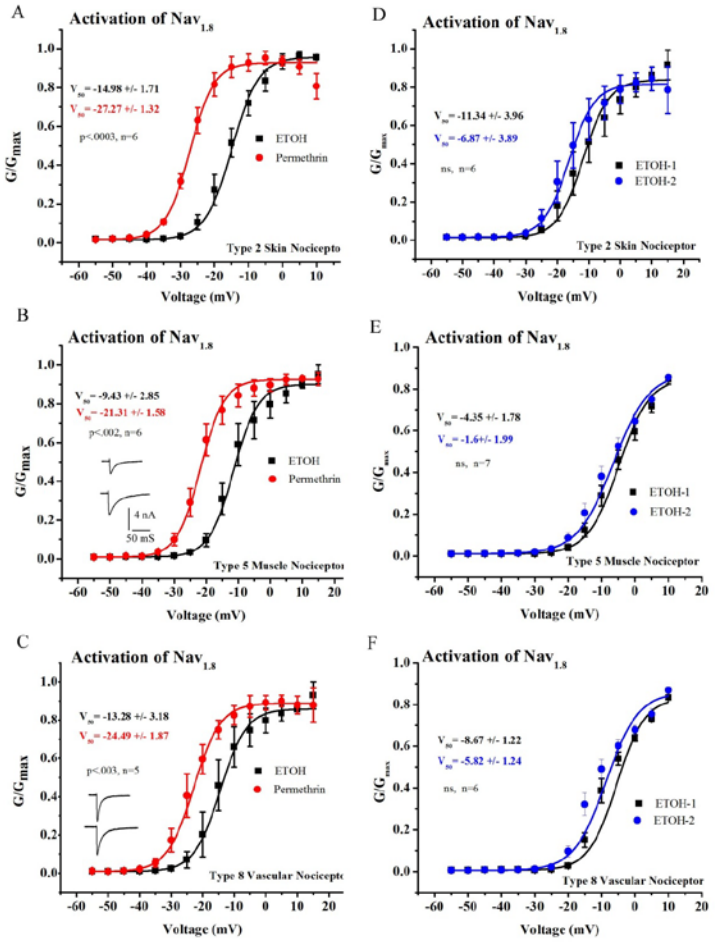

Figure 11. Permethrin Shifted the Voltage Dependent Activation of $Nav_{1.8}$. Depolarizing shifts in the activation $V_{.50}$ suggested greater excitability following permethrin exposure (10 μM): **A)** Skin nociceptors; **B)** Muscle nociceptors; Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post permethrin (0 mV activation step); **C)** Vascular nociceptors. Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post permethrin (0 mV activation step). Scale bars in 'B' apply. Control vehicle tests (ETOH-1 first exposure; ETOH-2 second exposure) did not produce any significant shifts of $V_{.5}$ (.001% ETOH; different cells from A, B, C): **D)** Skin nociceptors; **E)** Muscle nociceptors; **F)** Vascular nociceptors. Comparisons between the first ETOH-1 and 'Vehicle' tests (.001% ETOH) were non-significant.

Even though actual excitability was only increased in muscle nociceptors (figure 10B), permethrin affected skin, muscle and vascular nociceptors $V_{.5}$ in a similar manner and degree (figure 11). The $V_{.50}$ for activation shifted more than 10 mV in all nociceptor classes. Clearly nociceptors were highly vulnerable to the acute influence of permethrin.

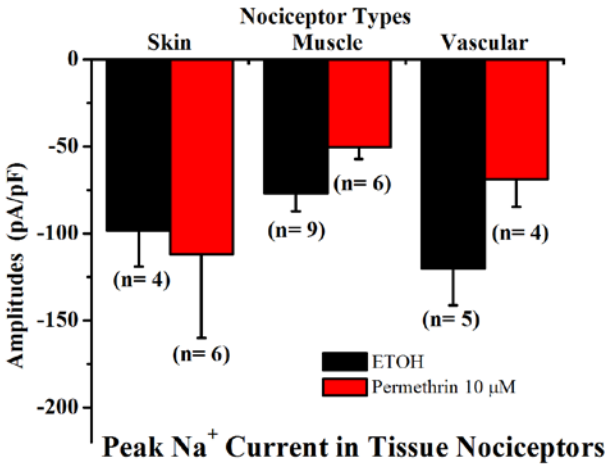

Figure 12. Acute Permethrin did not Increase the Amplitude of $Nav_{1.8}$. At the test voltage of 0 mV, the normalized peak amplitude of Nav1.8 in tissue specific nociceptors remained relatively stable compared to ETOH treated controls.

In a cell body, action potentials are terminated by voltage dependent deactivation. Action potential prolongation that we observed (figure 9) could have been due to poor deactivation of $Nav_{1.8}$. We directly assessed the influence of permethrin on deactivation of nociceptor $Nav_{1.8}$. Deactivation time constants (tau_{deact}) were determined by fits of exponential decay functions to Na^+ currents that were terminated 8 msec after a strong depolarizing voltage step (see Appendix, p. 53). As previously demonstrated with other type I pyrethroids (Soderlund, 2010), exposure to permethrin induced very large deactivation tail currents (figure 14). Relative to control cases, all permethrin treated nociceptors exhibited significant increases in the deactivation time constants consistent with a prolongation of action potential duration (e.g., tau_{deact} = 11.3 +/-1.4 vs 5.9 +/- 0.47 ms at -60 mV; type 5 muscle, $p<.01$). The influence of permethrin became strongly voltage dependent over the test voltages, and reached a maximum change near the holding potential (i.e., -40 mV test, V_H= -60 mV, figure 14). In order to evaluate the voltage dependent changes, exponential functions were fit to individual cell, voltage-tau, records and a constant '*k*' was derived from this equation that characterized the growth of the function with voltage (see

Appendix, p. 53). Comparison of *k* between ETOH and permethrin treated skin, muscle and vascular nociceptors were highly significant (p<.002, .008 and.02; figure 14).

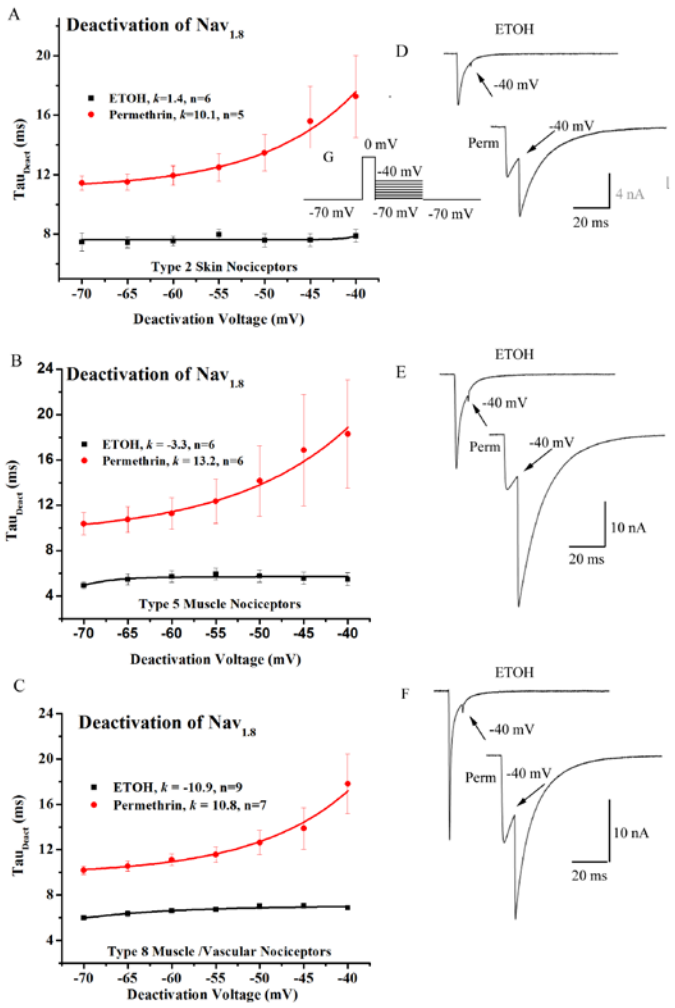

Figure 14. Permethrin Shifts Voltage Dependence of Deactivation in All Tissue Specific Nociceptors. A-C) ETOH exposed cells exhibit a relatively consistent pattern of deactivation across membrane voltages (black curves). Following treatment with permethrin, all deactivation time constants were increased and a strong voltage dependence appeared (red curves). There were no statistical differences amongst tissue specific nociceptors. **D-F)** Representative traces illustrate the deactivation tail current associated with permethrin and ETOH treated neurons. All traces taken from a single deactivation voltage step to -40 mV. The arrow signifies the point at which the voltage is abruptly clamped at -40 mV. **G)** A schematic of the deactivation protocol. The voltage step to 0 mV was 8 ms long. The deactivation voltage steps were 150 ms. 10 μMm permethrin applied for 2 minutes.

Nav proteins are highly expressed in cells and axons, but may not be 'available' for forming action potentials. Following activation, Nav proteins will enter an inactivated state from which they can be released (made available) by hyperpolarizing (very negative) potentials. Inactivation

also reflects the tendency for Nav proteins to close from the open (activated) state to a closed (inactivate) state despite the presence of activating voltage. It is contrasted by deactivation which reflects the tendency to close from the open state when the voltage shifts to one at which the protein is normally closed. This feature is classically studied by holding Nav proteins at strongly activating pre-pulse voltage (steady state) and then gradually releasing the inactivation that forms by systematically stepping the conditioning pre-pulse to hyperpolarizing voltages. A $V_{.50}$ for inactivation can be determined from Boltzman function fits to 'released' Nav currents (Appendix, p. 54). After performing tests of steady state inactivation in isolated Na^+ currents, we were unable to demonstrate any influence of permethrin on the voltage dependence of steady state inactivation of $Nav_{1.8}$ in tissue specific nociceptors (figure 15). The tau_{inact} was greatly increased (figure 15 inserts).

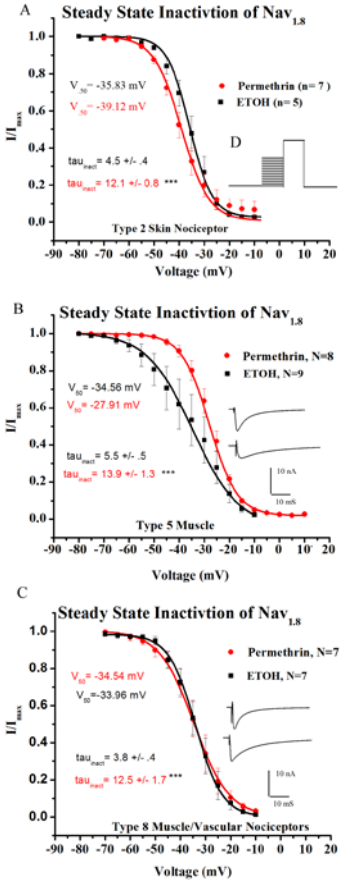

Figure 15. Permethrin and Fast Inactivation of $Nav_{1.8}$ A-C) Voltage dependent inactivation of $Nav_{1.8}$ was not significantly modified by application of 10 μM permethrin (2 minutes); The tau_{inact} (mS) was significantly increased in all cases. **D)** A schematic of the inactivation protocol. Inserts show representative traces (upper: ETOH; lower: Permethrin). Note the slowing of inactivation after permethrin. The cells were pre-pulsed in 5 mV steps from -10 to -80 mV. The test pulse was 0 mV, 150 ms.

Humans are highly resistant to pyrethroid poisoning. A number of factors (absorption, elimination, body temperature, innate sensitivity) contribute to the lower lethality of pyrethroids in humans (Ray and Frey, 2006; Narahashi et al., 2007). Pyrethroids are effective as insecticides

due to their actions on insect Nav proteins (Raymond-Delpech et al., 2005; Narahashi, 2000; Soderlund, 2010). However, mammalian Nav proteins are structurally diverse and anatomically specialized to a much greater extent than those expressed in invertebrates. Although the functional significance is obscure, the influence of pyrethroids on distinct mammalian Nav differ qualitatively and quantitatively (Ruigt, et al. 1987; Song et al., 1996a,b; Choi and Soderlund, 2006; Ray and Fry, 2006; Meacham, et al., 2008; Breckenridge et al., 2009; Tan and Soderlund 2009, Soderlund, 2010). Much significance has been attached to thermal modulation of Nav vulnerability. As the physiological temperature approaches 37 C, the actions of pyrethroids on the mammalian central nervous system (Song and Narahashi,1996; Motomura and Narahashi, 2000) diminished or abrogated (see also: Wang et al., 1972; van den Bercken et al., 1977; Cutkomp and Subramanyam, 1986; Salgado et al., 1989). This temperature dependence would seem to preclude pyrethroid actions on mammals whose body temperature hovers near 37 C. Nevertheless, it is well documented that exposure to permethrin formulations causes pain and paresthesia in humans lasting minutes to hours (Kolmodin-Hedman et al., 1982; Tucker and Flannigan, 1983; Flannigan and Tucker, 1985; Gotoh, et al., 1998; see Wolansky and Harrill, 2008). These conflicting pools of evidence are unresolved, but could indicate a broader spectrum of pyrethroid interaction with nervous system proteins contributing to spontaneous activity than previously thought. Accordingly, we examined the influence of temperature on permethrin initiated spontaneous activity and on non-Nav proteins that contribute to spontaneous activity in nociceptors.

Studies were conducted on muscle and vascular nociceptors. Following isolation of type 5 or type 8 nociceptors, the superfusion temperature was increased from 21 to 35.5 C. Action potentials features were tested with the resting membrane potential corrected to -60 mV. After preliminary characterization in an ETOH containing solution, permethrin was applied continuously for 10 minutes while AP features and spontaneous activity was assessed. Additional studies were performed in which permethrin induced spontaneous activity was first established at 21 C before the superfusion temperature was changed to 35.5 C.

When permethrin was applied at physiological temperatures, muscle nociceptors exhibited far less spontaneous activity than was observed at room temperature conditions (figure 16D; van den Bercken et al., 1977; see also Motomura and Narahashi, 2000). Similarly, when spontaneous

activity was initiated at 21 C and superfusion temperatures were subsequently increased to 35.5 C, action potential activity was greatly reduced or ceased altogether. In addition, the impressive permethrin-induced prolongation of action potential duration and abrogation of AHP were mitigated at mammalian physiological temperatures (figure 16A). Although AP duration was greatly reduced at 35.5 C, the permethrin treated neurons retained a significantly longer AP duration than the ETOH treated group. The associated obliteration of the afterhyperpolarization, documented in figure 2, was also largely reversed at 35 C in muscle nociceptors. Interestingly, the AHP of putative, type 8, vascular nociceptors did not recover at physiological temperatures (n=2).

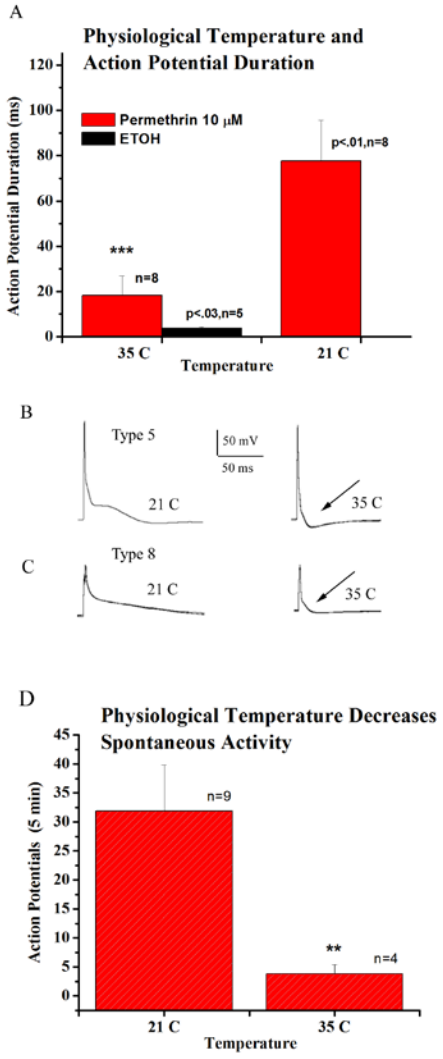

Figure 16. Thermal Modulation of Permethrin Influences on Action Potentials. A) Permethrin-induced increases in action potential duration was diminished at 35.5 C. Action potential duration remained significantly increased relative to ETOH treated neurons (type 5 and type 8 pooled). **B)** Representative action potentials of a type 5 muscle nociceptor at 21 and 35 C. Note the reappearance of an afterhyperpolarization period at 35 C (arrow). Three of 4 afterhyperpolarizations recovered in muscle nociceptors **C)** Representative action potentials of a type 8 nociceptor at 21 and 35 C. Note that the afterhyperpolarization did not recover in the two cases examined (arrow). **D)** Physiological temperatures significantly reduced the spontaneous action potentials evoked by permethrin (5 min observation; vascular nociceptors).

Acute Influence of Permethrin on Kv_7 Mediated Currents

Kv_7 refers to a family of voltage dependent proteins that are activated near the resting membrane potential. The flow of ions through Kv_7 proteins oppose Nav dependent activation while other K^+ channels, such as I_a, influence Nav deactivation. As a result, Kv_7 activation has the unique capacity to prevent the initiation of action potentials. A decline in Kv_7 activity could have a substantial influence on neuronal excitability and spontaneous discharge (Brown and Selyanko, 1985; Wang and McKinnon, 1995; Brown et al, 1997; Marrion, 1997; Robbins, 2001). Portions of the Kv_7 protein family have been indentified in sensory neurons (Passmore et al., 2003; Linley et al., 2008). Although the lethal influence of pyrethroids are manifested via Nav proteins, it is known that pyrethroids can also modulate function of a number of other neuronal ion channel proteins (Ray and Fry, 2006). In order to understand how permethrin initiated spontaneous activity in muscle nociceptors, we examined whether Kv_7 proteins were influenced by the acute application of permethrin.

Task 4: We will assess nociceptors for changes in K_{v7} function that could contribute to chronic and widespread pain.

Skin, muscle and vascular nociceptors were isolated as above. The K-iso solution containing .001% ETOH was applied during presentation of a protocol designed to isolate deactivating Kv_7 currents (see figure 17). Subsequently, ETOH or permethrin was applied for 2 min, followed by the Kv_7 specific inhibitor linopirdine or linopirdine+permethrin. Linopirdine sensitive Kv_7 currents were identified by subtraction. We were unable to identify any influence of permethrin on Kv_7 amplitude or voltage dependence in muscle or vascular nociceptors (figure 17). Type 2 nociceptors did not exhibit any linopirdine sensitive currents and therefore were not subjected to permethrin testing.

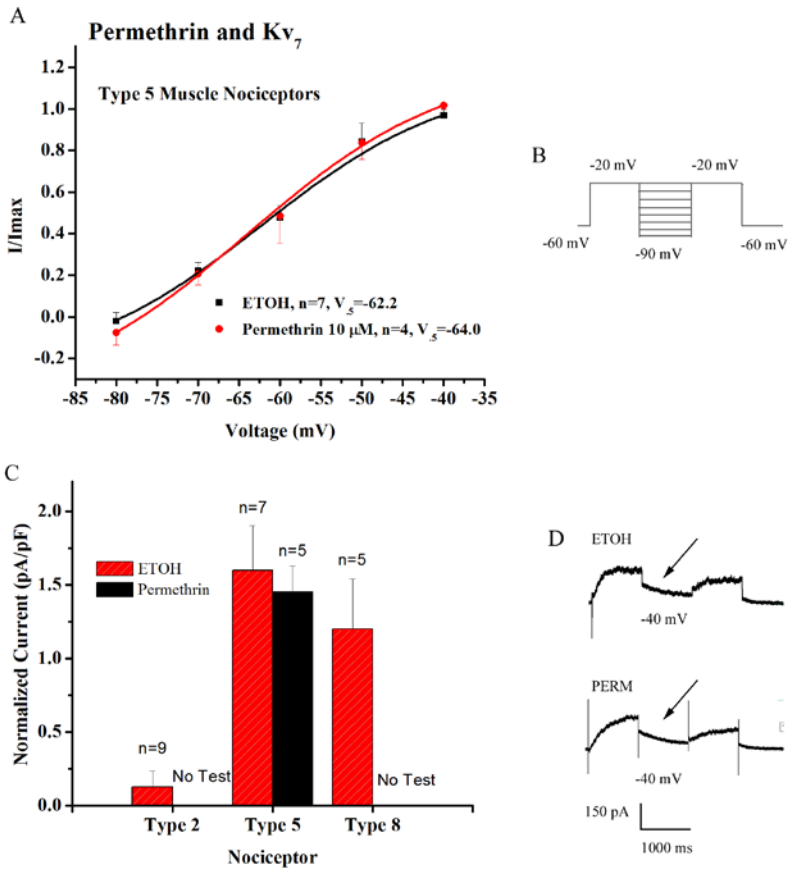

Figure 17. Permethrin did not Modify Currents Passing Through Kv_7 Proteins. A) Voltage dependence of Kv_7 is similar after 4 minute permethrin treatment (type 5 muscle nociceptors). **B)** A schematic representation of the voltage protocol used to evoke Kv_7 currents. Representative traces are shown in 'D'. **C)** Summary data of the averaged peak amplitude of permethrin and ETOH treated Kv_7 proteins expressed in Type 2 (skin), Type 5 (muscle) and type 8 (vascular) nociceptors. Skin nociceptors did not express a linopirdine sensitive current. No permethrin testing was conducted in skin or vascular nociceptors. **D)** Representative traces of linopirdine sensitive current (subtraction currents in the presence of ETOH or permethrin) evoked at -40 mV. The arrows indicate the linopirdine sensitive 'tail' currents.

Key Research Accomplishments:

Chronic Exposure to Permethrin, Chlorpyrifos and Pyridostigmine Bromide:

- Persistent voltage and amplitude changes in Kv_7 protein currents specifically in vascular nociceptors
- Persistent amplitude changes in K_{DR} protein currents specifically in vascular nociceptors
- Persistent increases in membrane resistance specifically in vascular nociceptors
- Muscle nociceptors unaffected ($Nav_{1.8}$, Kv_7, K_{DR})
- Skin, vascular and muscle nociceptor $Na_{v1.8}$ protein inactivation and deactivation unaffected
- Chronic effects of neurotoxicants/PB differ from acute effects of permethrin

Acute effects of permethrin on the pain system:

- Permethrin activates muscle nociceptors but not skin or vascular nociceptors via $Nav_{1.8}$
- Permethrin increases muscle nociceptor excitability, but not the excitability of skin or vascular nociceptors
- Permethrin increases skin, muscle and vascular nociceptor AP duration
- Permethrin accentuates skin, muscle and vascular nociceptor voltage dependent activation of $Na_{v1.8}$ protein (hyperpolarized $V_{.50}$)
- Permethrin retards skin, muscle and vascular nociceptor voltage dependent deactivation of $Na_{v1.8}$ protein
- Permethrin slows the rate (decay) of $Nav_{1.8}$ inactivation of muscle, skin or vascular nociceptors
- Permethrin has no influence on the normalized peak amplitude of skin, muscle or vascular nociceptor $Na_{v1.8}$
- Permethrin has no influence on voltage dependence of skin, muscle or vascular nociceptor inactivation of $Na_{v1.8}$
- Permethrin has no acute influence on Kv_7 amplitude or activation in muscle or vascular nociceptors
- At physiological temperatures (~35.5C) the acute influence of permethrin on spontaneous activity and action potential duration and afterhyperpolarization are greatly reduced but not eliminated.

Reportable Outcomes

Abstracts:

Jiang, N., Nutter T. J. and Cooper, B.Y. The Insecticide Permethrin Activates Muscle but not Skin Nociceptors. Accepted for Presentation at the International Association for the Study of Pain, August, 2012.

Cooper, B.Y., Nutter, T. and Jiang, N. Persistent K^+ channel dysfunction after chronic exposure to insecticides and pyridostigmine bromide. An Abstract submitted for presentation at the Annual Meeting of the Society for Neuroscience, November 2012.

Manuscripts:

Jiang, N., Nutter T. and Cooper, B.Y. Molecular and Cellular Interaction of Permethrin with Deep and Superficial Mammalian Nociceptors. Submitted to the *

Nutter T., Jiang, N., and Cooper, B.Y. Persistent Changes in K^+ Channel Protein Function Following Chronic Exposure to Insecticides and Pyridostigmine Bromide. In preparation*

Jiang, N., Nutter T. and Cooper, B.Y. The Chronic Influence of Insecticides and Pyridostigmine Bromide on $Nav_{1.8}$ Protein Function. In preparation.

Conclusions

We examined whether chronic exposure to combinations of two neurotoxicants (chlorpyrifos and permethrin) with pyridostigmine bromide could produce a delayed neuropathic pain condition in rats; and whether corresponding molecular changes would occur in neurons coding for pain in skin, muscle or vasculature. Following a 30 day exposure to neurotoxicants/PB we observed increased pain behavior only during period that coincided with neurotoxicant dosing. Pain indices faded after dosing ceased. As per the SOW (Task 1), we will extend treatments to 60 days to see if a chronic condition can be achieved.

Despite the absence of lasting behavioral changes, we discovered molecular alterations in the properties of Kv proteins (Kv_7, K_{DR}) that persisted at least 4 weeks after neurotoxicant/PB administration had ended. These changes were limited to a particular subpopulation of Aδ nociceptive neurons we have attributed to the vascular system (Rau et al., 2012). Other neuronal subclasses that innervate skin and muscle were not affected by neurotoxicants/PB. Despite the lack of demonstrable behavioral changes, we regard the observation of persistent changes in specific nociceptor protein properties as a highly significant finding with implications for the chronic pain of GWI.

One reported molecular alteration, a persistent decrease in Kv_7 function during NR1 dosing (Table 1), was consistent with the expectation of increased pain sensitivity from vascular tissues. The lack of a corresponding behavioral pain index (muscle pressure pain) might have been due to the inability of the pressure-pain method to probe for a vascular pain correlate that may have been present. Equally important, while Kv_7 sensitivity to chronic neurotoxicants was replicated during NR2 testing (Table 1), the direction of the effect was reversed. This finding suggests a dynamic interplay between neurotoxicants and molecular adaptations of Kv, and possibly other membrane proteins, that may not have been resolved 4 weeks after dosing. The ultimate balance achieved between Kv_7 and other K^+ and Na^+ channel proteins could prove to be a basis for a chronic pain condition sourced from a vulnerable subset of vascular nociceptors. <u>As described in the SOW, we will examine the status of molecular changes 8-12 weeks after dosing.</u>

Table I

	PB mg/kg, Oral	Permethrin mg/kg, Topical	Chlorpyrifos mg/kg, SC	Exposure days
NR1	13	1.3	60	30
NR2	13	2.6	120	30
NR3	13	2.6	120	60

Permethrin is applied every day

Chlorpyrifos is injected once every 2 weeks

PB is given by gavage every day for 2 weeks every 4 weeks

Some of the molecular changes that were observed, specifically in vascular nociceptors, were inconsistent with a path to chronic pain (increased Kv_7 after NR2; increased K_{DR} after NR1 and NR2). These shifts were documented 4 weeks after dosing and could have been reactive (compensatory) changes to molecular maladaptations that were present during dosing and the associated pain behaviors exhibited at that time. As noted above, the failure to reach an adaptive rebalancing between Na^+ and K^+ function could result in conditions favorable to the development of chronic pain. More importantly, with NR2 dosing, we replicated a specific interaction between the neurotoxicant/PB treatments and a subset of vascular nociceptors. The rules that are governing the dynamic interactions between the neurotoxicants, permethrin and chlorpyrifos, PB and K^+ channel function in these nociceptors are obscure, but the indications of the experiments of NR2 remain consistent with those of NR1 dosing: specific vascular nociceptor K^+ channel proteins are influenced by neurotoxicants 4 weeks after dosing has ceased. We will proceed with a determination of whether the ultimate status of these K^+ channel proteins, and other SOW

designated proteins, are consistent with a chronic pain disorder 8-12 weeks after dosing has ceased (SOW Tasks 2 and 4; see Appendix, p. 45).

In addition to behavioral and molecular studies of chronic neurotoxicant exposure, we also examined the acute affects of permethrin on nociceptor properties (Task 3). Studies of acute permethrin interactions revealed a powerful influence on nociceptor $Nav_{1.8}$ that impacted all classes of nociceptors. The observed increases of AP duration, loss of AHP, development of spontaneous activity, shift in activation $V_{.50}$ of $Nav_{1.8}$, and increased tau_{deact}, all predicted increased pain sensitivity across nociceptor populations. It was also noteworthy that Kv_7 properties were not altered by acute permethrin administration. Many of these findings were consistent with previous observations of permethrin and/or other pyrethroids with mammalian and insect Nav proteins (Narahashi et al., 1998; Narahashi, 2000; Ray and Fry, 2006; Soderlund, 2010); other observations offered new insights into the molecular and cellular targets of pyrethroid insecticides (e.g., Nav tau_{deact}, AHP, muscle nociceptor excitability and spontaneous activity; see Chin and Narahashi, 1986).

For GWI, the most important implications of the acute treatment experiments were the lack of a direct correspondence between the acute impact of permethrin and the chronic/synergistic influences of permethrin, chlorpyrifos and PB. If we contrast acute permethrin findings with outcomes from chronic treatments of NR1 and NR2, we do not find simple parallel outcomes. Instead, the molecular outcomes following chronic exposure were more consistent with a counter reaction of Kv proteins to the Nav targeted influences of permethrin. That is, acute influences on Nav that increase excitability, may have been counterbalanced during chronic treatment, by adaptive increases in Kv function that would bring excitability back toward appropriate levels. The pathways for such adaptive reactions are unknown at this time. The fact that acute effects of permethrin were relatively uniform across tissue specific nociceptor classes, but chronic effects specifically targeted vascular populations was highly significant. It should be considered that the physical location of vascular nociceptors renders them most exposed to concentrations of circulating neurotoxicants/PB as well as to any blood borne secondary influences (endocrine, immune) these agents might induce. As a result, vascular nociceptors could be the first nervous system component damaged by neurotoxicants. The implications of a neurovascular dysfunction could extend beyond that of pain system disorders to other vascular reflex deficiencies that have

been identified in the CNS of Gulf War veterans (Haley et al., 2009; Liu et al., 2010; Li et al., 2011). The molecular basis of these conditions might be similar.

Given the above considerations, we will continue with the SOW prescribed 60 day treatments and delayed molecular studies (4-12 weeks) to determine if lasting behavioral deficits occur and whether molecular changes in vascular nociceptors continue to evolve toward a maladaptive state.

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Appendix

Statement of Work

Task 1: We will examine the development, time course and persistence of a myalgia in rats exposed to combinations of neurotoxicants pyridostigmine bromide, permethrin and chlorpyrifos.

Time Frame: 10 months (4-14)

Young adult male rats (n=144-450) will be systematically treated with 3 neurotoxicants for periods of 30-60 days. On a weekly basis, we will test for pressure myalgia and general malaise (open field activity). Testing will continue for 3 months after termination of neuotoxicant dosing. Up to 450 rats will go through the dosing and behavioral testing that terminates at various stages with harvesting of DRG cells for electrophysiological studies. Only 144 rats are required for the behavioral studies.

Task 2: We will assess nociceptors for changes in Na_v function that could contribute to chronic and widespread pain following treatment with 3 neurotoxicants

Time Frame: 14 months (8-22)

Rats (n=225) are harvested from behavioral studies assessing myalgia following treatment with 3 neurotoxicants. Rats with and without behavioral changes are sacrificed at 1, 2 and 3 months following the 30-60 treatment regimen. For studies involving Na_v proteins, we will examine muscle, vascular and skin nociceptors that express $Na_v1.8$ and $Na_v1.9$. We will harvest DRG containing nociceptor cell bodies of skin, muscle and vascular nociceptors identified in DiI tracing studies and assess these for shifts in the physiology (voltage dependent activation and inactivation; kinetics of evoked and tail currents) of $Na_v1.8$ and $Na_v1.9$. Complementary current clamp studies assess action potential excitability. Delay and chronicity is assessed over a 3 month post-treatment period. Comparisons are made between behavioral tests and the time course of nociceptor malfunction. Acute effects are contrasted with chronic influences.

Task 2: We will assess nociceptors for changes in Na_v function that could contribute to chronic and widespread pain following treatment with 3 neurotoxicants

Task 2a: Assess Na_v Physiology in Muscle Nociceptors

Task 2b: Assess Na_v Physiology in Vascular Nociceptors

Task 2c: Assess Excitability in Muscle Nociceptors

Task 2d: Assess Excitability in Vascular Nociceptors

Task 2e: Assess Excitability in Skin Nociceptors

Task 2f: Assess Na_v Physiology in Skin Nociceptors:

Task 3: We will examine the acute effects of permethrin on Na_v proteins in skin, muscle and vascular nociceptors

Time Frame: 4 months (4-8)

Rats are used as experimental subjects (n=40). Although permethrin has demonstrated acute influence on Na_v in expression systems, and other type 1 pyrethroids are effective in DRG sensory neurons, no one has examined the influence of permethrin on specific nociceptor populations (muscle, skin, vascular). In these neurons the structural phenotype of $Na_v1.8$ may be the same, yet the pathways that regulate excitability and expression may be distinct. Moreover, the effect of permethrin on $Na_v1.9$ has never been examined.

Task 3a: Assess the Acute Influence of Permethrin on Na_v Physiology in Muscle Nociceptors

Task 3b: Assess the Acute Influence of Permethrin on Na_v Physiology in Vascular Nociceptors

Task 3c: Assess the Acute Influence of Permethrin on Na_v Physiology in Skin Nociceptors

Task 4: We will assess nociceptors for changes in K_v7 function that could contribute to chronic and widespread pain.

Time Frame: 16 months (6-22)

Muscle and vascular nociceptors express a K_v7-like current that is sensitive to Oxo-M and linopirdine. We will use optical recording methods to assess spontaneous activity in nociceptors.

We will use whole cell patch methods to identify the type of nociceptor exhibiting spontaneous activity and determine the status of K_v7 in these nociceptors. We will use agents that activate/suppress K_v7 (e.g., retigibine; XE911; linopirdine) and down stream pathways (PIP_2, $IP_3/Ca^{++)}$ to assess the contribution of this voltage sensitive protein to the spontaneous activity. Chronicity is assessed over a 3 month post-treatment period. DRG are harvested from PB and permethrin and chlorpyrifos treated rats (n=225) exhibiting myalgic symptoms as determined in Task 1. Control groups receive vehicle pretreatments or single agents over the same time course.

Task 4a: Assess K_v7 Physiology in Muscle Nociceptors

Task 4b: Assess K_v7 Physiology in Vascular Nociceptors

Task 4c: Assess Spontaneous Activity in Nociceptors

Task 4d: Assess the Nociceptor Phenotype Exhibiting Spontaneous Activity

Task 5: We will assess the capacity of pharmaceutical agents to reverse a neurotoxicant dependent myalgia.

Time Frame: 6 months (18-24)

After identification of an effective dose to produce a persistent myalgia and the molecular dysfunction(s) that parallel this myalgia (Tasks 1, 2 and 4), we will treat groups of rats (n=48) with the specific dosing procedure required. Following manifestation of the delayed behavioral change, we will begin a treatment series using agents targeting those proteins that were identified in Tasks 2 and 4. Behavior scores will be tracked during the treatment period to assess recovery. If rats return to normal levels, treatment dosing will discontinue after 3 weeks. Behavioral studies will continue after treatment to determine if behavior scores remain stable post-treatment

Methods

Acute Studies of Permethrin

Preparation of Cells. Young adult male Sprague-Dawley rats (90-110 g) were anesthetized (Isoflurane) and euthanized by decapitation. The dorsal root ganglia were excised, digested in a solution containing type 1 collagenase and Dispase II. The procedure has been described in detail previously (Petruska et al., 2002). Isolated neurons were plated on 8-10, 35 mm Petri dishes. 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. Electrodes (2-5 MΩ) were formed from boroscilate 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 noted. 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.

Recording and Characterization of Skin, Muscle and Vascular Nociceptors. For electrical recordings, conventional patch techniques were used to achieve the whole cell mode. Whole cell recordings were made with an Axopatch 200B or 700 (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). Borosilicate pipettes were used (Sutter Instruments; uncoated) and pulled to a tip resistance of 2-4 MΩ.

Cells were classified as skin, muscle or vascular nociceptors according to the pattern of voltage activated currents evoked by three protocols (figure A1; Petruska et al., 2002; Rau et al., 2007). All reported data was derived from recordings made on type 2 (C nociceptor), type 5 (C or type IV nociceptor), and type 8 (Aδ or type IV nociceptor). Type 2 and type 5 were identified as skin and muscle nociceptors, respectively in prior tracing studies (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2012). Type 8 nociceptors were traced form all tissue sites (skin, muscle, colon,

mucosa; Rau et al., 2007; Rau et al., 2012) and are presumed to be vascular in origin. Consistent with their vascular designation, type 8 nociceptors co-express SP and CGRP (Petruska et al., 2002; Edvinsson et al., 1990; Jansen et al., 1992). We refer to these as 'putative' vascular nociceptors because we have yet to positively confirm their vascular innervation. It is possible this class innervates other widely spread tissues in addition to, or to the exclusion of, blood vessels (e.g., fascia, nerve sheath). Although the nociceptors we have tested in these studies are important classes of capsaicin (heat) sensitive nociceptors, additional classes of nociceptors can be traced from muscle and skin (Rau et al., 2012).

Classification of nociceptors into 'types' was performed by presentation of a series of depolarizing or hyperpolarizing characterization protocols as described previously in detail (Petruska et al., 2002). The technique is based upon original studies by Scroggs that were subsequently extended to describe more than 17 classes (Cardenas et al., 1995; Rau et al., 2012). Briefly, nociceptors differentially express multiple forms of voltage activated Na^+ (Na_v), hyperpolarization activated (I_H) and 'A-type' K^+ currents that can be distinguished by their voltage dependence, threshold, amplitude and kinetics. The identification of these currents by depolarizing or hyperpolarizing protocols permits classification of neurons into uniform subclasses on electrophysiological criteria alone. Subclassified nociceptors have been shown to exhibit distinct and internally uniform expression of serotonergic, cholinergic, purinergic, opiate, ASIC, TRP and K2p receptors (acid sensing ion channels, transient receptor potential channels, 2 pore potassium channels) as well as neuropeptides (Pertruska et al., 2002; Rau et al., 2005; Jiang et al., 2006; Rau et al., 2012).

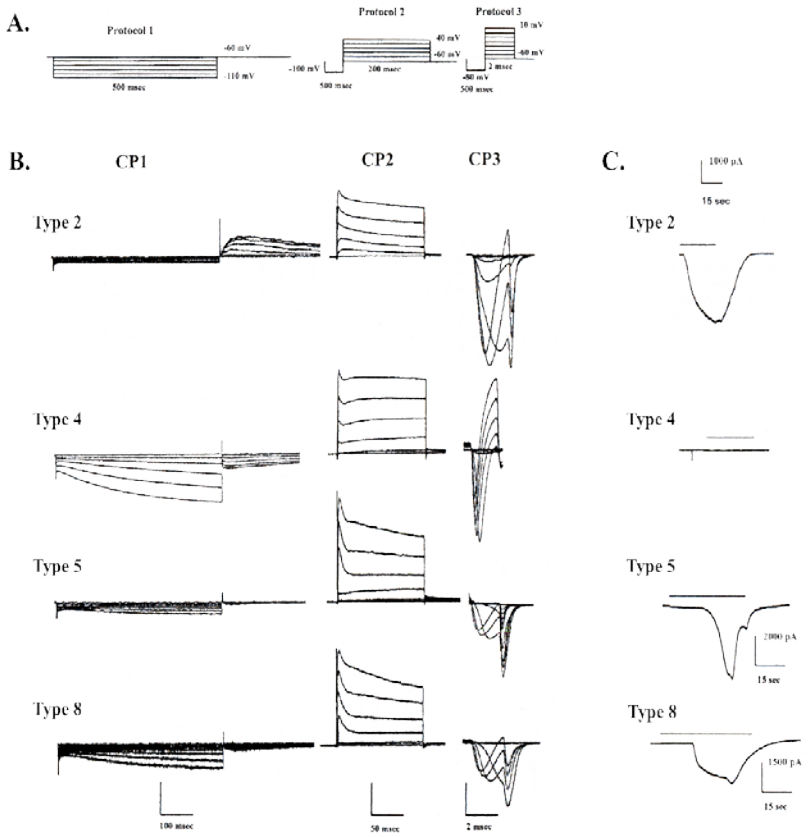

Figure A1. Classification of type 2, 4, 5 and 8 cells by patterns of voltage-activated currents. **A)** Classification protocols 1, 2 and 3 (CP1-3) for cell classification (from Petruska et al., 2000, 2002). **B)** Examples of current signatures for skin (type 2), muscle (type 5) and vascular nociceptors (type 8). Signatures for type 4 (skin nociceptors) are presented for comparison. **C)** Capsaicin (1 μM) response profiles evoked in representative cell types 2, 4, 5 and 8. Vertical scale bars from CP1 represent 500 pA (type 2), 500 pA (type 4), 200 pA (type 5), and 300 pA (type 8). Vertical scale bars from CP2 represent 15,000 pA (type 2), 15,000 pA (type 4), 10,000 pA (type 5), and 10,000 pA (type 8). Vertical scale bars from CP3 represent 5,000 pA (type 2), 10,000 pA (type 4), 15,000 pA (type 5), and 15,000 pA (type 8).

Acute Studies

In the acute studies, we examined how permethrin affected a number of proteins that were expressed in tissue specific nociceptors. This involved isolation a variety of currents in distinct experiments.

Action Potential, Excitability and Spontaneous Activity Experiments. Following classification as a skin, muscle or vascular nociceptor, cells were brought into current clamp mode. The resting membrane potential was identified (<-50 mV excluded from study). A Tyrode solution

containing .001 % ETOH was applied for 2 minutes. Current was injected to bring the cell to -60 mV. Subsequently an action potential was evoked by a brief, high amplitude, current injection (1 ms, 3 nA; 3 replications; 21 C). 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. Subsequently, a Tyrode solution containing 10 μM permethrin (racemic mixture of 26.4% cis and 71.7% trans in ETOH vehicle; Sigma Aldrich) was applied and excitability and individual action potential were assessed for 10 minutes (1 minute test intervals). The resting membrane potential was adjusted to -60 mV for these tests. If spontaneous activity was detected during the test intervals, excitability testing was terminated and spontaneous activity assessments were begun.

After excitability tests were concluded, the resting membrane adjustments were removed and the cell was permitted to find its natural resting potential. The cell was observed for at least 5 minutes in these conditions in which ETOH or permethrin solutions were continuously applied. In distinct groups of cells, these studies were replicated using: 1) only ETOH vehicle; 2) permethrin + tetrodotoxin (TTX, 1 μM); 3) ETOH vehicle at 35.5 C. When permethrin was applied, we used an 'all glass' superfusion system to avoid binding to plastic tubing (Shafer and Hughes, 2010). The application system is described in the attached manuscript (Jiang et al., 2012; Appendix, p. 65).

Action Potential Measures

Action potential duration. 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 fell below the original resting membrane potential.

Action potential excitability. The number of action potentials evoked during all ten stepped depolarization were counted and totaled.

Spontaneous activity. The number of action potentials observed during the 5 minute observation period were counted.

Isolation and Characterization of Kv_7 Protein Currents.

Voltage activated K^+ channels were characterized in a 'K-Iso' solution containing (in mM) 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with KOH. The reversal potential for K^+ was calculated to be -86 mV. The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

A current subtraction method was used to isolate Kv_7 mediated currents from other K^+ currents that were present as tail currents. The Kv_7 voltage deactivation protocol tests were first carried out in the K-Iso solution containing .001% ETOH (pre-applied for 2 min). This was followed by application of the K-Iso solution containing the Kv_7 specific antagonist linopirdine (10 μM in ETOH; 2 min application by close superfusion) followed by the Kv_7 voltage deactivation protocol. The linopirdine sensitive Kv_7 current was isolated by subtraction. The permethrin sensitive Kv_7 current was determined by comparisons between cells receiving the sequence: ETOH-ETOH-Linopirdine and ETOH-Permethrin-Linopirdine. The deactivation protocol was repeated following each 2 minute exposure. For the Kv_7 deactivation protocol: a 1000 ms step command to -20 mV was followed by a series repolarizing 10 mV steps from -10 to -90 mV (1000 ms; V_H = -60 mV). A tail current could be measured during the repolarization step.

The amplitude of the tail current was measured for 1000 ms from a point 10 ms after the repolarizing voltage step to the point 10 ms prior to the return step to -20 mV. The amplitude of linopirdine sensitive Kv_7 current was plotted against the repolarization voltage to obtain a current-voltage relationship.

For contrasts between peak ETOH and permethrin treated currents, we normalized the peak Kv_7 linopirdine sensitive currents for cell capacitance (pA/pf), where the cell capacitance is known to be directly proportional to cell volume. The normalized peak current (from -30 mV to -70 mV) was pooled across voltage and tested for significance using a one way ANOVA. A significant 'F' value was followed by contrasts between normalized amplitude at various deactivation

voltages for ETOH and linopirdine treated groups. T-tests were conducted as follow-up tests. The alpha level was set at .05.

Na^+ Current Isolation and Characterization in Acute Studies.

In studies of deactivation and inactivation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-1' solution containing (in mM) 50 NaCl, 92.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH. As needed, 500 nM TTX was added to isolate $Nav_{1.8}$ from other Nav that were present (Jiang et al., 2011). The pipette solution contained 140 CsF, 10 NaCl, 10 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH. In studies of activation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-2' solution containing (in mM) 30 NaCl, 122.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH.

Acute Studies of $Nav_{1.8}$ Deactivation. Cells were held at -70 mV. An 8 ms step command to 0 mV was used to evoke a maximal Na^+ current. The cell was repolarized progressively in 10 mV steps ranging from -40 to -70 mV. As 8 ms was insufficient for full inactivation, a tail current could be measured during the repolarization step. In ETOH treated cases, tests were carried out in the Nav-Iso solution containing .001% ETOH. In treated cases, the tests were carried out in the Nav-Iso solution containing 10 μM permethrin in .001% ETOH. Tail currents were fit to exponential decay functions. A time constant, tau_{deact}, was obtained by a successful fit to individual skin, muscle and vascular nociceptors decay currents. The mean and standard deviation of the tau_{deact} were determined by pooling from various cell and treatment conditions. Comparisons were made between the tau_{deact} determined at each voltage step. T-tests for independent groups were used for these comparisons.

The tau_{deact} was subsequently plotted against the deactivation voltage for each cell. An exponential growth function of the form:

$$y = Ae^{(x/k)} + y0$$

was fit to the curve formed from the voltage and decay constant data. A constant *'k'* was determined from a successful fit of this function. These *'k'* values were then used to characterize the voltage dependence of deactivation. The *k* values were pooled by groups and tests were

conducted comparing the ETOH and permethrin treated cells in each nociceptor group. A T-test for independent groups to compare the *k* values. A significant effect was considered to have occurred when $p<0.05$.

Acute Studies of $Nav_{1.8}$ Activation

The $Nav_{1.8}$ current was isolated from other Nav by application of the Na-Iso external solution with 500 nM TTX (2 min). In the fluoride based internal solution, the persistent currents ($Nav_{1.9}$) were inactivated at a holding potential of –70 mV. The remaining, slow, TTXr current could be characterized directly. Using a V_h of –70 mV, in the presence of TTX (500 nM), cells were stepped to 20 mV in 5 mV steps (150 ms duration). In distinct cells, evoked currents were measured in either the presence of .001% ETOH or permethrin (10 μM). The P/4 procedure was applied during data collection to adjust for leak current contributions. All characterizations were performed at room temperature with a series resistance correction of 70-80%.

Individual currents were measured at their peak and transformed into a conductance:

$$G=I_{peak}/(V_m-V_{rev}),$$

where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be 27.8 mV for Na-Iso-2. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzman function of the form:

$$G=G_{max}/(1+\exp((V_{.50}-V_m)/K))$$

where $V_{.50}$ is the voltage at which G is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.5}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{.50}$s were then contrasted using two tailed T-Tests. The alpha level was set at .05.

Acute Steady-State Inactivation of $Nav_{1.8}$. The Na-Iso-1 solution containing 500 nM TTX was used to study the voltage dependence of inactivation of $Nav_{1.8}$. Tissue specific nociceptive neurons were held at -70 mV. Starting at an inactivation conditioning voltage of -10 mV, progressive command steps were made to -70 mV in 5 mV steps (150 ms). Each of these

commands steps were followed by a fully depolarizing step to 0 mV (150 ms). The peak current evoked at 0 mV was measured and normalized against the maximum evoked current for that cell (I/I_{MAX}). The normalized current was plotted against the conditioning step voltage and fit to a Boltzman type curve of the form:

$I=I_{max}/(1+\exp((V_{.50}-V_m)/K))$

where $V_{.50}$ is the voltage at which I is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.50}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{.50}$ represents the voltage at which half of the $Nav_{1.8}$ channels are inactivated and K reflects the rate of growth of the relationship. A T-test for independent groups was used to compare the $V_{.50}$ values. A significant effect was considered to have occurred when $p< 0.05$.

Chronic Treatment Studies

In the chronic studies, we examined how permethrin, chlorpyrifos and PB affected a number of proteins that were expressed in tissue specific nociceptors. This involved isolation of a variety of currents in distinct experiments that were similar but not identical to those used in acute studies.

Dosing. Juvenile rats (90-110 g) were used in all studies. Rats were acclimated to the behavioral procedures for 2-5 weeks before dosing began. Animals were treated with neurotoxicants/PB in three regimens (NR1, NR2 and NR3; see Table A1). In NR1 and NR2, neurotoxicants and PB were administered over a 30 days period. During this 30 day period, permethrin (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 (Sigma) was applied by subcutaneous injection (corn oil) once every 14 days. PB was given by oral gavage (water) for 14 consecutive days, beginning at day 1. Animals were sacrificed for electrophysiological experiments 4 weeks after the dosing period had ended. Groups of rats consisting of animals receiving equal volumes of ETOH, corn oil and water over the identical time period served as controls. In NR3, the dosages were the same as NR2 but the treatments continued for 60 days and the delays for molecular tests extended to 8-12 weeks. The NR3 animals have not yet been sacrificed.

Table AI

	PB mg/kg, Oral	Permethrin mg/kg, Topical	Chlorpyrifos mg/kg, SC	Exposure days
NR1	13	1.3	60	30
NR2	13	2.6	120	30
NR3	13	2.6	120	60

Permethrin is applied every day

Chlorpyrifos is injected once every 2 weeks

PB is given by gavage every day for 2 weeks every 4 weeks

Action Potential, Excitability and Spontaneous Activity Experiments. Following classification as a skin, muscle or vascular nociceptor, cells were brought into current clamp mode. The resting membrane potential was identified (<-50 mV excluded from study). Current was injected to bring the cell to -60 mV. Subsequently, an action potential was evoked by a brief, high amplitude, current injection (1 ms, 3 nA; 3 replications; 21 C). 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. After excitability tests were concluded, the resting membrane adjustments were removed and the cell was permitted to find its natural resting potential. The superfusion temperature was then increased to ~35 C (TC_{bip}; Cell Microsytems). Cells were examined for the presence of spontaneous activity over a 3 minute observation period. Measures of action potential duration (APD), action potential afterhyperpolarization duration (AHD), excitability threshold, excitability, resting membrane potential and membrane resistance were made in NR1 and NR2 treated cells.

Action Potential Measures

Action potential duration. 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 resting membrane potential.

Action potential excitability. The number of action potentials evoked during 15 stepped depolarizations were counted and totaled.

Action potential afterhyperpolarization duration. The time in milliseconds from the point in which the repolarizing action potential fell below the previous resting membrane potential until it recovered to within 80% of the previous resting membrane potential.

Spontaneous activity. The number of action potentials observed during a 3 minute observation period were counted.

Isolation and Characterization of Kv_7 Protein Currents in Chronic Treatment Studies.

Voltage activated K^+ channels were characterized in a 'K-Iso' solution containing (in mM) 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with KOH. The reversal potential for K^+ was calculated to be -86 mV. The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

A current subtraction method was used to isolate Kv_7 mediated currents from other K^+ currents that were present as tail currents. Following application of a K-Iso solution containing .001% ETOH (2 min application), the Kv_7 voltage protocol was presented. Subsequently, a K-Iso solution containing the Kv_7 inhibitor, linopirdine (10 μM) was presented for 2 minutes. The Kv_7 voltage protocol was repeated. Linopirdine treated currents were subtracted from the ETOH treated currents to obtain the linopirdine sensitive, Kv_7, current. The total K_{DR} current was also obtained from the linopirdine treated data as the mean of current evoked from 3 consecutive steps to +20 mV. These data were collected 4 weeks after dosing had concluded from rats that

had been neurotoxicant or vehicle treated for 30 days with NR1 or NR2 (Table 1). The voltage protocol was the same as described in acute studies (see also figure 14).

The amplitude of the Kv_7 tail current was measured for 1000 ms from a point 10 ms after the repolarizing voltage step to the point 10 ms prior to the return step to the deactivation step voltage (see figure 14). Subsequently, the amplitude of linopirdine sensitive Kv_7 current was plotted against the repolarization voltage to obtain a current-voltage relationship.

For contrasts between peak ETOH and permethrin treated currents, we normalized the peak Kv_7 linopirdine sensitive currents for cell capacitance (pA/pf) where the cell capacitance is directly proportional to cell area. The normalized tail current (from -30 mV to -70 mV) was pooled across voltage and tested for significance using a one way ANOVA. A significant 'F' valued was followed by contrasts between normalized amplitude at various deactivation voltages for ETOH and linopirdine treated groups. T-tests were used as follow-up tests. The alpha level was set at .05.

<u>Na^+ Current Isolation and Characterization in Chronic Treatment Studies.</u>

For assessment of deactivation and inactivation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-1' solution containing (in mM) 50 NaCl, 92.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH. As needed, 500 nM TTX was added to isolate $Nav_{1.8}$ from other Nav that were present (Jiang et al., 2011). The pipette solution contained 140 CsF, 10 NaCl, 10 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH.

For assessment of activation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-3' solution containing (in mM) 20 NaCl, 122.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH.

<u>Studies of $Nav_{1.8}$ Deactivation following Chronic Neurotoxicants/PB</u>.

For the deactivation test protocol, cells were held at -70 mV. An 8 ms step command to 0 mV was used to evoke a maximal Na^+ current. The cell was repolarized progressively in 10 mV steps

ranging from -40 to -70 mV. As 8 ms was insufficient for full inactivation, a tail current could be measured during the repolarization step. Following characterization of candidate neurons as muscle, skin or vascular nociceptors, the Nav-Iso-1 solution containing 500 nM TTX was applied, by close superfusion, for 2 minutes. This was continued during the presentation of the deactviation test protocol. All characterizations were performed at room temperature with a series resistance correction of 70-80%.

A time constant, tau_{deact}, was obtained by a successful fit to individual skin, muscle and vascular nociceptors decay currents. The mean and standard deviation of the tau_{deact} were determined by pooling from various cell, vehicle and treatment conditions. Comparisons were made between the tau_{deact} determined at each voltage step. T-tests for independent groups were used for these comparisons.

The tau_{deact} was subsequently plotted against the deactivation voltage for each cell. An exponential growth function of the form:

$$y = Ae^{(x/k)} + y0$$

was fit to the curve formed from the voltage and decay constant data. A constant *'k'* was determined from a successful fit of this function. These *'k'* values were then used to characterize the voltage dependence of deactivation. The *k* values were pooled by groups and tests were conducted comparing the neurons from the chronically neurotoxicant and vehicle treated animals in each nociceptor group. A T-test for independent groups to compare the *k* values. A significant effect was considered to have occurred when the probability was less than 0.05.

Studies of $Nav_{1.8}$ Activation following Chronic Treatment with Neurotoxicants

The $Nav_{1.8}$ current was isolated from other Nav by application of the Na-Iso-3 external solution in the presence of 500 nM TTX (2 min). Using a V_h of –70 mV, tissue specific nociceptive neurons were stepped to 20 mV in 5 mV steps (150 ms duration; 'activation protocol'). The P/4 procedure was applied during data collection to adjust for leak current contributions. All characterizations were performed at room temperature with a series resistance correction of 70-80%.

Individual currents were measured at their peak and transformed into a conductance:

$G=I_{peak}/(V_m-V_{rev})$, where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be 16.7 mV for Na-Iso-3. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzman function of the form:

$$G=G_{max}/(1+\exp((V_{.50}-V_m)/K))$$

where $V_{.50}$ is the voltage at which G is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.50}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{.50}$s were then contrasted using two tailed T-Tests. The alpha level was set at .05.

Studies of Steady-State Inactivation of $Nav_{1.8}$. Following Chronic Neurotoxicant Treatments. The Na-Iso-1 solution containing 500 nM TTX was used to study the voltage dependence of inactivation of $Nav_{1.8}$. Tissue specific nociceptive neurons were held at -70 mV. Starting at an inactivation conditioning step voltage of -10 mV, progressive command steps were made to -70 mV in 5 mV steps (150 ms). Each of these commands steps were followed by a fully depolarizing step to 0 mV (150 ms; 'inactivation protocol'). The peak current evoked at 0 mV was measured and normalized against the maximum evoked current for that cell during the protocol (I/I_{MAX}). The normalized current was plotted against the conditioning step voltage and fit to a Boltzman type curve of the form:

$$I=I_{max}/(1+\exp((V_{.50}-V_m)/K))$$

where $V_{.50}$ is the voltage at which I is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.50}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{.50}$ represents the voltage at which half of the $Nav_{1.8}$ channels are inactivated and K reflects the rate of growth of the relationship. A T-test for independent groups was used to compare the $V_{.5}$ values. A significant effect was considered to have occurred when the probability of a type 1 error was less than 0.05.

Manuscripts and Abstracts

An Abstract Submitted to the International Association for the Study of Pain,

August, 2012

Title: The Insecticide Permethrin Activates Muscle but not Skin Nociceptors

Jiang, N., Nutter, T. and Cooper, B.

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Aim of Investigation:

Veterans of the 1991 Gulf War report a variety of serious sensory, cognitive and motor deficits. Widespread joint and muscle pain occurred at far higher incidence (~3:1) in Gulf War veterans than in veterans returning from other theaters. The Research Advisory Committee on Gulf War Veterans Illnesses, (GWI; 2008) concluded that exposure to insecticides was a major contributor to GWI. We examined, for the first time, the interaction of the insecticide permethrin with skin and muscle nociceptors.

Methods:

Young male adult rats (90-150 g) were anesthetized and decapitated. Neurons of the dorsal root ganglia were dispersed and plated on 35 mm Petri dishes. Muscle/vascular and skin nociceptors were categorized using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2000, 2002; Rau et. al., 2007). Excitability was assessed, and whole cell patch clamp records were obtained on selected isolated currents before and after exposure to permethrin or alcohol vehicle (10 uM; $Nav_{1.8}$; Kv_7; Ia). All methods were consistent with the Ethical Guidelines for Pain Research.

Results:

Following exposure to permethrin, action potential (AP) duration was greatly increased in type 2 (skin) and type 5 (muscle), and type 8 (skin muscle and putative vascular) nociceptors (6.96 +/- 0.8 vs. 163.7 +/- 44.2, 8.8 +/- 0.7 vs. 105.9 +/- 12.2, and 6.9 +/- 0.6 vs. 82.7 +/- 12.4 ms for types 2, 5 and 8; $p<.001$, n=4-6). No afterhyperpolarization was present. Type 5 muscle nociceptors developed spontaneous activity following exposure to permethrin (34.4 +/- 12.2 APs/5 min permethrin vs. 0.0 APs/5min ETOH; n=5 and 5; $p<.0001$). Application of TTX did

not prevent the development of spontaneous activity (TTX 1 uM: 51.6 +/- 27.5 APs/5min; n=6). We subsequently examined the influence of permethrin on currents that could contribute to action potential duration or spontaneous activity. Permethrin (10 uM) greatly increased the tau of deactivation (tau_{deact}) of $Nav_{1.8}$ in all three nociceptor types in a voltage dependent manner (tau_{deact} = 11.3 +/-1.4 vs 5.9 +/- 0.47 ms; type 5, $p<.003$; 11.1 +/- 0.5 vs. 5.9 +/- 0.5 ms, type 8, $p<.001$; 12.1 +/- 1.5 vs. 7.5 +/- 0.3 ms, type 2, $p<.005$; n=6 to 9; at -60 mV). We did not observe any permethrin dependent influences on amplitude or current voltage relationships of Kv_7 (n=8 and 5, type 5); however the amplitude of A-current was significantly increased in permethrin treated type 5 nociceptors (184.6 +/- 30.3 pA vs 437.3 +/- 43.0 pA; n=5 at -30 mV; $p<.02$).

Conclusion:

The insecticide permethrin shifts the tau_{deact} = of $Nav_{1.8}$ in both cutaneous and muscle nociceptors in a manner consistent with influences on action potential duration and the potentiation of multiple AP discharges. However, only one class of muscle nociceptors exhibited spontaneous discharge after exposure to permethrin. Additional, cell specific, currents contribute to the development of spontaneous activity in one class of muscle nociceptor.

Acknowledgements:

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An Abstract Submitted to the Society for Neuroscience, November, 2012

Title:

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

Cooper, B.Y., Nutter, T. J. and Jiang, N.

Introduction: A disproportionate number of soldiers that served in the 1991 Gulf War subsequently developed widespread chronic pain that was not associated with trauma (Stimpson et al., 2006; Thomas et al., 2006). Exposure to insecticides and the nerve gas prophylactic pyridostigmine bromide (PB) were identified as risk factors by the Research Advisory Committee on Gulf War Veterans' Illnesses (GWI). We examined whether synergisms between neurotoxicants/PB could lead to behavioral and molecular indices of chronic pain in the rat.

Methods: Over a period of 30 days, male rats were exposed to two neurotoxicant regimens (NR1 and NR2) composed of chlorpyrifos (60 and 120 mg/kg; SC), permethrin (1.3 and 2.6 mg/kg; topical), and PB (13.0 and 13.0 mg/kg; oral) or their respective vehicles (corn oil, ethanol, water). During and after exposure, we assessed muscle pressure pain thresholds and open field activity. Four weeks after exposures, we used whole cell patch electrophysiology to examine the function of tissue specific DRG nociceptor proteins expressed in skin, muscle, and putative vascular nociceptors.

Results: Rest times increased during NR1 and NR2 but subsided when exposure ceased ($p<.001$ and .03; n=23 and 9). Muscle pain thresholds were decreased during, but not after, NR2 treatment only ($p<.04$, n=6 and 12). Four weeks after treatments, K_{DR} and Kv_7 currents were significantly altered by NR1 and NR2 ($p <.04$-.04 and.002-.008; n= 6-10). The voltage dependence of $Nav_{1.8}$ (inactivation, deactivation), spontaneous activity or action potential excitability were unchanged. The significant increases (K_{DR}, NR1 and NR2; Kv_7 in NR2) and decreases (Kv_7 in NR1) in the normalized amplitude of channel currents were only present in putative vascular nociceptors; muscle and skin nociceptors were unaffected.

Conclusions: Putative vascular nociceptors manifested special vulnerability to chronic insecticide exposure. Although various behavioral and molecular changes were not all consistent with a pain condition, the persistent molecular alterations and unique susceptibility of this nociceptor population could be important for the etiology of GWI pain.

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Manuscripts

Molecular and Cellular Interaction of Permethrin with Mammalian Deep and Superficial Nociceptors

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Abstract

Veterans of the 1991 Gulf War frequently developed widespread joint, muscle and other chronic pain. The Research Advisory Committee on Gulf War Veterans Illnesses, (GWI; Binns et al., 2008) concluded that exposure to multiple insecticides was a contributor to GWI. Using whole cell patch clamp techniques, we examined the interaction of the pyrethroid insecticide permethrin on skin, muscle and putative vascular nociceptors of the rat DRG (dorsal root ganglion). Following permethrin application (10 μM), action potential (AP) duration was increased in all nociceptor populations, but only muscle nociceptors developed spontaneous activity or increased excitability (tests at 21 C). TTX (tetrodotoxin) did not prevent the development of spontaneous activity or reduce excitability. We examined the influence of permethrin on TTX resistant proteins that control excitability and spontaneous activity ($Nav_{1.8}$; Kv_7). In all nociceptor populations, permethrin increased the tau of deactivation (tau_{deact}), in a voltage dependent manner, and shifted the $V_{.50}$ for activation over 10 mV. There were no permethrin dependent influences on $Kv_{7,}$ or on the voltage dependence of inactivation of $Nav_{1.8}$. The influence of permethrin on AP duration, spontaneous activity, $V_{.50}$ and tau_{deact} were greatly reduced but not fully reversed when tests were conducted at 35.5 C. In conclusion, permethrin greatly modifies the voltage dependent activation and deactivation of skin, muscle and vascular nociceptors. These influences remain significant at 35.5 C. One population of muscle nociceptors exhibited a unique vulnerability to the acute administration of permethrin manifested as increased excitability and spontaneous activity. The implications for chronic exposure are unclear at this time.

Keywords: Pain, Neurotoxicant, Nociceptor, Gulf War, Deactivation, $Nav_{1.8}$

Introduction

Widespread joint and muscle pain is a common complaint associated with GWI (Gulf War Illness; Stimpson et al., 2006; Thomas et al., 2006). Despite the years that have passed, the etiology of this disorder has remained obscure. 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, GW veterans were exposed to 13 or more pesticides (DOD Environmental Exposure Report: Pesticides, 2003). It is noteworthy that the pyrethroid class of pesticides can have direct interactions with the pain system (permethrin, phenothrin), and as such, their potential role in the development of GW sensory pathology is necessarily magnified.

In humans, topical or oral pyrethroids cause pain and paresthesia (Kolmodin-Hedman et al., 1982; Tucker and Flannigan, 1983; Flannigan and Tucker, 1985; Gotoh, et al., 1998; see Wolansky and Harrill, 2008). Pyrethroids alter the activity of mammalian proteins that are closely related to insect proteins by which they exert their lethal effects (voltage gate sodium channels, VGSC, or Nav). One of these mammalian proteins (i.e., $Nav_{1.8}$) is prominent in nociceptor (pain) coding (Djouhri et al., 2003a,b; Jiang and Cooper, 2011). While stationed in the Gulf region, GW veterans made liberal use of the type 1 pyrethroid insecticide, permethrin.

Early investigations of pyrethroid interactions with mammalian sensory neurons of the DRG (dorsal root ganglion), confirmed powerful influences between several pyrethroids (allethrin, tetramethrin, deltamethrin) and Nav proteins (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996; Tabarean and Narahashi, 1998; Tabarean and Narahashi, 2001). While there have been few studies on the influence of permethrin on neural protein function, the influence of structurally similar pyrethroid neurotoxicants has been studied extensively. In insects, pyrethroids induce or obstruct neural activity via modulation of activation, steady-state inactivation and deactivation of Nav proteins (Narahashi et al., 1998; Narahashi, 2000; Ray and Fry, 2006; Soderlund, 2010). This array of functional interaction has been confirmed in mammalian neurons across the diverse family of Nav (e.g., $Nav_{1.2}$, $Nav_{1.3}$, $Nav_{1.6}$, $Nav_{1.8}$), but the specifics of the modulation differ both qualitatively and quantitatively (Song and Narahashi, 1996a; Song et al., 1996; Smith and Soderlund 1998; Motomura and

Narahashi, 2001; Dekker, et al., 2005; Choi and Soderlund, 2006; Meacham, et al., 2008; Breckenridge et al., 2009; Tan and Soderlund, 2009).

When $Nav_{1.8}$ is expressed in Xenopus, permethrin produces delays in deactivating currents that are typical of other pyrethroids but manifests only minor use dependence reported for others of this class (Choi and Soderlund, 2006). The specific interactions between permethrin and functional nociceptor physiology is not known. There are important functional differences between nociceptors innervating superficial (skin) and deep tissues (e.g., muscle) that could qualitatively determine the consequences of permethrin poisoning (Rau et al., 2012).

As the chronic pain of GWI remains a significant clinical issue, and as the interaction of pyrethroids with the pain system has some general public safety interest, we initiated a series of studies to examine how permethrin interacts with specific skin, muscle and vascular nociceptors that express the pyrethroid sensitive protein $Nav_{1.8}$, Additional studies were conducted to examine whether permethrin might also modify the function of other nociceptor proteins that contribute to excitability and spontaneous activity (Kv_7).

Methods

Preparation of Cells. Young adult male Sprague-Dawley rats (90-110 g) were anesthetized (Isoflurane) and euthanized by decapitation. The dorsal root ganglia were excised, digested in a solution containing type 1 collagenase and Dispase II. The procedure has been described in detail previously (Petruska et al., 2002). Isolated neurons were plated on 8-10, 35 mm Petri dishes. 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. Electrodes were formed from boroscilate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm. 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 noted. 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.

Recording and Characterization of Skin, Muscle and Vascular Nociceptors. Whole cell recordings were made with an Axopatch 200B or Multiclamp 700 (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).

Classification of nociceptors into 'types' was performed by presentation of a series of depolarizing or hyperpolarizing characterization protocols originally developed by Scroggs and extended by Cooper (Cardenas et al., 1995; Petruska et al., 2000; Petruska et al 2002; figure 1). All reported data were derived from recordings made on type 2 (C nociceptor), type 5 (C or type IV nociceptor), and type 8 (Aδ or type IV nociceptor). Type 2 and type 5 were identified as skin and muscle nociceptors, respectively, in tracing studies (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2012). Type 8 nociceptors were traced form all tissue sites (skin, muscle, colon, mucosa; Rau et al., 2007; Rau et al., 2012) and are presumed to be vascular in origin. Consistent with their vascular designation, type 8 nociceptors co-express SP and CGRP (Petruska et al., 2002; Edvinsson et al., 1990; Jansen et al., 1992). We refer to these as 'putative' vascular nociceptors because we have yet to positively confirm their vascular innervation. It is possible this class innervates other widely spread tissues in addition to, or to the exclusion of, blood vessels (e.g., fascia, nerve sheath). Although the nociceptors we have tested in these studies are important classes of capsaicin (heat) sensitive nociceptors, additional classes of nociceptors can be traced from muscle and skin (Rau et al., 2012).

Figure 1 about here*

Permethrin Application. Agents were applied by close superfusion (sewer pipe). An all glass superfusion system was devised 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 ID, 1.5mm OD; 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. At the termination of each experiment, the plastic toggle that was exposed to permethrin was discarded and flexible glass tubing was rinsed with 10 ml of 100% ETOH. To equalize flow rates, the glass application system was used to apply all agents. Stock solutions of permethrin were prepared and maintained in glass bottles. In studies in which heated solutions were used, a feedback controlled TC_{bip} bath controller and HPRE applicator (Cell MicroControls, Norfolk, VA) was used to pre-heat the superfused solution to the desired temperature.

Action Potential, Excitability and Spontaneous Activity Experiments. In current clamp mode, a action potential was evoked by a brief, high amplitude, current injection (1 ms, 3 nA; 3 replications). Excitability was assessed by a series of stepped current injections (0.1 to 1 nA; 250 ms; 10 consecutive steps). Subsequently, a Tyrode solution containing 10 μM permethrin (racemic mixture of 26.4% cis and 71.7% trans in ETOH vehicle; Sigma Aldrich) was applied and excitability and individual action potential characteristics were assessed for 10 minutes (1 minute test intervals). The resting membrane potential was adjusted to -60 mV for these tests. If spontaneous activity was detected during the test intervals, excitability testing was terminated and spontaneous activity assessments were begun.

After excitability tests were concluded, the resting membrane adjustments were removed and the cell was permitted to find its natural resting potential. The cell was observed for at least 5 minutes in these conditions in which ETOH or permethrin solutions were continuously applied.

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 fell below the original resting membrane potential. Action potential excitability, was assessed as the total number of action potentials evoked during ten stepped depolarizations.

Na^+ Current Isolation and Characterization. In studies of deactivation and inactivation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-1' solution containing (in mM) 50 NaCl, 92.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH. As needed, 500 nM TTX was added to isolate $Nav_{1.8}$ from other Nav that were present (Jiang and Cooper, 2011). The pipette solution contained 140 CsF, 10 NaCl, 10 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH. In studies of activation, voltage activated Na^+ channels were characterized in a 'Nav-Iso-2' solution containing (in mM) 30 NaCl, 112.5 TEA-Cl, 4 $MgCl_2$, 0.1 $CaCl_2$, 0.1 $CdCl_2$, 10 glucose and 10 HEPES, adjusted to pH 7.4 with TEA-OH.

$Nav_{1.8}$ Deactivation. Cells were held at -70 mV. An 8 ms step command to 0 mV was used to evoke a maximal Na^+ current. The cell was repolarized progressively in 10 mV steps ranging from -40 to -70 mV. As 8 ms was insufficient for full inactivation, a tail current could be measured during the repolarization step. In vehicle cases, tests were carried out in the Nav-Iso solution containing .001% ETOH. In treated cases, the tests were carried out in the Nav-Iso solution containing 10 μM permethrin in .001% ETOH.). The P/4 procedure was applied during data collection to adjust for leak current contributions. Following experiments, $Nav_{1.8}$ tail currents were fit to exponential decay functions. A time constant, tau_{deact}, was obtained by a successful fit to individual skin, muscle and vascular nociceptors decay currents. The mean and standard deviation of the tau_{deact} were determined by pooling from various cell and treatment conditions. An ANOVA was performed on the Treatment x Voltage data for each tissue nociceptor class. A significant interaction was followed with individual comparisons between the permethrin and ETOH tau_{deact} at each voltage step. The Bonferroni correction procedure was used to protect against type 1 error ($p<.009$).

$Nav_{1.8}$ Activation. The $Nav_{1.8}$ current was isolated from other Nav by application of the Na-Iso external solution with 500 nM TTX (2 min). In the fluoride based internal solution, the persistent currents ($Nav_{1.9}$) were inactivated at a holding potential of –70 mV (Jiang and Cooper,

2011). The remaining, slow, TTXr current could be characterized directly. Using a V_h of –70 mV, in the presence of TTX (500 nM), cells were stepped to 20 mV in 5 mV steps (150 ms duration). In distinct cells, evoked currents were measured in either the presence of .001% ETOH or permethrin (10 μM in ETOH). The P/4 procedure was applied during data collection to adjust for leak current contributions. All characterizations were performed at room temperature. Liquid junction potentials errors were not corrected.

Individual currents were measured at their peak and transformed into a conductance:

$G=I_{peak}/(V_m-V_{rev})$,

where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be 27.8 mV for Na-Iso-2. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzman function of the form:

$G=G_{max}/(1+\exp((V_{.50}-V_m)/K))$

where $V_{.50}$ is the voltage at which G is half maximal, and K is a slope factor.

From the curve fits made to each individual cell, a $V_{.50}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). The $V_{.50}$s were then contrasted using two tailed T-Tests. The alpha level was set at .05.

Steady-State Inactivation of $Nav_{1.8}$. The Na-Iso-1 solution containing 500 nM TTX was used to study the voltage dependence of inactivation of $Nav_{1.8}$. Starting at an inactivation conditioning voltage of -10 mV, progressive command steps were made to -70 mV in 5 mV steps (150 ms). Each of these commands steps were followed by a fully depolarizing step to 0 mV (150 ms). The peak current evoked at 0 mV was measured and normalized against the maximum evoked current for that cell (I/I_{MAX}). The normalized current was plotted against the conditioning step voltage and fit to a Boltzman type curve:

$I=I_{max}/(1+\exp((V_{.50}-V_m)/K))$

From the curve fits made to each individual cell, a $V_{.50}$ was determined and then pooled for the various conditions (ETOH, permethrin, cell type). A T-test for independent groups was used to compare the $V_{.50}$ values. A significant effect was considered to have occurred when $p < 0.05$.

Isolation and Characterization of Kv_7 Protein Currents. Kv_7 mediated tail currents were characterized in a 'K-Iso' solution containing (in mM) 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with KOH. The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

A current subtraction method was used to isolate Kv_7 mediated currents from other K^+ currents that were present as deactivation tail currents. For the Kv_7 deactivation protocol: a 1000 ms step command to -20 mV was followed by a series repolarizing 10 mV steps from -10 to -90 mV (1000 ms; V_H = -60 mV). A tail current could be measured during the repolarization step.

The Kv_7 voltage deactivation protocol tests were first carried out in the K-Iso solution containing .001% ETOH (pre-applied for 2 min). This was followed by application of the K-Iso solution containing the Kv_7 specific antagonist linopirdine (10 μM in ETOH; 2 min application by close superfusion) followed by the Kv_7 voltage deactivation protocol. The linopirdine sensitive Kv_7 current was isolated by subtraction. The permethrin sensitive Kv_7 current was determined by comparisons between cells receiving the sequence: ETOH-ETOH-Linopirdine and ETOH-Permethrin-Linopirdine.

The amplitude of the tail current was measured for 1000 ms from a point 10 ms after the repolarizing voltage step to the point 10 ms prior to the return step to -20 mV. The amplitude of linopirdine sensitive Kv_7 current was plotted against the repolarization voltage to obtain a current-voltage relationship. As described above, a Boltzman function was fit and a $V_{.50}$ determined for each individual cell. A T-test was used to compare the pooled $V_{.50's}$ for ETOH and permethrin treated cells. The normalized amplitude of the linopirdine sensitive current was determined as the measured amplitude (pA) divided by the cell size parameter (pF). The

normalized amplitudes were pooled across deactivation voltages (-30 to -70 mV) to obtain a mean normalized current. T-tests were performed as described above.

Results

Action Potential Features and Excitability

Plated neurons were characterized and identified as skin (type 2), muscle (type 5) or putative, type 8, vascular nociceptors (Rau et al., 2007; Rau et al., 2012). Cells were brought into current clamp mode and the influence of permethrin (10 μM) on nociceptor action potentials was assessed. Consistent with the influence of type II pyrethroid, deltamethrin (Tabarean and Narahashi, 1998), the type I pyrethroid, permethrin, significantly increased action potential duration. Skin, muscle and vascular nociceptors were affected in a similar manner (figure 2). The increase in duration was remarkable and so extensive as to completely obscure the afterhyperpolarization period (6.96 +/- 0.8 vs 163.7 +/- 44.2; 8.8 +/- 0.7 vs. 105.9 +/- 12.2, and 6.9 +/- 0.6 vs. 82.7 +/- 12.4 ms for types 2, 5 and 8; $p<.001$, n=4-6; figure 2).

We assessed whether permethrin-induced changes in AP characteristics would be associated with altered action potential excitability. Current was injected to maintain an RMP of -60 mV. A series of current injections were then performed on ETOH and permethrin treated nociceptors (10 current injection steps of 0.1-1.0 nA; 225 ms/test). Over an observation period of 10 minutes (1 min test intervals), the number of action potentials evoked by a series of current injections were increased in type 5 muscle nociceptors (13.0 +/- 4.5 APs; n=5; figure 3) but not in skin or vascular nociceptors. Often excitability tests on type 5 muscle nociceptors did not last the full 10 min period due to the development of spontaneous activity.

Place figure 2 about here*

With the application of permethrin, all type 5 muscle nociceptors depolarized and exhibited spontaneous discharge (34.4 +/- 12.2 APs/5 min permethrin vs. 0.0 APs/5min ETOH; figure 3). This activity sometimes took the form of high frequency bursts that were interrupted by periods on inactivity; more often, discharges occurred in conventional unitary streams of single APs (see figure 3). Discharges continued even when permethrin application had ceased. In contrast, despite gradual depolarization of all cells in these conditions, neither vascular nor skin nociceptor populations exhibited spontaneous activity following permethrin application (n=16). ETOH vehicle did not produce spontaneous activity in any nociceptor class over equivalent application periods (n=8). We initiated studies to identify the molecular basis of spontaneous activity in muscle nociceptors.

To examine whether TTXs (TTX sensitive) currents were essential to the development of spontaneous activity, we treated a group of type 5 muscle nociceptors to a combination of permethrin and TTX (10 and 1 μM; n=6). Using identical procedures as above, 5 of 6 treated nociceptors depolarized and became spontaneous in the presence of permethrin+TTX (51.6 +/- 27.5 APs/5min; n=6; figure 3d). All TTX treated cases also exhibited increased excitability (figure 3b). Action potential duration was not significantly reduced by co-application of TTX (105.9 +/-12.2 vs. 115.0 +/- 6.1 ms with TTX (not shown). We can conclude from these studies that TTXr (TTXresistant) Nav proteins, such as $Nav_{1.8}$, were sufficient to maintain permethrin influences on AP duration, excitability and spontaneous activity of muscle nociceptors.

Place figure 3 about here*

Voltage Dependent Activation, Inactivation and Deactivation of $Nav_{1.8}$

Muscle nociceptors, as well as many other nociceptor types, express the pyrethroid sensitive TTXr $Nav_{1.8}$ protein (Djouhri et al., 2003a,b; Amir et al., 2006; Jiang and Cooper, 2011). Accordingly, we applied protocols to examine the activation, inactivation and deactivation of $Nav_{1.8}$ in the presence of permethrin.

The tendency to form action potentials (excitability) is determined by the threshold of Nav protein activation. We examined whether the activation of $Nav_{1.8}$ was influenced by the acute application of permethrin (10 μM). Skin, muscle and vascular nociceptors were identified in the usual manner. A Na-Iso-2 solution was used to separate Na^+ currents from other voltage sensitive membrane currents. Permethrin was applied for 2 minutes. Subsequently, a $V_{.50}$ was derived from Boltzman functions fit to the normalized voltage-conductance (G) plots. Contrasts were then made between the $V_{.50}$ of ETOH/ETOH treated cases and ETOH/Permethrin treated cases. Consistent with increased excitability of muscle nociceptors, permethrin shifted the midpoint of excitation ($V_{.50}$) more than 10 mV in the hyperpolarizing direction (figure 4b). Although excitability changes were limited to muscle nociceptors, large hyperpolarizing shifts in $V_{.50}$ were observed in skin and vascular nociceptors as well. No changes in normalized peak amplitude were observed in any nociceptor class (0 mV test; figure 5).

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Place figure 5 about here

The prolongation of action potential duration (figure 2) could have been due to poor deactivation of $Nav_{1.8}$. We directly assessed the influence of permethrin on deactivation of nociceptor $Nav_{1.8}$. Deactivation time constants (tau_{deact}) were determined by fits of exponential decay functions to Na^+ currents that were terminated 8 msec after a strong depolarizing voltage step (0 mV). Other type I pyrethroids greatly increase deactivation tail currents of Nav proteins (Soderlund, 2010). Permethrin treated skin, muscle and vascular nociceptors exhibited significant increases in the deactivation time constants consistent with a prolongation of action potential duration (e.g., tau_{deact} = 11.3 +/-1.4 vs 5.9 +/- 0.47 ms at -60 mV; type 5 muscle, $p<.01$). In addition, permethrin induced strongly voltage dependence on the deactivation tau that reached a maximum change near the holding potential (i.e., -40 mV test, figure 6). Comparison of time constants between ETOH and permethrin treated skin, muscle and vascular nociceptors were highly significant at virtually every deactivation voltage.

Insert figure 6 about here

Additional tests were performed to assess whether a neurotoxicant would alter the steady state, fast, inactivation of nociceptor $Nav_{1.8}$. A $V_{.50}$ for inactivation was determined from Boltzman function fits to $Nav_{1.8}$ currents that were released from inactivation following stepped reductions in conditioning voltages. Muscle, skin and putative vascular nociceptors were exposed to either ETOH or permethrin. Two minutes following application, we were unable to demonstrate any influence on the voltage dependence ($V_{.50}$); however, time constants fit to the decay phase of the evoked current (tau_{inact}) were profoundly increased by permethrin. As with activation and deactivation, there were no differences between the tau_{inact} determined in superficial and deep nociceptors (figure 7).

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Humans are highly resistant to pyrethroid poisoning. As the temperature approaches 37 C, the actions of pyrethroids on the mammalian central nervous system are diminished or abrogated (Song and Narahashi, 1996c). Nevertheless, it is well documented that exposure to permethrin formulations causes pain and paresthesia in humans lasting minutes to hours (Soderlund et al., 2002; Ray and Fry, 2006). These conflicting pools of evidence are unresolved, but could indicate a broader spectrum of pyrethroid interaction with nervous system proteins contributing to spontaneous activity than previously thought. Accordingly, we examined the influence of temperature on permethrin initiated spontaneous activity and on non-Nav proteins that contribute to spontaneous activity in nociceptors.

Studies were conducted mainly on muscle nociceptors as they were the only nociceptor population to exhibit spontaneous activity in our investigations. Following isolation of type 5 or type 8 nociceptors, the superfusion temperature was increased from 21 to 35.5 C. Action

potentials features were tested with the resting membrane potential corrected to -60 mV and cells were observed for the development of spontaneous activity without adjustment of RMP. After preliminary characterization in Tyrode's solution containing ETOH (.001%), permethrin was applied continuously for 10 minutes while AP features were assessed at 1 minute intervals. Nociceptors were then observed for 5 minutes for the development of spontaneous activity. Additional studies were performed in which permethrin induced spontaneous activity was first established at 21 C before the superfusion temperature was changed to 35.5 C.

When permethrin was applied at physiological temperatures, muscle nociceptors exhibited far less spontaneous activity than was observed at room temperature conditions (figure 8D; van den Bercken et al., 1977; see also Motomura and Narahashi, 2000). Similarly, when spontaneous activity was initiated at 21 C and superfusion temperatures were subsequently increased to 35.5 C, action potential activity was greatly reduced or ceased altogether. In addition, the impressive permethrin-induced prolongation of action potential duration and abrogation of AHP were mitigated at mammalian physiological temperatures (figure 8A). Although AP duration was greatly reduced at 35.5 C, the permethrin treated neurons retained a significantly longer AP duration than the ETOH treated group. The associated obliteration of the afterhyperpolarization, documented in figure 2, was also largely reversed at 35 C in muscle nociceptors. Interestingly, the AHP of putative, type 8, vascular nociceptors did not recover at physiological temperatures (n=2).

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Influence of Permethrin on Kv_7 Mediated Currents

Although the lethal influence of pyrethroids are manifested via Nav proteins, it is known that pyrethroids can also modulate function of other neuronal proteins (Ray and Fry, 2006). Activation of Kv_7 proteins opposes the initiation of action potentials. A decline in Kv_7 activity could have a substantial influence on neuronal excitability and spontaneous discharge (Marrion, 1997; Robbins, 2001). In order to understand how permethrin initiated spontaneous activity in

muscle nociceptors, we examined whether Kv_7 proteins were influenced by the acute application of permethrin.

Skin, muscle and vascular nociceptors were isolated as above. The K-iso solution containing .001% ETOH was applied during presentation of a protocol designed to isolate deactivating Kv_7 currents (see figure 9). Subsequently, ETOH or permethrin was applied for 2 min, followed by the Kv_7 specific inhibitor linopirdine or linopirdine+permethrin. Linopirdine sensitive Kv_7 currents were identified by subtraction. We were unable to identify any influence of permethrin on Kv_7 amplitude or voltage dependence in muscle or vascular nociceptors (figure 9). Type 2 skin nociceptors did not exhibit any linopirdine sensitive currents and therefore were not subjected to permethrin testing.

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Discussion

Following the 1991 Persian Gulf War, many returning veterans developed chronic, widespread joint and muscle pains that were not related to any known physical injury sustained during the conflict (Stimpson et al., 2006; Thomas et al., 2006). The events leading to the development of GWI pain are still obscure, but an extensive review has concluded that the use of multiple insecticides/repellants (neurotoxicants) co-varied with the occurrence of symptoms (Binns et al., 2008; Steele et al., 2011). While stationed in the Gulf region, warfighters made ample use of the pyrethroid insecticides permethrin and allethrin. A better understanding of the interaction of pyrethroids, and other insecticides, with the mammalian nervous system may lead to a fuller understanding of the etiology of GWI and the development o f improved strategies to treat these multisymptom conditions.

Pyrethroid agents are a natural starting point for investigations into the etiology of GWI pain syndromes. Given the known interactions of pyrethroids with Nav proteins, it is not surprising that they could cause acute pain and paresthesia. In humans, 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). At the least, these acute sensory disturbances indicate that a simple thermal mitigation of pyrethroid-Nav effects may not be as straightforward as generally assumed.

The influence of type I and type II pyrethroids (allethrin, tetramethrin, deltamethrin) on TTXs and TTXr, Nav proteins of the DRG suggest a relatively direct pathway to nociceptor activation and pain (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Song et al., 1996a,b; Tabarean and Narahashi, 1998; Tabarean and Narahashi 2001). Our studies represent the first systematic attempt to determine how the type I pyrethroid, permethrin, interacted with identified nociceptor subpopulations that innervate superficial or deep tissues. We described a wide range of potent interactions, including several cellular and molecular effects which were uniformly manifested in both superficial and deep nociceptors. These included: 1) A TTXr dependent increase in AP duration; 2) Elimination of the AHP phase of the AP; 3) A >10 mV hyperpolarization of the $V_{.50}$ for $Nav_{1.8}$ activation; 4) A two fold increase in the tau_{deact} of $Nav_{1.8}$; and 5) A nearly 3 fold increase in the tau_{inact} of $Nav_{1.8.}$ Both the range and degree of these interactions were impressive and certainly much greater than would accompany exposure to an endogenous pro-inflammatory mediator (e.g., England et al., 1996; Gold et al., 1996; Gold et al., 1998).

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The remarkable, 10 fold, prolongation of the action potential duration appeared in both superficial and deep tissue nociceptors, and at the molecular level, reflected substantial shifts in the time constants of inactivation and deactivation of $Nav_{1.8}$. The dramatic amplification of Nav deactivation tail currents is the most widely and consistently reported influence of pyrethroids on neuronal Nav protein in insects or mammals (Soderlund, 2010). The slowing of deactivation leads to massive tail current rebounds that can initiate action potential discharge. This alone has the potential to create spontaneous activity. Bursts matching this description of events could be seen directly in the spontaneous discharge records of muscle nociceptors (figure 3), but many APs were generated that appeared to be independent of tail current regeneration. The tendency for spontaneous discharge was no doubt promoted by a considerable shift in the voltage dependent activation of nociceptor $Nav_{1.8}$ manifested as a 10 mV, or greater, hyperpolarizing

shift in the activation $V_{.50}$. Changes in voltage dependent activation have been reported to occur with several, but not all pyrethroids (Ginsburg and Narahashi, 1993) in various mammalian preparations (Song and Narahashi, 1996; Tabarean and Narahashi, 1998; Tabarean and Narahashi 2001).

Permethrin produces depolarization and spontaneous activity in mammalian neurons in various species and preparations (Carlton 1977; Parkin and Le Quesne 1982, Takahashi and Le Quesne 1982; Staatz-Benson & Hosko, 1986; Meyer et al., 2008). Yet it is important to note, that while shifts in deactivation and activation were present across representative superficial and deep nociceptors, only type 5 muscle nociceptors depolarized and produced spontaneous discharge; and only type 5 nociceptors exhibited increased excitability in the presence of permethrin. This functional distinction might be due to the influence of permethrin on channel proteins, other than $Nav_{1.8}$, that are expressed in type 5 nociceptors, but absent in skin and vascular nociceptors. Pyrethroids do influence other neuronal proteins, including inhibition of voltage gated Ca^{++} and the Na^{+}/K^{+} ATPase (Hagiwara et al., 1988; Kakko et al., 2003; Hildebrand et al., 2004; Shafer and Mayer, 2004; Ray and Fry, 2006). Given the general distribution of these proteins, it is not clear how their inhibition could account for the unique influence of permethrin on type 5 muscle nociceptors.

With this in mind, we specifically probed the involvement of another family of proteins, Kv_7 (KCNQ), that are known to be important in the generation of spontaneous activity (Marrion, 1997; Robbins, 2001), and that exhibit a selective distribution in DRG (Passmore et al., 2003; Linely et al., 2008). Consistent with this hypothesis, we could not identify Kv_7 mediated currents in type 2 skin nociceptors that failed to exhibit any spontaneous discharge. However both type 8 and type 5 neurons did express a linopirdine sensitive Kv_7 current, yet only type 5 neurons became spontaneous. In any case, we were unable to detect any direct effects of permethrin on voltage dependent Kv_7 currents in muscle nociceptors that could have contribute to spontaneous activity or excitability. It is possible that those factors that render muscle nociceptors uniquely vulnerable to permethrin are more quantitative than qualitative. Perhaps the balance between ionic fluxes that initiate ($Nav_{1.7}$, $Nav_{1.8}$) or oppose action potential formation (Kv_7, K_{ca}, K_A) are more important than the presence and permethrin vulnerability of any given ion channel protein.

Given the often reported presence of chronic muscle pain in GWV (Unwin et al., 1999; Gray et al., 1999; Steele et al., 2000; Kang et al., 2000; Gray et al., 2002), the special vulnerability of type 5 muscle nociceptors to acute permethrin was an intriguing finding. In contrast, cutaneous pain and hyperesthesia is a rarely reported symptom of GWI (Dunphy et al., 2003). The existence of a special vulnerability of deep tissue nociceptors to chronic pyrethroid exposure has implications for the development and maintenance of GWI pain. Certainly, the capacity of a nociceptor to transition into a spontaneously active mode following exposure to a neurotoxicant, and to maintain that activation after the application has ceased, could be the basis for tonic pain of either a peripheral or central origin. The peptidergic nature of these muscle afferents also comports with notions of amplification of nociceptive signaling at a spinal level following release of SP (substance P; Willis et al., 1996; Willis et al., 2001; Petruska et al., 2002). Many believe these spinal mechanisms are crucial in the development and maintenance of chronic pain of many varieties (Willis, 2002).

Whether secondary to trauma, disease or neurotoxicants, it is not clear why pain related neuronal activity would continue, either peripherally or centrally, for years after a critical exposure/event incident. Within 90 minutes after application, permethrin accumulates within cells, *in vitro*, to levels 50 fold higher than that found in surrounding media (Shafer and Hughes, 2010). The intracellular accumulation of permethrin opens the door to the possibility of residual activity and the amplification of any concentration dependent effects during chronic exposure. Despite this, there is no specific basis to tie chronic permethrin exposure to a chronic pain condition following a prolonged topical contact with pyrethroids (Appel et al., 2008; Rossbach et al., 2009). Recent reports that permethrin can instigate DNA damage (striatum) in mammalian CNS neurons or peripheral muscle suggests a mechanism for chronic molecular derangements, but not necessarily those that would create spontaneous nociceptor activity. The specific functional consequences of this damage and how it would impact on pain perception remains unclear (Vadhana et al., 2010; Falcioni, et al., 2011; see also Imamura et al., 2000).

Humans are highly resistant to pyrethroid poisoning. A number of factors (absorption, elimination, body temperature, innate sensitivity) contribute to the lower lethality of pyrethroids in humans (Ray and Frey, 2006; Narahashi et al., 2007). At ~20 C, pyrethroids exert profound effects on Nav proteins that are fatal to insects; but at mammalian physiological temperatures,

some of these influences are abrogated (Song and Narahashi, 1996c; see also Motomura and Narahashi, 2000). The inability to account for pain and paresthesia of human exposure to pyrethroids makes it clear that key elements of pyrethroid interactions with the mammalian nervous system are not fully understood. In rat nociceptors, we were able to demonstrate the reduction of a substantial portion of permethrin effects on nociceptor AP duration, AHP and spontaneous activity; however, physiological temperatures did not fully reverse either the AP duration (type 5), AHP suppression (type 8 cells) or spontaneous activity of muscle nociceptors induced by permethrin. Regardless of temperature, the range and type of functional deficits might differ substantially in chronic exposure conditions, or when coupled with exposure to additional agents that increase the functional concentration or otherwise synergize with permethrin to affect multiple nociceptor proteins.

In conclusion, the type I pyrethroid permethrin exerts profound effects on nociceptors via its interaction with $Nav_{1.8}$. While the influence is mainly uniform across nociceptor populations, permethrin especially impacted functional activity in one population of muscle nociceptors. Although effects were greatly diminished at mammalian physiological temperatures, a portion of permethrin induced alterations were retained at 35.5 C. It is uncertain whether these residual effects are important factors contributing to the development of chronic widespread pain of GWI. Chronic exposure studies are currently under way.

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Figure Captions

Figure 1. Classification of type 2, 4, 5 and 8 cells by patterns of voltage-activated currents. **A)** Classification protocols 1, 2 and 3 (CP1-3) for cell classification (from Petruska et al., 2000, 2002). **B)** Examples of current signatures for skin (type 2), muscle (type 5) and vascular nociceptors (type 8). Signatures for type 4 (skin nociceptors) are presented for comparison. **C)** Capsaicin (1 μM) response profiles evoked in representative cell types 2, 4, 5 and 8. Vertical scale bars from CP1 represent 500 pA (type 2), 500 pA (type 4), 200 pA (type 5), and 300 pA (type 8). Vertical scale bars from CP2 represent 15,000 pA (type 2), 15,000 pA (type 4), 10,000 pA (type 5), and 10,000 pA (type 8). Vertical scale bars from CP3 represent 5,000 pA (type 2), 10,000 pA (type 4), 15,000 pA (type 5), and 15,000 pA (type 8).

Figure 2. Permethrin Increases AP Duration in Nociceptors. A-C) Action potentials in tissue specific nociceptors following exposure to ETOH vehicle. The arrow highlights the afterhyperpolarization. **D-F)** Action potentials in the same nociceptors following exposure to 10 μM permethrin for 6-10 minutes. After-hyperpolarizations are absent in all treated neurons. **G)** Summary data for 3 nociceptive classes. Duration increases were highly significant. Action potentials were evoked by a 3 nA injection for 1 ms.

Figure 3. Permethrin Increases Excitability and Induces Spontaneous Activity in Muscle Nociceptors. A) Excitability testing in a type 5 muscle nociceptor. Ten consecutive current injection tests in an ETOH (upper panel) and Permethrin treated case (lower panel; 10 μM, 10 min). Vertical green bars separate consecutive tests. Substantial increases in action potential discharge were evident. **B)** Summary data for excitability testing indicated that only type 5 muscle nociceptors manifested excitability shifts. TTX did not block shifts in AP excitability (** $p<.03$ vs ETOH). **C)** Representative spontaneous activity in a type 5 muscle nociceptor after exposure to ETOH or permethrin (10 μM). Five minutes of observation are shown. Exposure to ETOH for 5 minutes (upper panel) did not result in spontaneous activity. **D)** Summary data of

the influence of permethrin on spontaneous activity. Only type 5 muscle nociceptors exhibited spontaneous activity. TTX did not impair the development of spontaneous activity. * p<.03 vs ETOH (Mann-Whitney U test; ties were not corrected). *** p<.001

Figure 4. Permethrin Shifted the Voltage Dependent Activation of $Nav_{1.8}$. Depolarizing shifts in the activation $V_{.50}$ suggested greater excitability following permethrin exposure (10 μM): **A)** Skin nociceptors; **B)** Muscle nociceptors; Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post permethrin (0 mV activation step); **C)** Vascular nociceptors. Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post permethrin (0 mV activation step). Scale bars in 'B' apply. Control vehicle tests (ETOH-1 first exposure; ETOH-2 second exposure) did not produce any significant shifts of $V_{.50}$ (.001% ETOH; different cells from A, B, C): **D)** Skin nociceptors; **E)** Muscle nociceptors; **F)** Vascular nociceptors. Comparisons between the first ETOH-1 and 'Vehicle' tests (.001% ETOH) were non-significant.

Figure 5. Acute Permethrin did not Increase the Amplitude of $Nav_{1.8}$. At the test voltage of 0 mV, the normalized peak amplitude of $Nav_{1.8}$ in tissue specific nociceptors remained relatively stable compared to ETOH treated controls.

Figure 6. Permethrin Shifts Voltage Dependence of Deactivation in All Tissue Specific Nociceptors. A-C) ETOH exposed cells exhibit a relatively consistent pattern of deactivation across membrane voltages (black curves). Following treatment with permethrin, deactivation time constants were increased: (skin: F=22.1, DF=1.9; muscle: F=10.2, DF=1,11; vascular: F=28.4, DF=1,14) and a strong voltage dependence appeared (red curves). The interaction between voltage and time constants were highly significant for all tissue nociceptors (skin: F=6.04, p<.0001; muscle: F=3.49, p<.005; vascular: F=5.49, p<.0001). There were no statistical differences between tissue specific nociceptors. **D-F)** Representative traces illustrate the deactivation tail current associated with permethrin and ETOH treated neurons. All traces taken

from a single deactivation voltage step to -40 mV. The arrow signifies the point at which the voltage is abruptly clamped at -40 mV. **G)** A schematic of the deactivation protocol. The voltage step to 0 mV was 8 ms long. The deactivation voltage steps were 150 ms. 10 μMm permethrin applied for 2 minutes.

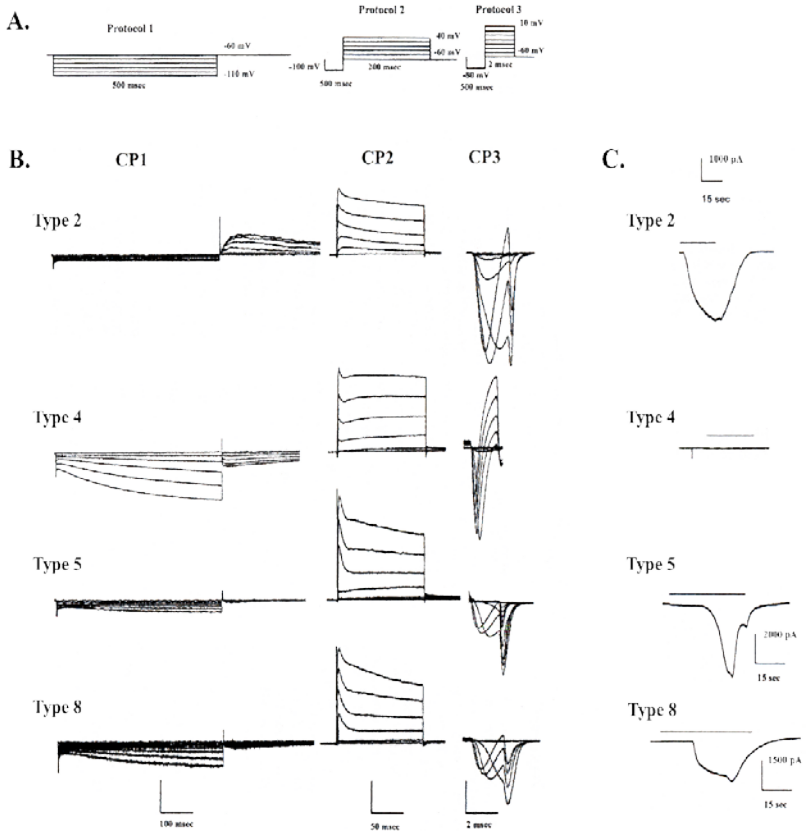
Figure 7. Permethrin and Fast Inactivation of $Nav_{1.8}$ A-C) Voltage dependent inactivation of $Nav_{1.8}$ was not significantly modified by application of 10 μM permethrin (2 minutes); The tau_{inact} (mS) was significantly increased in all cases. **D)** A schematic of the inactivation protocol. Inserts show representative traces (upper: ETOH; lower: Permethrin). Note the slowing of inactivation after permethrin. The cells were pre-pulsed in 5 mV steps from -10 to -80 mV. The test pulse was 0 mV, 150 ms.

Figure 8. Thermal Modulation of Permethrin Influences on Action Potentials. A) Permethrin-induced increases in action potential duration was diminished at 35.5 C. Action potential duration remained significantly increased relative to ETOH treated neurons (type 5 and type 8 pooled). **B)** Representative action potentials of a type 5 muscle nociceptor at 21 and 35 C. Note the reappearance of an afterhyperpolarization period at 35 C (arrow). Three of 4 afterhyperpolarizations recovered in muscle nociceptors **C)** Representative action potentials of a type 8 nociceptor at 21 and 35 C. Note that the afterhyperpolarization did not recover in the two cases examined (arrow). **D)** Physiological temperatures significantly reduced the spontaneous action potentials evoked by permethrin (5 min observation; vascular nociceptors).

Figure 9. Permethrin did not Modify Currents Passing Through Kv_7 Proteins. A) Voltage dependence of Kv_7 is similar after 4 minute permethrin treatment (type 5 muscle nociceptors). **B)** A schematic representation of the voltage protocol used to evoke Kv_7 currents. Representative traces are shown in 'D'. **C)** Summary data of the averaged peak amplitude of permethrin and ETOH treated Kv_7 proteins expressed in Type 2 (skin), Type 5 (muscle) and type 8 (vascular) nociceptors. Skin nociceptors did not express a linopirdine sensitive current. No

permethrin testing was conducted on vascular nociceptor Kv_7. **D)** Representative traces of linopirdine sensitive current (subtraction currents in the presence of ETOH or permethrin) evoked at -40 mV. The arrows indicate the linopirdine sensitive 'tail' currents.

Figures



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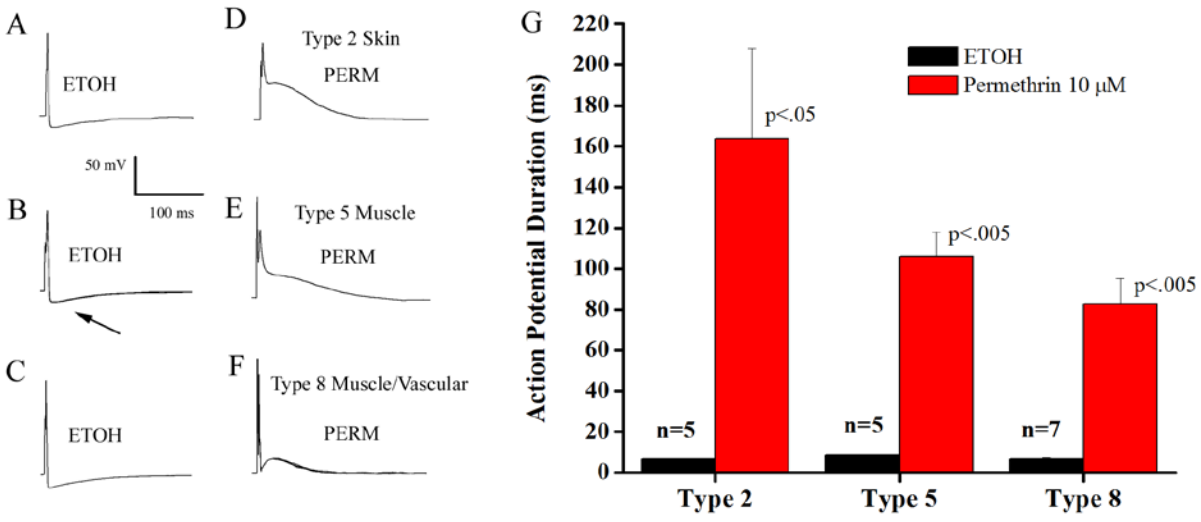

Figure 2. Permethrin Increases AP Duration in Nociceptors. A-C) Action potentials in tissue specific nociceptors following exposure to ETOH vehicle. The arrow highlights the afterhyperpolarization. **D-F)** Action potentials in the same nociceptors following exposure to 10 μM permethrin for 6-10 minutes. After-hyperpolarizations are absent in all treated neurons. **G)** Summary data for 3 nociceptive classes. Duration increases were highly significant. Action potentials were evoked by a 3 nA injection for 1 ms.

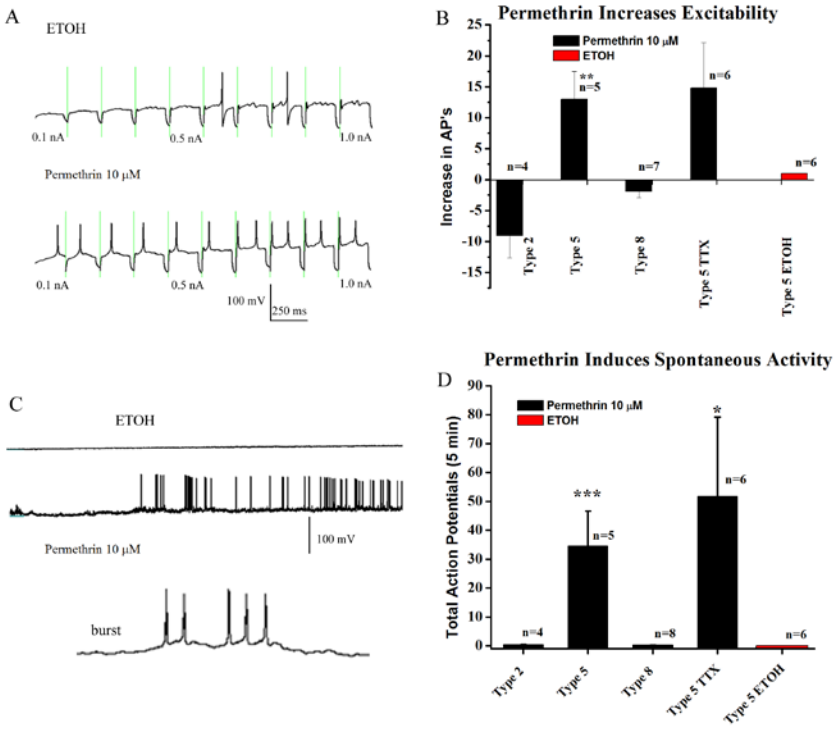

Figure 3. Permethrin Increases Excitability and Induces Spontaneous Activity in Muscle Nociceptors. A) Excitability testing in a type 5 muscle nociceptor. Ten consecutive current injection tests in an ETOH (upper panel) and Permethrin treated case (lower panel; 10 μM, 10 min). Vertical green bars separate consecutive tests. Substantial increases in action potential discharge were evident. **B)** Summary data for excitability testing indicated that only type 5 muscle nociceptors manifested excitability shifts. TTX did not block shifts in AP excitability (** p<.03 vs ETOH). **C)** Representative spontaneous activity in type 5 muscle nociceptors after exposure to ETOH (upper) or permethrin (middle trace; 10 μM). Five minutes of observation are shown. A burst of APs from a permethrin treated case expanded for display. **D)** Summary data of the influence of permethrin on spontaneous activity. Only type 5 muscle nociceptors exhibited spontaneous activity. TTX did not impair the development of spontaneous activity. * p<.03 vs ETOH (Mann-Whitney U test; ties were not corrected). *** p<.001

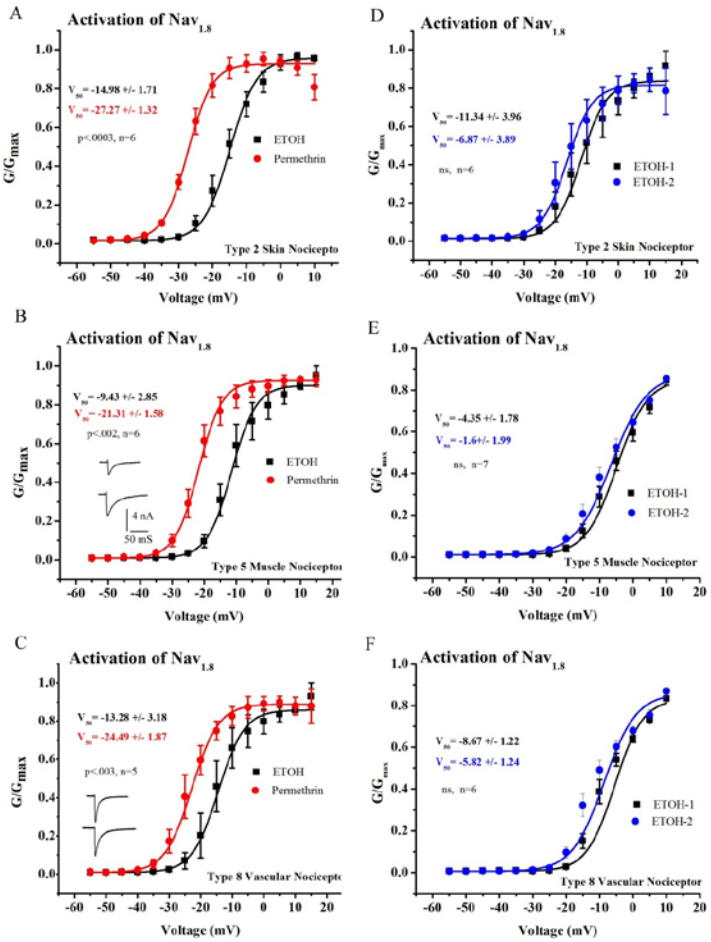

Figure 4. Permethrin Shifted the Voltage Dependent Activation of $Nav_{1.8}$. Depolarizing shifts in the activation $V_{.50}$ suggested greater excitability following permethrin exposure (10 μM): **A)** Skin nociceptors; **B)** Muscle nociceptors; Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post permethrin (0 mV activation step); **C)** Vascular nociceptors. Representative traces are presented as inserts. Upper trace: pre-permethrin; lower trace: post permethrin (0 mV activation step). Scale bars in 'B' apply. Control vehicle tests (ETOH-1 first exposure; ETOH-2 second exposure) did not produce any significant shifts of $V_{.50}$ (.001% ETOH; different cells from A, B, C): **D)** Skin nociceptors; **E)** Muscle nociceptors; **F)** Vascular nociceptors. Comparisons between the first ETOH-1 and 'Vehicle' tests (.001% ETOH) were non-significant.

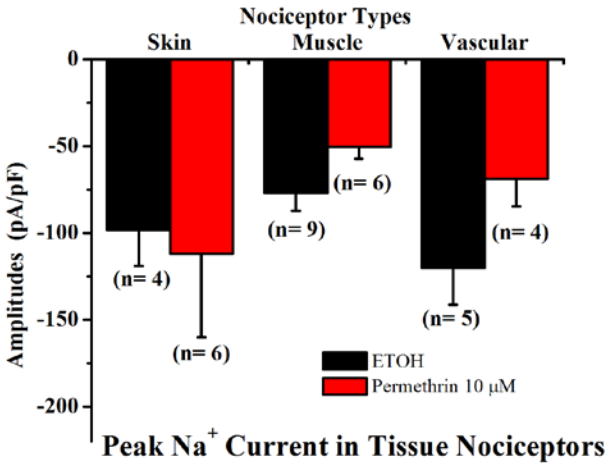

Figure 5. Acute Permethrin did not Increase the Amplitude of $Nav_{1.8}$. At the test voltage of 0 mV, the normalized peak amplitude of Nav1.8 in tissue specific nociceptors remained relatively stable compared to ETOH treated controls.

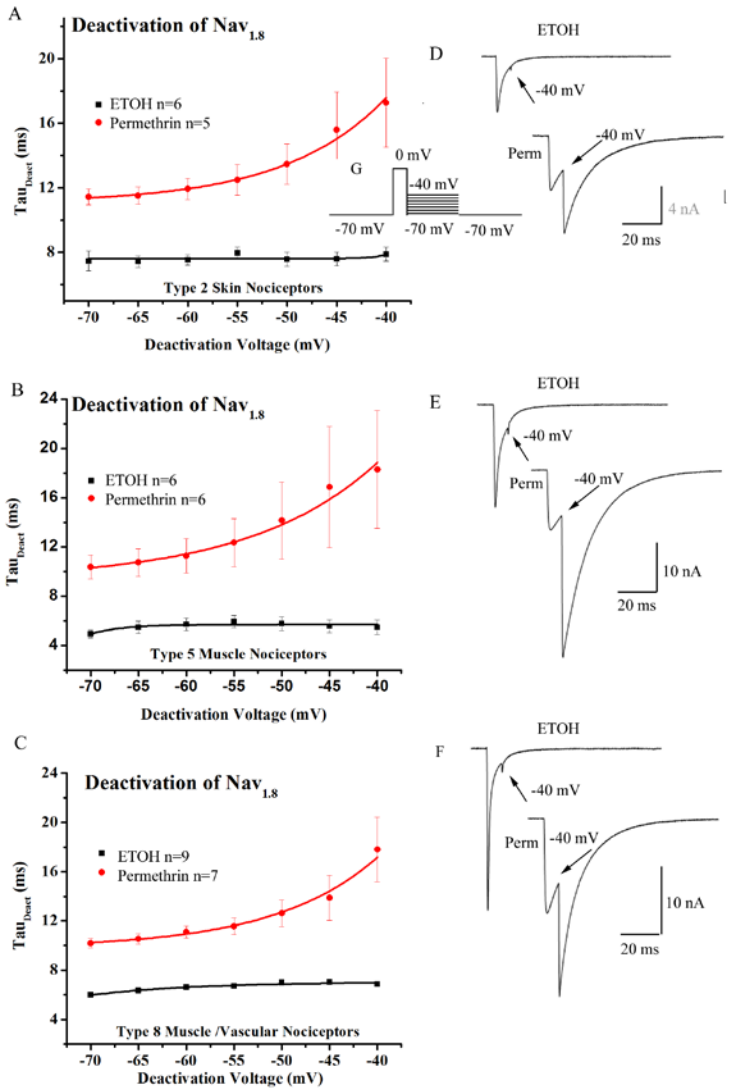

Figure 6. Permethrin Shifts Voltage Dependence of Deactivation in All Tissue Specific Nociceptors. A-C) ETOH exposed cells exhibit a relatively consistent pattern of deactivation across membrane voltages (black curves). Following treatment with permethrin, deactivation time constants were increased: (skin: F=22.1, DF=1.9; muscle: F=10.2, DF=1,11; vascular: F=28.4, DF=1,14) and a strong voltage dependence appeared (red curves). The interaction between voltage and time constants were highly significant for all tissue nociceptors (skin: F=6.04, p<.0001; muscle: F=3.49, p<.005; vascular: F=5.49, p<.0001). There were no statistical differences between tissue specific nociceptors. **D-F)** Representative traces illustrate the deactivation tail current associated with permethrin and ETOH treated neurons. All traces taken from a single deactivation voltage step to -40 mV. The arrow signifies the point at which the voltage is abruptly clamped at -40 mV. **G)** A schematic of the deactivation protocol. The voltage step to 0 mV was 8 ms long. The deactivation voltage steps were 150 ms. 10 μMm permethrin applied for 2 minutes.

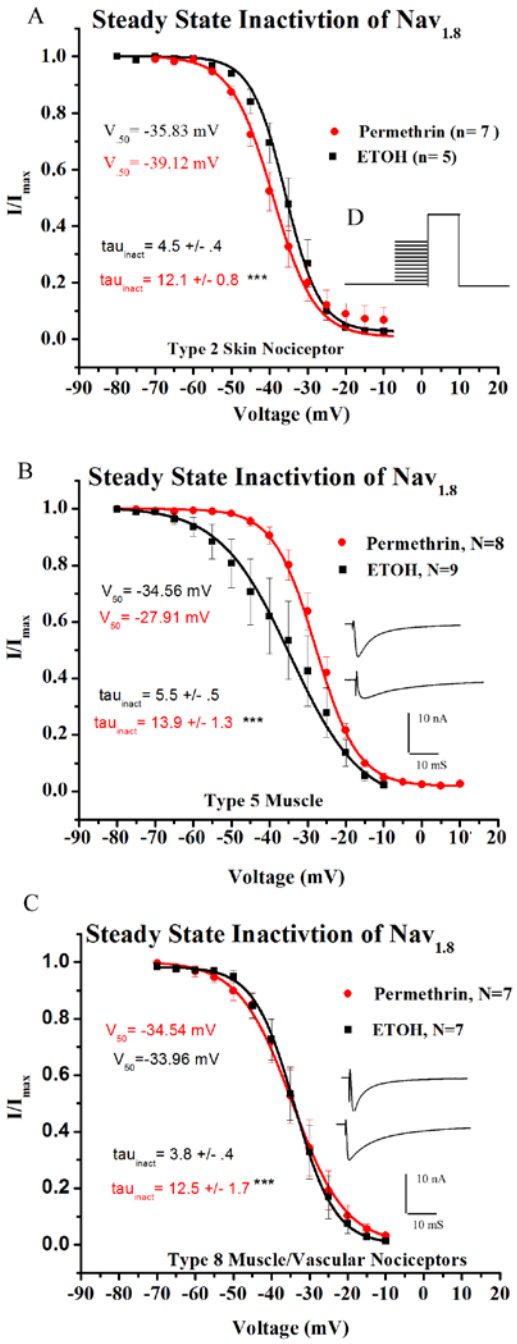

Figure 7. Permethrin and Fast Inactivation of $Nav_{1.8}$ A-C) Voltage dependent inactivation of $Nav_{1.8}$ was not significantly modified by application of 10 μM permethrin (2 minutes); The tau_{inact} (mS) was significantly increased in all cases. **D)** A schematic of the inactivation protocol. Inserts show representative traces (upper: ETOH; lower: Permethrin). Note the slowing of inactivation after permethrin. The cells were pre-pulsed in 5 mV steps from -10 to -80 mV. The test pulse was 0 mV, 150 ms.

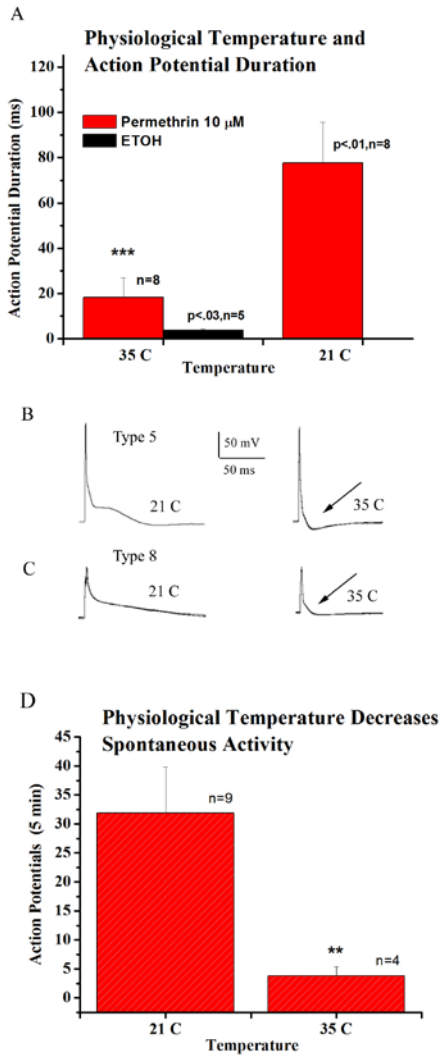

Figure 8. Thermal Modulation of Permethrin Influences on Action Potentials. A) Permethrin-induced increases in action potential duration was diminished at 35.5 C. Action potential duration remained significantly increased relative to ETOH treated neurons (type 5 and type 8 pooled). **B)** Representative action potentials of a type 5 muscle nociceptor at 21 and 35 C. Note the reappearance of an afterhyperpolarization period at 35 C (arrow). Three of 4 afterhyperpolarizations recovered in muscle nociceptors **C)** Representative action potentials of a type 8 nociceptor at 21 and 35 C. Note that the afterhyperpolarization did not recover in the two cases examined (arrow). **D)** Physiological temperatures significantly reduced the spontaneous action potentials evoked by permethrin (5 min observation; vascular nociceptors).

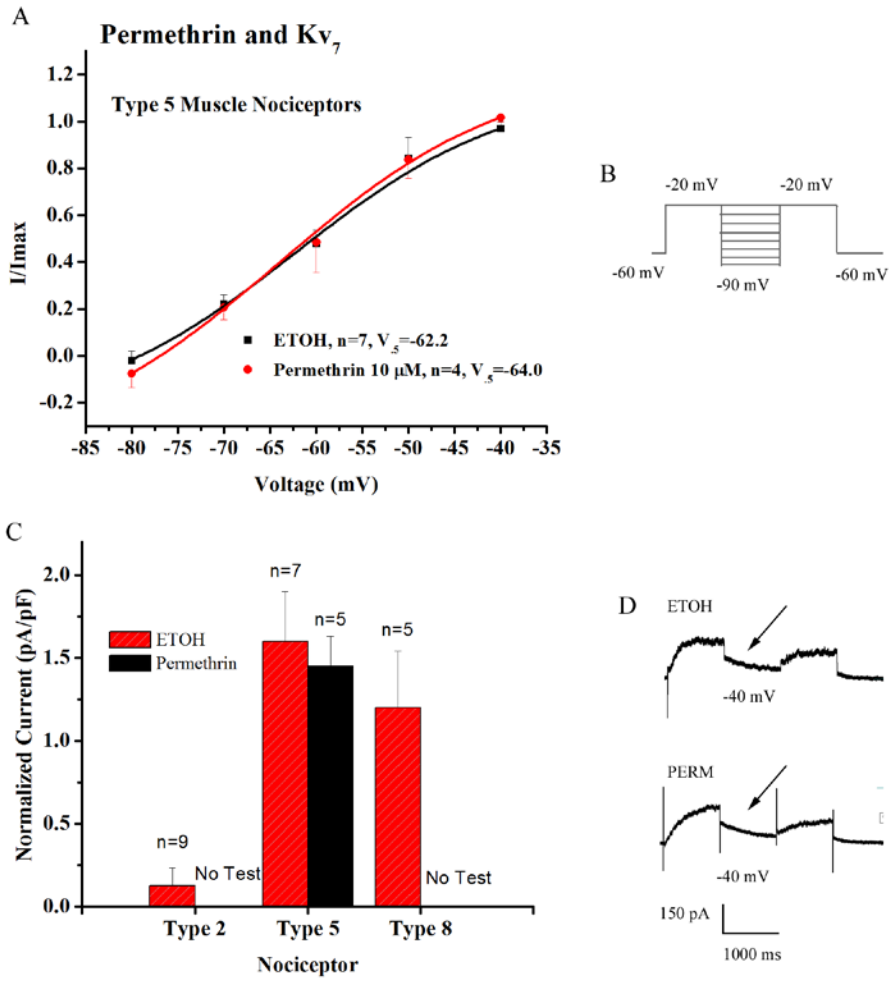

Figure 9. Permethrin did not Modify Currents Passing Through Kv_7 Proteins. A) Voltage dependence of Kv_7 is similar after 4 minute permethrin treatment (type 5 muscle nociceptors). **B)** A schematic representation of the voltage protocol used to evoke Kv_7 currents. Representative traces are shown in 'D'. **C)** Summary data of the averaged peak amplitude of permethrin and ETOH treated Kv_7 proteins expressed in Type 2 (skin), Type 5 (muscle) and type 8 (vascular) nociceptors. Skin nociceptors did not express a linopirdine sensitive current. No permethrin testing was conducted on vascular nociceptor Kv_7. **D)** Representative traces of linopirdine sensitive current (subtraction currents in the presence of ETOH or permethrin) evoked at -40 mV. The arrows indicate the linopirdine sensitive 'tail' currents.