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14. ABSTRACT

Our laboratory developed an animal model that replicates some of the symptoms of the chronic pain of GWI. Through this animal model we identified molecular targets in pain sensing neurons, which appear to have been chronically altered following exposure to insecticides and repellants that were present in the GW theater. K_v7 openers (KVOs) have known analgesic/palliative capacities that could serve as treatments for the chronic pain associated with GWI. We have previously shown that Retigabine (KVO) can temporarily reverse signs of GWI pain. Building on year 1 progress, we tested whether a combination KVOs (Retigabine and Diclofenac) could reverse behavioral pain-signs in our GWI pain model. Some positive outcomes were found but were compromised by serious side effects. Molecular experiments in year 2 indicated that the combination of Retigabine and Meclofenamate could be more effective. Behavioral studies on this treatment approach are ongoing.

15. SUBJECT TERMS
Pain, Nociceptor, Retigabine, Pesticides, kv7, Gulf War Illness, Treatment

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Introduction

Over the last several years, our laboratory developed and refined an animal model of GWI pain (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al. 2015; Flunker et al., 2017). We ultimately demonstrated that a 4 week exposure to permethrin, chlorpyrifos, DEET and PB (pyridostigmine bromide) could produce a delayed manifestation of pain-like signs that could persist up to 24 weeks after the initial exposure to the four GW chemicals.

K_v7 is a nociceptor ion channel that, when activated, diminishs or prevents the neuronal discharges that mediate pain sensations (Brown and Passmore, 2009; Du et al., 2017). Retigabine and Flupirtine are classic K_v7 openers (KVOs) that have been effective for treatment of some forms of chronic pain in humans (Luben et al., 1994; Worz et al., 1996; Uberall et al., 2011; Mishra et al., 2013; Uberall et al., 2013; Worz, 2014).

In an effort to identify drugs for treatment of GWI pain, we tested the capacity of the K_v7 opener (KVO), Retigabine, to reverse an established pattern of chronic pain behavior, in rats, that developed 9 weeks following exposure to GWI chemicals (Cooper et al., 2018). We reported that a single daily dose of Retigabine (7 mg/kg) reversed established ambulation deficits that were present 13-16 weeks post-exposure. Although Retigabine significantly and rapidly diminished pain behaviors, the improvement was transitory. Pain signs gradually returned over the last 2 weeks of the 4 week treatment period.

It has been shown that certain FDA approved NSAID drugs also have the capacity to open K_v7 channels (Diclofenac; Meclofenamate; Celecoxib; Peretz et al., 2005; Peretz et al., 2007; Bruggermann et al., 2010; Frolov and Singh, 2011; Du et al., 2011). There is evidence that combinations of Retigabine with the NSAID KVOs can be more effective at opening K_v7 ion channels than either drug singly (Peretz et al., 2005; Khattab et al., 2018). If dose combinations can be shown to be more effective activators of K_v7 in deep tissue nociceptors, this could substantially improve the capacity of KVOs to be effective analgesics for the widespread deep tissue pain associated with GWI. There would likely be other applications to pain control, as well. These would include chronic and traumatic pain associated with military service.

In the experiments funded for budget year 2, we continued our examination of the capacity of combinations of NSAID KVOs (Diclofenac, Meclofenamate) and a classic K_v7 opener (Retigabine) to amplify signaling through K_v7, and depress the excitability of deep tissue nociceptors associated with the development of GWI pain. Based upon the findings of these molecular and cellular studies we examined whether KVO combinations could reverse pain-like signs observed in rats exposed to GWI neurotoxicants.

Keywords: pain, nociceptor, K_v7, Retigabine, pesticides, Gulf War Illness, treatment

3. Accomplishments

What were the major goals of the project in year 2?

Objectives: Year 2

1) **Determine the influence of Retigabine + Meclofenamate on voltage dependent opening of K_v7**
2) **Determine the influence of Retigabine + Diclofenac on voltage dependent opening of K_v7**
3) **Determine the influence of Retigabine + Diclofenac on Nociceptor Reactivity**
4) **Determne the influence of Retigabine + Diclofenac on pain-like behaviors**

What was accomplished under these goals?

A summary of accomplishments for years 1 and 2 (see below for details):

KVOs can be effective analgesics, but over a long term course of treatment, adverse side effects can be an issue. We examined whether we could lower the analgesic dose required (thus reducing side effects) by using a combination (adjunctive) therapy approach that took advantage of evidence that different families of KVOs act at distinct K_v7 binding sites (the principle of cooperativity). If KVOs were used in combination, we hypothesized that the effective doses could be substantially reduced and/or performance of the KVO could be boosted beyond that achievable by a single drug.

We characterized selected single and combination KVOs for their capacity to modify deep tissue nociceptor K_v7 activity. These classes of nociceptors were previously shown to develop

maladaptations following exposure to GW neurotoxicants. There are three forms of K_v7 activity that would be important for it to function as an analgesic: 1) Voltage dependent activation; 2) Dose dependent opening of the channel; and 3) Maximal current (conductance). We found that the three KVOs we studied differentially modified voltage dependent and dose dependent opening of K_v7; they also differed with respect to maximal current potency. Two of the NSAID inhibitor KVOs (Diclofenac and Meclofenamate) appeared to be good candidates for combination therapies with Retigabine (RET). In those combination experiments performed in years 1 and 2, we demonstrated that sub-threshold doses of RET (2.5 or 5 μM) greatly enhanced the effect of sub- and near threshold doses of DIC (50 μM) and MEC (30 μM). K_v7 activation, with these combinations, evoked currents that substantially exceeded those produced by RET, DIC or MEC alone. This outcome confirmed the fundamental concept that multiple KVOs acting independently at different binding sites could greatly enhance the potency of KVO activity.

In further proof of the concept, we also reported that combinations of RET and DIC could substantially reduce or block deep tissue nociceptor action potential activity following a challenge with an acetylcholine agonist (Oxotremorine-M; OXO).

Using molar ratios determined in the above studies (RET to DIC) we conducted behavioral studies on rats exhibiting pain like behaviors following exposure to GW chemicals (permethrin, chlorpyrifos, DEET and pyridostigmine bromide (Specific Aim 1). We observed successful reduction in pain-like behaviors at a dose ratio of 1:4 (2.5 mg/kg RET: 10 mg/kg DIC). However repeated dosing at these concentrations were not tolerated by these rats, with several rats becoming

ill or dying. Reduced concentration at this molar ratio and other molar ratios of RET and DIC were ineffective in additional behavioral studies. Detailed results are presented below.

As many experiments began in year one but were completed in year 2, we combined the year 2 findings with those of year 1 in order to present a more logical sequence of ideas and findings.

Specific Aim 2. Optimizing Molecular Synergisms of KVOs on Deep Tissue Nociceptor K_v7 Hypothesis: Combinations of KVOs will increase maximum voltage dependent current through K_v7

Major Task 1: KVO Dose Response Testing on Nociceptor K_v7 Activity (in vitro molecular studies)

Background: There are three important ways in which a KVO can modify reactivity of a nociceptor that would result in reduction or elimination of pain symptoms: 1) depolarized shift in voltage dependent activation; 2) reduce dose dependent (direct) activation; and 3) increase in the maximal conductance. K_v7 is a voltage activated channel whose activation opposes formation of action potentials. The amount of opposing current that flows is proportional to the applied voltage. That is, the more channels that are opened, the more opposition current is realized and the less likely nociceptor action potentials are generated and pain is experienced. On the other hand, with direct (dose dependent) activation by a KVO, all the channels can be opened at once providing an optimal effect on nociceptor discharge by keeping nociceptor membrane strongly hyperpolarized.

If maximal conductance is increased, a supramaximal effect on pain should result. We will determine whether combination KVO treatments can achieve one or more of these outcomes.

Subtask 2: Diclofenac Dose Response

Timeline: Months 1-4

Using the protocol described in 'Methods' (Appendix, p. 42) we assessed the influence of the NSAID KVO, Diclofenac (DIC), on deep tissue nociceptor K_v7. As described above, voltage shifts from -55 to -40 mV were applied in the presence and absence of DIC. The KVO or vehicle-induced shift in voltage activated current was assessed in deep tissue nociceptors known to be affected by GWI neurotoxicants. Direct activation of K_v7 current, by DIC, was also assessed in each experiment.

Shifts in Voltage Sensitivity. We tested whether DIC could alter voltage sensitivity. Doses of 50 to 140 μM were applied in distinct experiments. There was a strong influence of DIC on voltage sensitivity at a threshold dosage of 60 μM, thereafter little influence on voltage dependent opening was detected (figure 1A, B, C, D). It is likely that once K_v7 channels are directly opened by a KVO it can no longer be activated by voltage shifts, because they were removed from the voltage sensitive pool. However, this was clearly not the case when MEC was the KVO tested. Clearly, in nociceptors, distinct mechanisms are involved in the actions of these two NSAID/KVOs.

Direct Activation. At doses at which voltage shifts were realized (60 μM), little direct activation of the channel was apparent. Above this dose, DIC proved to be a reliable activator of K_v7. At 80 μM, significant K^+ currents developed and grew steadily through 120 μM (figure 2). At 120 μM K_v7 current appeared to be maximal (~1 pA/pF). Dose-current data fit to a Hill function indicated an ED_{50} of 93.2 +/- 8.99 μM.

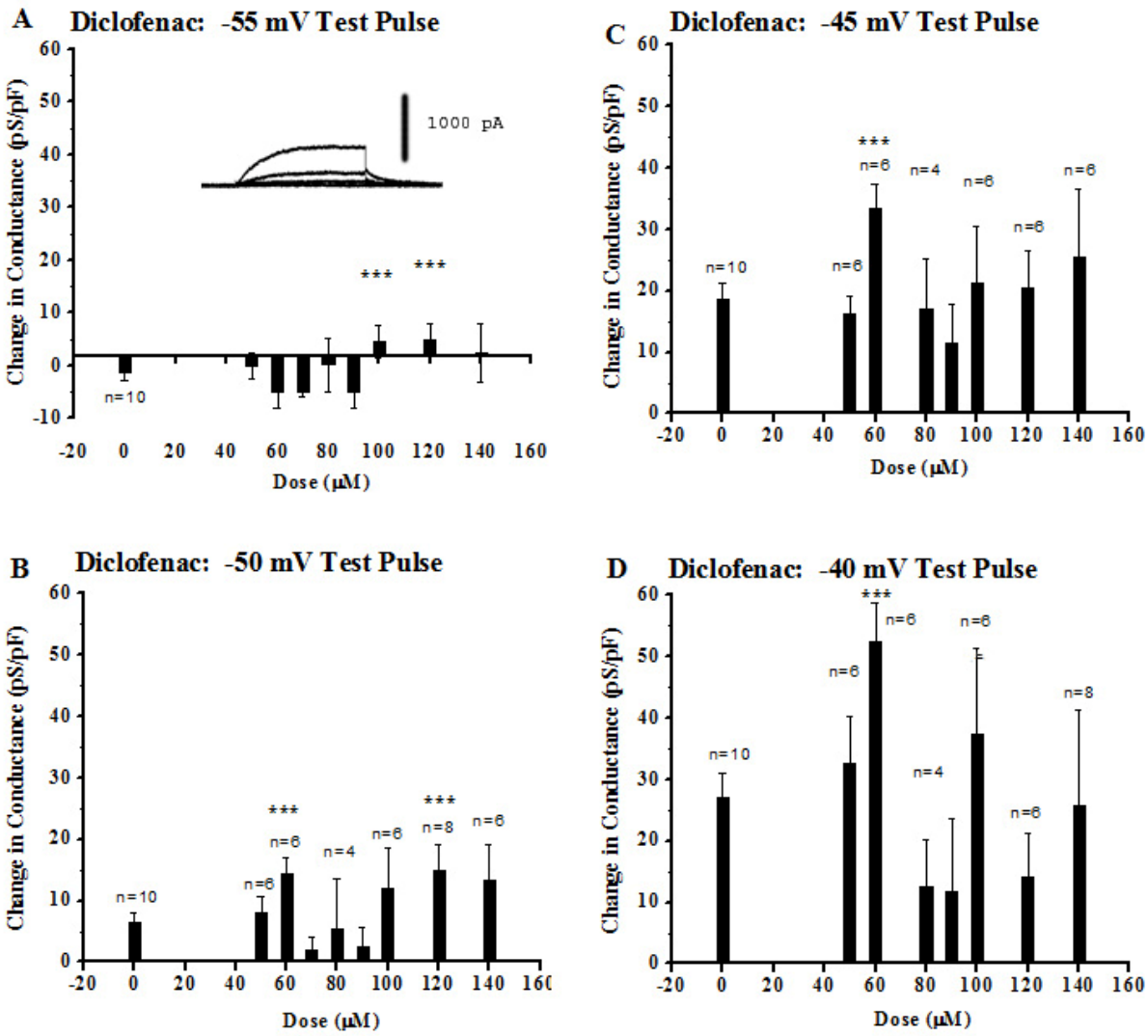

Figure 1. The Influence of Diclofenac on K_v7 Voltage Sensitivity. DIC had a narrow effect on the voltage sensitivity of K_v7. **A)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -55 mV (V_h=-60 mV). **B)**. The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -50 mV (V_h=-60 mV). **C)**. The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -45 mV (V_h=-60 mV). **D)**. The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -40 mV (V_h=-60 mV). Data collected from voltage clamp experiments in a K^+ isolation solution. Only one dose was applied per cell. Statistical comparisons were made between vehicle-vehicle and vehicle-DIC tests. Vehicle: DMSO; *** p<.05 or greater

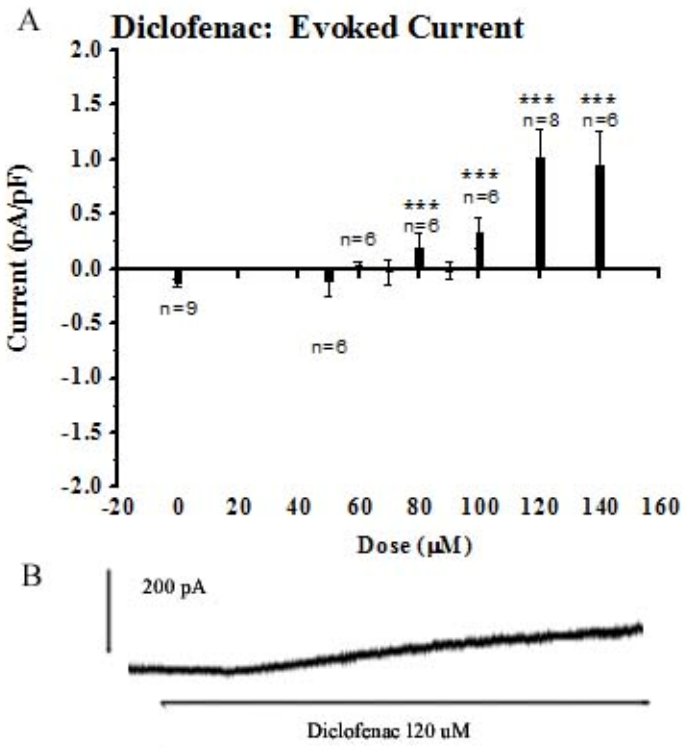

Figure 2. The Effect of Diclofenac on Resting Currents Mediated by K_v7. DIC opened K_v7 channels without a shift in applied voltage. Relatively small outward (hyperpolarizing) currents were observed. **A)** Average normalized current produced at -60 mV (V_h=-60 mV) following a 2 minute application of DIC. Evoked currents increased in proportion to the dose applied, but reached a maximum at 120 uM. Current was measured at the end of the 2 min application of DIC. **B)** A typical current trace produced by DIC. Currents grew slowly but were stable within 2 minutes. Data collected from voltage clamp experiments conducted in a K^+ isolation solution. Each neuron was exposed to only 1 dose. Statistical comparisons were made between vehicle and DIC tests. Vehicle: DMSO; *** p<.05 or greater.

Subtask 3: Celecoxib Dose Response

Timeline: Months 5-9

Experiments were conducted in a manner identical to those described above.

There was little evidence that Celecoxib (CEL) could influence the voltage dependence of K_v7. Although CEL was an effective opener of K_v7 channels at 20-40 μM, it was clear there was a strong non-specific effect on other voltage sensitive K^+ channels. This non-K_v7 specific effect, on other voltage activated currents, could impair the effectiveness of CEL as a deep tissue nociceptor analgesic. We decided to forego further experiments on this NSAID/KVO.

Subtask 1: Meclofenamate Dose Response

Timeline: Months 1-4

While many experiments on Meclofenamate were conducted in year 1, some were greatly expanded in year 2 in order to deal with statistical problems derived from high variability.

Using the protocol described in 'Methods' (Appendix, p. 56) we assessed the influence of the cyclooxygenase inhibitor, Meclofenamate (MEC), on deep tissue nociceptor K_v7. Briefly, voltage shifts from -55 to -40 mV were applied in the presence and absence of a KVO. The KVO influence on voltage activated current was assessed in deep tissue nociceptors implicated in GWI pain. Direct, dose dependent, activation of K_v7 was also assessed.

Using deep tissue nociceptors implicated in our GWI pain models, we observed a dose dependent influence of MEC on voltage dependent activity of K_v7 (figure 3A, B, C, D). Current measures

were converted to conductance (pS; PicoSiemens) in order to control for 'driving force'. Conductance was normalized (divided) by a measure of cell volume (pF; picofarads) in order to account for differences in the size of individual nociceptors. Comparisons were made to vehicle exposed cases.

Shifts in Voltage Sensitivity. Doses of 20 to 100 μM were applied in separate studies. When the dose of MEC reached 70 μM, we observed a significant increase of K_v7 conductance at all test voltages (figure 3). As MEC doses increased through 100 μM, conductance increases were observed at nearly all levels that were tested (-40, -45, -50 and -55 mV). The influence of MEC on voltage dependent conductance was very different from that of DIC or RET. With DIC, voltage-conductance effects occurred mainly at low doses of the KVO that were near evoked current threshold. In contrast, voltage dependent shift induced by MEC occurred mainly at the highest doses applied. As we will see below, voltage-conductance effects induced by RET were observed across virtually the full range of KVO doses.

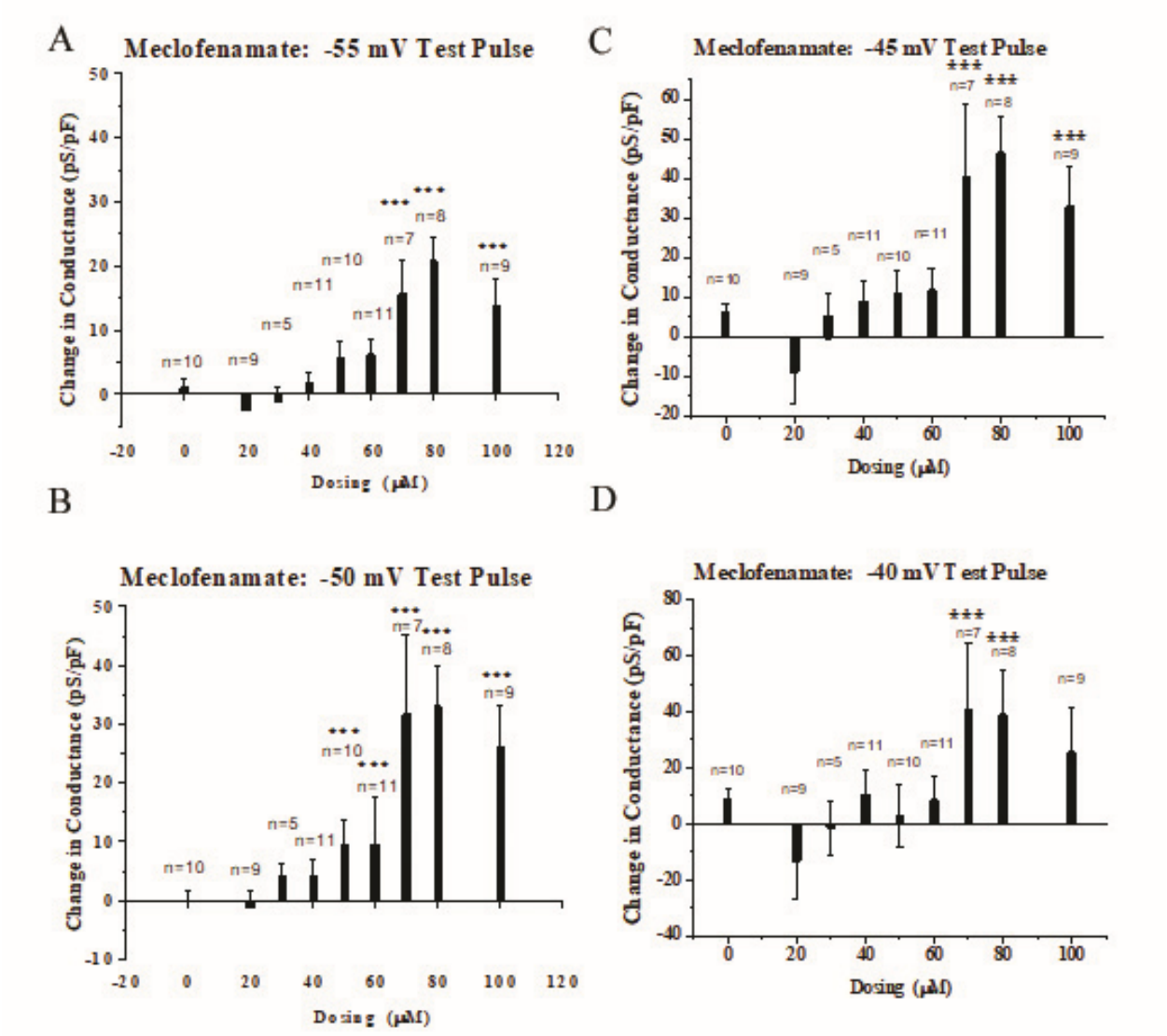

Figure 3. The Influence of Meclofenamate on K_v7 Voltage Sensitivity. MEC reliably increased the voltage sensitivity of K_v7 following a 2 minute application in a manner that generally paralleled direct activation (see figure 4). **A)** The change in normalized conductance of deep tissue nociceptors following exposures to test agents (vehicle-vehicle or vehicle-MEC). Conductance was derived from currents evoked by a voltage step to -55 mV (V_h=-60 mV). **B)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-MEC). Conductance was derived from currents evoked by a voltage step to -50 mV (V_h=-60 mV). **C)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-MEC). Conductance was derived from currents evoked by a voltage step to -45 mV (V_h=-60 mV). **D)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-MEC). Conductance was derived from currents evoked by a voltage step to -40 mV (V_h=-60 mV). Data collected from voltage clamp experiments in a K^+ isolation solution. The number of cells contributing to each bar is indicated by 'n='. Statistical comparisons were made between vehicle-vehicle and vehicle-MEC tests. Vehicle: Water; *** p<.05 or greater

Direct Activation. When MEC was applied in the absence of any voltage shifts (i.e., at the holding potential of -60 mV), it proved to be a very effective direct activator of K_v7. At doses as low as 30 μM, a week K_v7 current could be detected and grew substantially with increasing concentration. A maximum outward current was reached at 80 μM (6.3 +/- 1.2 pA/pF; figure 4). The ED_{50} (effective dose for 50% activation) for MEC was determined to be 62.2 +/- 3.2 μM.

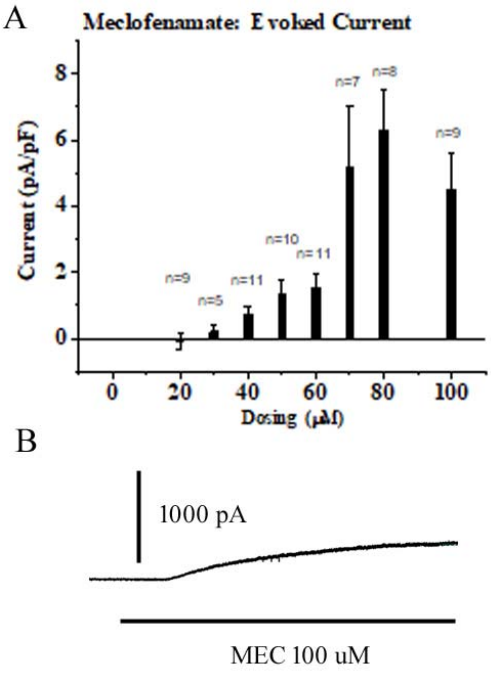

Figure 4. The Effect of Meclofenamate on Resting Currents Mediated by K_v7. MEC opened K_v7 channels without a shift in applied voltage. Relatively large outward (hyperpolarizing) currents were observed. **A)** Average normalized current produced at the holding potential (V_h=-60 mV) following a 2 minute application of MEC. Evoked currents increased in proportion to the dose applied, but reached a maximum at 80 uM. Current was measured at the end of the 2 min application of MEC. Outward currents were never observed during vehicle applications. Data collected from voltage clamp experiments in a K^+ isolation solution. The number of cells contributing to each bar is indicated by 'n='. Statistical comparisons were made between vehicle and MEC tests. Vehicle: Water; *** p<.05 or greater

In summary, MEC strongly opened K_v7 channels. Threshold doses for direct activation were lower (30 vs 50 μM), and maximal currents were much greater with MEC compared to DIC (6 vs 1 pA/pF). Direct activation began at 30 μM, and voltage dependent shifts in conductance were present, but these were limited to the high dose range (>70 μM). The relatively more powerful direct effect of MEC on K_v7 activity suggested it would be superior to DIC in this application; especially when low doses were desired. We examine the influence of MEC on nociceptor excitability in Specific Aim 3.

Subtask 4: Retigabine Dose Response

Timeline: Months 5-9

Subtask 4 was completed in year 1.

Shifts in Voltage Sensitivity. RET amplified the voltage sensitivity of K_v7 at doses as low as 5 μM (figure 5). No directly activated current could be demonstrated at this dose. Voltage effects were observed above the threshold of 5 μM, and across a wide range of concentrations. This pattern contrasted to that observed for DIC or MEC (figures 1 and 3, above).

Direct Activation. As doses increased from 20 to 100 μM, evoked currents steadily increased reaching a peak of 80 pA (1.14 +/- .15 pA/pF; figure 6). An ED_{50} of 64.3 +/- 12.4 μM was determined by fit to a sigmoidal curve. Maximal maintained currents, evoked by RET, were similar in amplitude to those evoked by DIC.

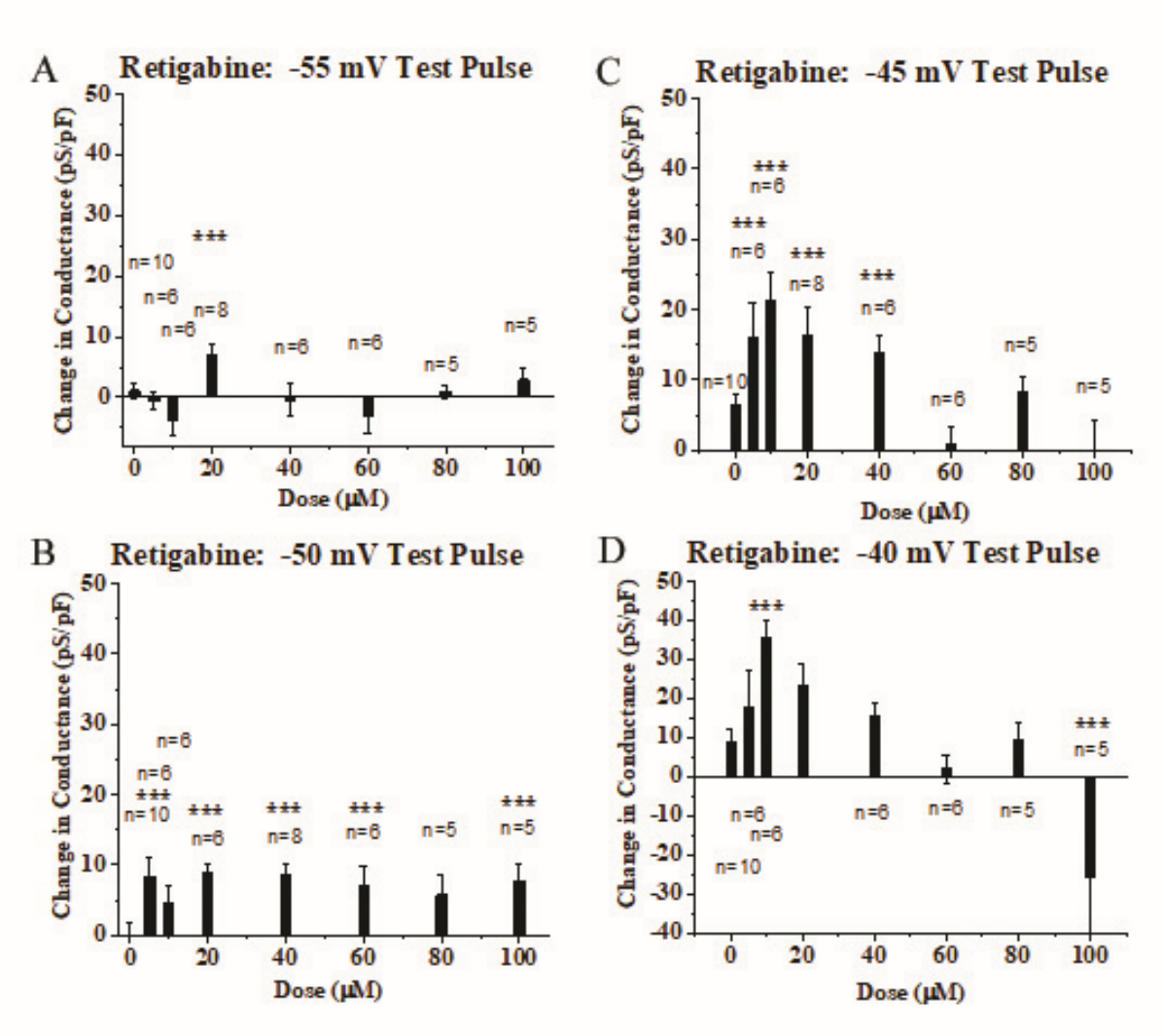

Figure 5. Retigabine Shifts K_v7 Voltage Sensitivity. A-D) The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-RET). Conductance was derived from currents evoked by a voltage step to -55, -50, -45, and -40, respectively. Data collected from voltage clamp experiments in a K^+ isolation solution. One dose was applied per cell. Statistical comparisons were made between vehicle-vehicle and vehicle-RET tests. Vehicle: Tyrode's; *** p<.05 or greater.

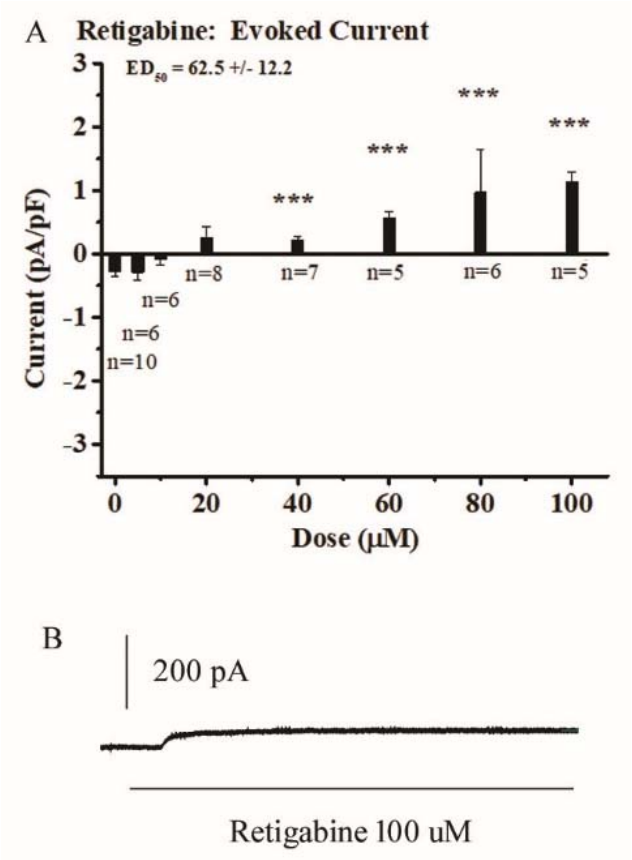

Figure 6. Resting K^+ Currents Evoked by Retigabine. A) Average normalized current produced at -60 mV (V_h=-60 mV) following a 2 minute application of RET. Evoked currents increased in proportion to the dose applied, but reached a maximum at 100 uM. **B)** A typical current trace produced by RET. Currents grew rapidly and were stable. ED_{50}=64.3+/-12.4 uM; *** p<.05 or greater. Vehicle: Tyrode's.

In summary, the three KVO's exhibited distinct patterns of activity against nociceptor K_v7. The classic KVO, Retigabine was an effective, relatively low threshold opener of nociceptor K_v7 channels. RET and MEC exhibited similar ED50's, while RET and DIC had similar peak evoked currents. However, RET manifested a lower threshold and wider range of voltage dependent effects that were not seen with DIC or MEC. The peak currents were dramatically larger in MEC, as well as consistently larger throughout the range of effect. Target capacities, for analgesic effectiveness, would include a low threshold for current and voltage effects (RET) and large evoked currents (MEC). In Specific Aim 3, we examined how combinations of RET/ DIC and RET/MEC could be optimized to fit these goals.

Major Task 2: KVO Synergisms on Nociceptor K_v7 Activity (in vitro molecular studies)

Subtask 1: Retigabine and Meclofenamate

Timeline: Months 10-11

Subtask 2: Retigabine and Diclofenac

Timeline: Months 10-11

Subtask 3: Retigabine and Celecoxib

Timeline: Months 10-11

Major Task 2

In sensory neurons, $K_v7.2$ combines with $K_v7.3$ to mediate the M-current (Jiang et al., 2013; Du et al., 2017). The M-current derives its name from the muscarinic acetylcholine receptor whose activation results in the closing of K_v7 channels, via a cytoplasmic pathway, and increased neuron excitability. KVOs are agents that open K_v7 channels and reduce excitability via direct binding to

$K_v7.2$ or $K_v7.3$. KVOs with differential affinities for $K_v7.2$ or $K_v7.3$ could produce greater net hyperpolarizing M-current when acting in combination because they differ in binding sites and subunit affinity (Tatulian et al., 2001; Peretz et al., 2005; Huang et al., 2013). In fact, such demonstrations were made (RET and MEC) in non-neuronal host cells (CHO; Chinese hamster ovary; Peretz et al., 2005).

In this project, we examined whether combination KVOs would be effective in mammalian nociceptor neurons that we have shown to be influenced by exposure to GW neurotoxicants (Cooper et al., 2018). Below, we demonstrate that combinations of RET with MEC or DIC can substantially amplify currents through K_v7 ion channels. This finding suggest that combination therapies could be effective analgesics in GW veterans. The lowered doses made possible with combination therapies could reduce the risk of deleterious side effects sometimes associated with long term use of single KVO agents (Singh et al., 1994; Fries, 1996; Ulubay et al., 2018; Bock and Link, 2019).

Major Task 2, Subtask 1: Retigabine and Meclofenamate

Timeline: Months 10-11

These studies were carried out mainly in year 2. Several combinations of RET and MEC were tested, but only one is presented below, in detail. All the data will be included in the Appendix of the final report.

Sub-threshold doses of RET and MEC were tested using procedures identical to those described in Major Task 1. When deep tissue nociceptors were exposed to combinations of sub-threshold doses of RET (5 μM) and MEC (20 μM), there was little evidence of any amplification of evoked current (not shown). Nor did combination of RET 2.5 μM with 70 μM MEC show any significant amplification. When the MEC concentration was raised to 30 μM (barely above threshold), very substantial current amplification was observed. That amplification far exceeded what could be observed with RET DIC combinations (see below). As shown in figure 7, subthreshold and near threshold combination of 5 μM RET and 30 μM MEC produce outward currents in excess of 250 pA. That greatly exceeded the maximum current evoked by any dose of RET or DIC (<100 pA; see figures 2 and 4). As shown in figure 7, the combination of RET 5 μM and MEC 30 μM produced currents significantly greater than 80 μM RET, or 60 μM MEC alone, and were roughly equivalent to 100 μM RET or 70 μM MEC. We did not observe any shifts in the voltage dependent conductance at any of the combinations tested.

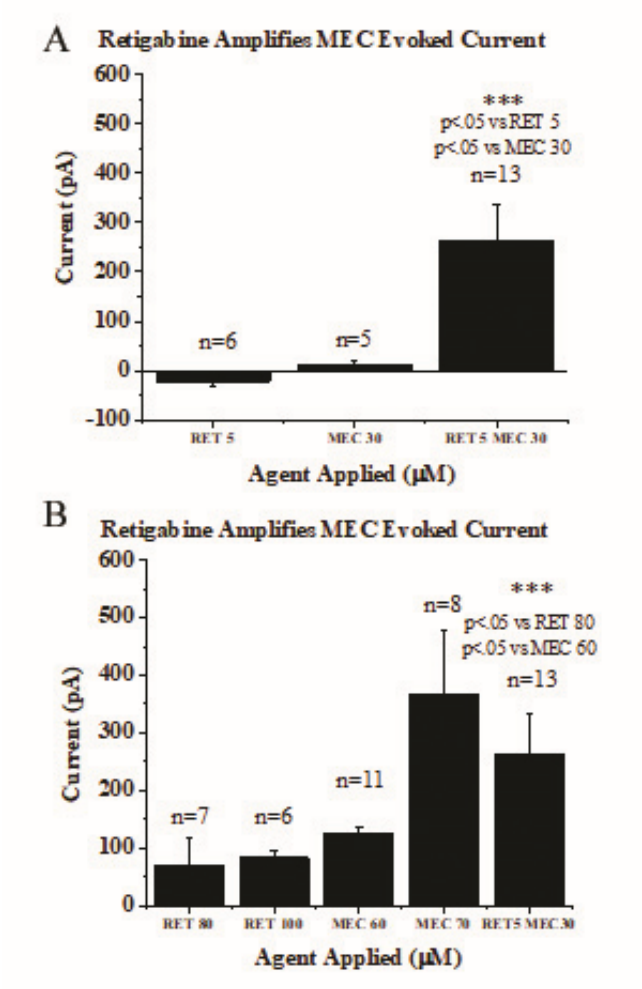

Figure 7. The combination of doses of RET 5 uM and MEC 30 uM produced currents exceeding those of RET or MEC alone. A) Currents measured at the end of the 2 min combined application greatly exceed those evoked by individual applications of threshold doses (RET 5 uM or MEC 30 uM). **B)** Currents measured at the end of the 2 min combined application also exceeded those evoked by supra-threshold applications of REC 80 uM or MEC 60 uM. RET 100 uM or MEC 70 uM evoked currents that were similar to those of RET 5 uM and MEC 30 uM combined. Data were collected from voltage clamp experiments conducted in a K^+ isolation solution. Statistical comparisons were made between RET/MEC, RET and MEC tests on different cells. For statistical tests, the currents were normalized for cell size (pA/pF). Mann-Whitney U tests were employed due to non-normal distributions.

Summary

The combination of RET and MEC exhibited superior potential for analgesic capacity. This was evidenced by the massive amplification of K_v7 activity at very low doses of the two KVOs. Although evoked current was greatly amplified, the voltage dependence was not similarily affected.

Major Task 2, Subtask 2: Retigabine and Diclofenac

Timeline: Months 12-14

These studies were mainly presented as part of the year 1 report. However, a number of additional studies were conducted in year 2.

We examined whether combinations of RET and DIC could improve voltage sensitivity or amplitude of resting currents over those observed following single application studies. Two strategies were employed: 1) amplifying DIC response with a low dose of RET; and 2) amplifying RET responses with a low dose of DIC

Sub-threshold combinations of RET and DIC were tested using procedures identical to those described above. Several combinations were investigated, and three are presented in detail. Combining 5 μM RET with 50 μM DIC significantly improved resting current amplitudes (figure 8). The average evoked currents by 5 μM RET or 50 μM DIC (applied singly) were -22.7 + 3.7 pA and -26.7 + 20.2 respectively, the combination of 5 μM RET and 50 μM DIC generated

outward currents that averaged 59.5 + 18.1 pA; an increase of over 70 pA. The combination of 5 μM RET and 50 μM DIC produce significantly more current than 70 μM DIC or 40 μM RET alone and was roughly equivalent to a dose of 80 μM DIC or 60 μM RET (see figures 2 and 6). However, co-application of 5 μM RET with 50 μM DIC did not significantly improve voltage sensitivity beyond that which could be obtained by single application of DIC or RET.

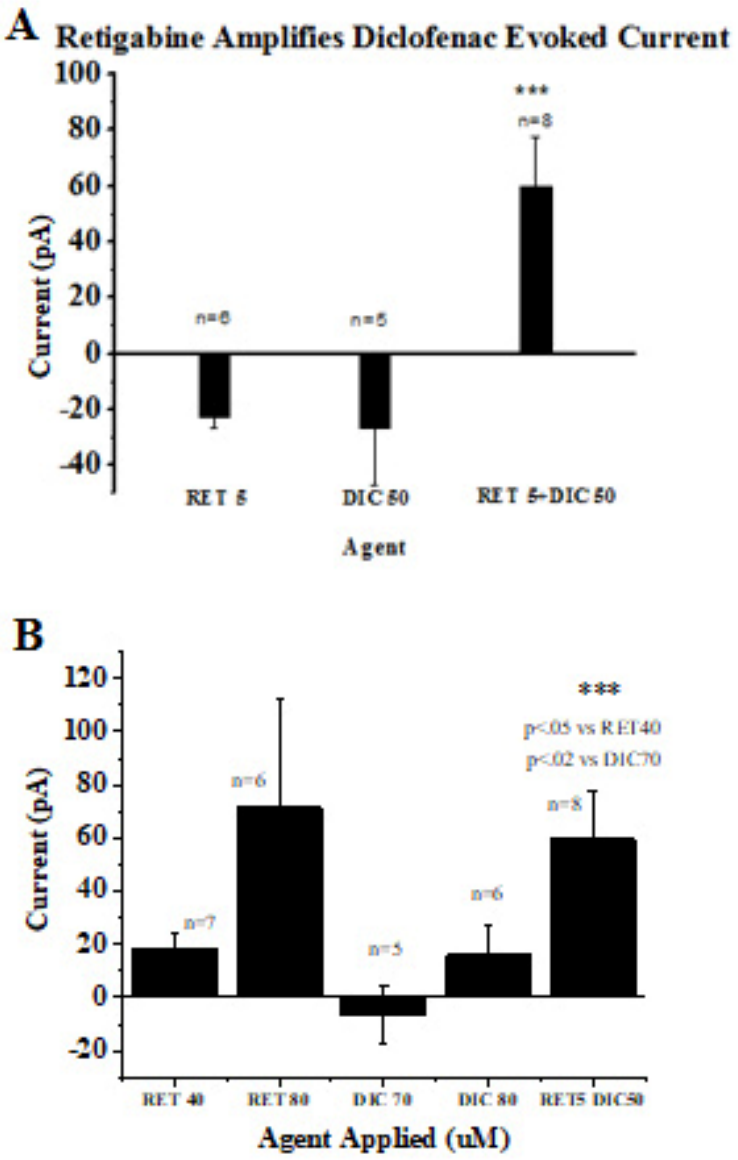

Figure 8. The combination of subthreshold doses of RET 5 uM and DIC 50 uM produced currents exceeding those of RET or DIC alone. A) Currents measured at the end of the 2 min combined application greatly exceed those evoked by individual applications of sub-threshold doses (RET 5 uM or DIC 50 uM). **B)** Currents measured at the end of the 2 min combined application also exceeded those evoked by supra-threshold applications of RET 40 uM or DIC 70 uM. RET 80 uM or DIC 80 uM evoked currents that were similar to those of RET 5 uM and DIC 50 uM combined. Data were collected from voltage clamp experiments conducted in a K^+ isolation solution. Statistical comparisons were made between RET/DIC, RET and DIC tests on different cells. For statistical tests, the currents were normalized for cell size (pA/pF). *** p<.05 or greater.

Based upon these outcomes we examined whether we could lower the DIC dose further and retain the amplifying effects of a combination with RET. Combining 5 μM RET with 20 μM DIC produced a similar pattern of outcomes at a lower dose (figure 9). The combination of 5 μM RET with 20 μM DIC generated outward currents that averaged 125.7 +/- 59.7 pA. Although highly variable, the average current only nominally exceeded currents that were observed with the combination of 5 μM and 50 μM DIC. Despite the high variability, the combination of 5 μM RET and 20 μM DIC produced significantly more current than 80 μM DIC or 40 μM RET alone and was roughly equivalent to a dose of 100 μM DIC or 60 μM RET.

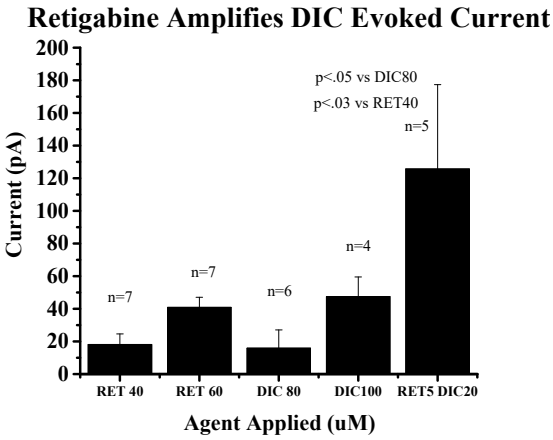

Figure 9. The combination of doses of RET 5 uM and DIC 20 uM produced currents exceeding those of RET or DIC alone. Currents measured at the end of the 2 min combined application exceeded those evoked by supra-threshold applications of RET 40 uM or DIC 80 uM. RET 60 uM or DIC 100 uM evoked currents were similar to those of RET 5 uM and DIC 20 uM combined. Data collected from voltage clamp experiments conducted in a K^+ isolation solution. Statistical comparisons were made between RET/DIC, RET and DIC tests on different cells. For statistical tests, the currents were normalized for cell size (pA/pF).

Subtask 3: Retigabine and Celecoxib

Timeline: Months 10-11

Due to the substantial non-specific effects of CEL on K^+ currents, we have not conducted the scheduled test of RET and CEL combination studies. Rather we will focus on improving RET+ DIC and RET+MEC outcomes.

Summary for Major Task 2

Combination doses of RET and MEC or RET and DIC greatly amplified K_v7 currents of deep tissue nociceptors relative to individual applications of each KVO. Opening of these ion channels will produce strong hyperpolarization of nociceptor membranes and thereby prevent the action potential discharge experienced as pain. Importantly, the doses of the combination agents were, by themselves, below or at threshold for current activation. An adjunctive approach to pain control minimizes the likelihood of any side effects that might occur from long term use of high doses of KVOs, but may not fully exploit the hyperpolarizing potential of KVO combinations.

Specific Aim 3. Optimizing KVO Functional Synergisms

The GW neurotoxicant Chlorpyrifos can produce long term changes in the function of acetylcholine esterase and the expression of muscarinic receptors (see discussion, Cooper et al., 2016). We have shown, in our rat model, that maladaptations in this muscarinic receptor pathway is related to the pathophysiology of GWI pain (Cooper et al., 2018). Muscarinic dependent burst

discharges (MDBD) is a feature somewhat unique to deep tissue nociceptors (Cooper et al., 2016, 2018). Although initiated by acetylcholine binding, MDBD is a complex series of intracellular events that include the activation of TRPA1, the closing of K_v7, activation of $Na_v1.8$, $Na_v1.9$, and other muscarine dependent events. Muscarine dependent depolarization and burst discharges are amplified in rats that have been exposed to GWI chemicals. K_v7 modulates MDBD burst duration in GW agent exposed rats, but does not appear to be central to the pathophysiology (Nutter et al., 2015; Flunker et al., 2017).

Some have suggested that GWI is a variant of OPIDN (organophosphate induced delayed neuropathy). Recent studies on OPIDN have demonstrated that chlorpyrifos is a direct activator of TRPA1 (Ding et al., 2017). As we have shown, muscarinic dependent burst discharges are fundamentally mediated by TRPA1 activation, and that these TRPA1 depolarizations in deep tissue nociceptors are increased in rats exhibiting pain-like behaviors following GW neurotoxicant exposure. We have also shown that Chlorpyrifos is a necessary component of the exposure protocol that produces a delayed chronic pain documented in our rat model (Flunker et al., 2017). For this reason, the capacity of candidate analgesics (i.e., KVOs) to block muscarinic nociceptor activation was deemed a high priority pre-clinical test.

Accordingly, in Specific Aim 3, we will determine whether KVO combinations are able to block muscarinic depolarization and action potential discharges. These studies will further refine KVO selected agents for behavioral studies of Specific Aim 1.

Hypothesis: Combinations of KVOs will prevent nociceptor discharges evoked by muscarinic agonists

Major Task 1: Cellular Synergisms of KVOs

Subtask 1: KVO combination Dose 1

Timeline: Months 12-14

Subtask 2: KVO combination Dose 2

Timeline: Months 12-14

Subtask 3: KVO combination Dose 3

Timeline: Months 12-14

We examined whether the powerful current amplifications that we observed with combinations of KVOs were effective at reducing action potential bursting in deep tissue nociceptors. These effects were contrasted with single KVOs tests. A neuron has a characteristic resting membrane potential of about -65 mV (RMP). Agents that depolarized the RMP move the neuron closer to action potential threshold (AP) and discharge. Given the capacity of KVOs to open K_V7, we would expect the RMP to hyperpolarize; that is, get further away from action potential threshold (more negative). Thereby, reducing or blocking AP's and preventing pain perception. We examined whether our combination strategy could hyperpolarize membranes (make RMP more negative) and block APs subjected to a cholinergic challenge test.

Following cell characterization, cells were brought into current clamp mode. Exposure of deep tissue nociceptors to a single KVO produced relatively rapid hyperpolarization that remained constant through the 2 minute application period. Significant hyperpolarizations were observed with DIC at 50, and 20 μM test doses (figure 10). Further testing revealed that DIC

hyperpolarizations abated at 2.5 μM (0.54 +/- 1.03 mV; n=5). Significant hyperpolarization was also observed with RET at 5 and 10 μM (-2.00 +/- 0.60 mV; n=6 and -1.84 +/- 0.56 mV; n=6, 5 and 10 μM, respectively; figure 10). At a dose of 2.5 μM, RET produced a depolarization that was non-different from vehicle controls.

Following the application of 5 μM RET and 50 μM DIC in combination, the RMP was significantly hyperpolarized (-4.4 +/- 1.4 mV; $p<.003$, n=8), and exceeded that observed with DIC alone ($p<.02$, n=6). The combination of 5 μM RET and 20 μM DIC produced significantly less hyperpolarization (-2.83 +/- 0.37; $p<.05$, n=5), but still exceeded that produced in control tests ($p<.001$, n=8; see figure 10A and B).

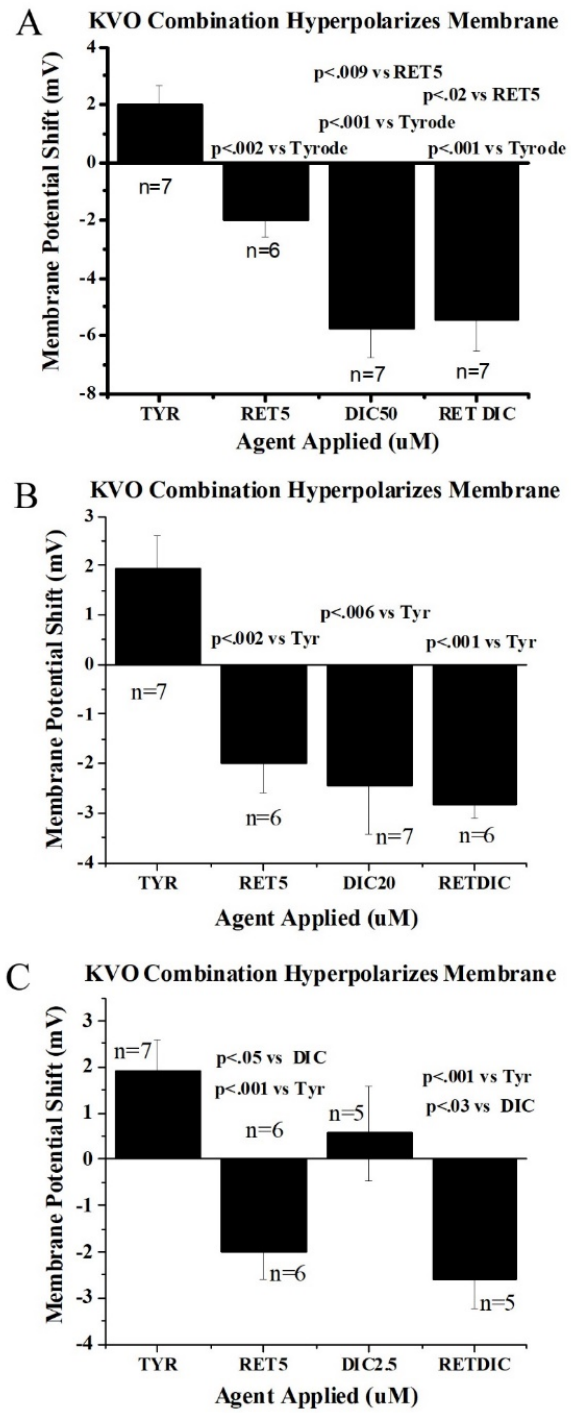

Figure 10. Combinations of RET and DIC Hyperpolarized Nociceptor Membranes. A) RET 5uM and DIC 50 uM produce powerful shifts in the membrane potential that exceeded that of RET alone. **B)** Hyperpolarization by RET 5 uM and DIC 20 uM were significantly less than that of RET 5 and DIC 50 uM. The combination dose was not greater than either agent alone. **C)** RET 5 uM and DIC 2.5 uM produced significantly less hyperpolarization than combinations with 20 or 50 uM DIC, and significantly more than DIC 2.5 alone.

We expanded analgesic testing using a cholinergic challenge test. We had previously demonstrated that deep tissue nociceptors responded to a muscarinic cholinergic with powerful action potential bursts, and that these bursts were potentiated in nociceptors harvested from rats that were exposed to GW chemicals (Cooper et al., 2018). Because breakthrough responses tended to bias means, we present both the mean and median values below. All statistical tests were carried out on means.

Immediately following a 2 minute KVO exposure (or vehicle), 10 μM Oxotremorine, OXO was applied in the presence of the test KVOs. In control tests, without the presence of a KVO, OXO produced a rapid depolarization (23.3 +/- 2.13 mV, n=10) and burst discharge (91.1 +/-21.6 APs; median: 75.5 APs; figure 11A). The combination of 5 μM RET with 50 μM DIC significantly reduced burst discharge (10.86 +/- 8.94; median: 1 AP; $p<.007$, n=7) while significantly *increasing* OXO-induced depolarization (32.2 +/- 2.81 mV, $p<.05$, n=8). Applied singly, DIC 50 μM was also capable reducing OXO dependent bursting (26.75 +/- 19.8; median= 8 APs; n=8). This pattern indicated that, despite the voltage clamp data, the combination of RET 5 with DIC 50 μM did not increase the analgesic potency of DIC as defined by the cholinergic challenge test. The interpretation of the test was complicated by the significant increase in OXO dependent depolarizations observed with this combination.

Further experiments examined whether analgesic potency could be retained when reducing DIC concentrations. The combination of 5 μM RET with 20 μM DIC significantly reduced burst discharges (27.2 +/- 20.6, median: 3.5 APs, $p<.02$, n=6) without effecting OXO induced depolarization (26.3 +/- 4.6 mV). Neither 5 μM RET (89.8 + 26.8 APs; median 94.5 APs; n=6) nor 20 μM DIC alone (45.3 + 18.9; median 31 APs; n=7) reduced AP bursting (figure 11B). This was an improvement over the effect pattern of RET 5/DIC 50 μM; however, the combination of RET 5/DIC 20 μM was not significantly better at reducing AP discharges than DIC 20 μM alone. The better performance achieved with a lower level of DIC may have resulted from the significantly larger OXO dependent depolarizations observed when 50 μM DIC was used as the combination dose. In this combination, hyperpolarizations were significantly greater ($p<.05$).

Ultimately, the best overall results were obtained with the combination dose of 5 μM RET and 2.5 μM DIC. Significant hyperpolarizations were retained with the combination (figure 11C), but neither of these doses could reduce AP discharge when presented individually. The combination dose proved to be effective (figure 11C). Average AP bursts were significantly reduced to 10.2 +/-4.3 (n=5; median 14 APs; $p<.02$ vs OXO). OXO-induced depolarizations were unaffected (21.3 +/- 3.4 mV). Importantly, the AP reducing influence of the RET 5/DIC 2.5 μM treatment combination was significantly superior to either RET 5 or DIC 2.5 μM single applications and did not differ from those of the above combinations (RET 5/DIC 50 μM; RET 5/DIC 20 μM).

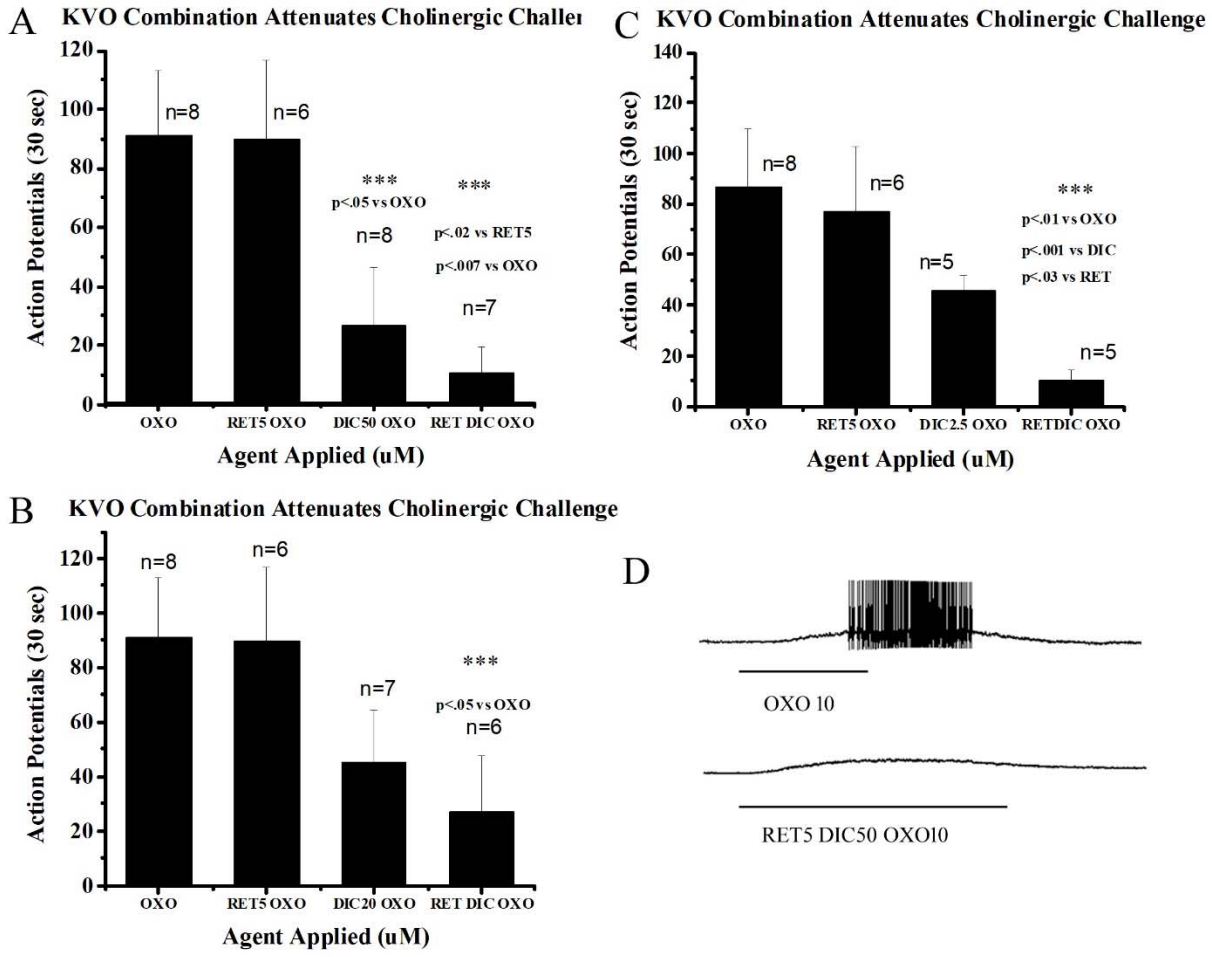

Figure 11. Combination KVOs Reduce Deep Tissue Nociceptor Discharge Consequent to a Cholinergic Challenge. A) The number of AP's evoked by 10 uM OXO were significantly reduced by RET 5 uM and DIC 50 uM. RET 5 uM had no influence. **B)** RET 5 uM and DIC 20 uM significantly reduced APs evoked by 10 uM OXO. Neither 5 uM or DIC 20 uM had any effect on AP discharge. **C**) RET 5 uM and DIC 2.5 uM significantly reduced APs evoked by 10 uM OXO. Neither 5 uM or DIC 2.5 uM had any effect on AP discharge **D)** Representative traces illustrate a powerful burst evoked by 10 uM OXO (upper trace). AP bursting was completely abrogated by the application of RET 5 uM and DIC 50 uM (2 min pretreatment; lower trace). Both RET and DIC were present during the OXO test.

As the combination of RET 5 and DIC 2.5 proved highly successful, we made additional tests in which RET was the main agent in the combination. The combination of RET 7.5 μM and DIC 1.25 μM was also very effective. This combination both significantly hyperpolarized nociceptor membranes (-3.06 +/- 0.76 mV; $p<.001$ vs Veh control) and drastically reduced action potential discharges (5.28 +/- 5.12 APs; median of 0 APs; $p<.003$ vs Veh control). Despite the further reduction of DIC concentration, there was no statistical difference between these two combinations (5/2.5 and 7.5/1.25) in either membrane hyperpolarization or action potential suppression. As pilot studies later revealed, it would be important to reduce DIC levels as much as possible during the behavioral testing phase of the project.

Specific Aim 1: Recovery from Pain Behaviors Using Multiple K_v7 Ion Channel Openers
Hypothesis: Multiple K_v7 openers will synergize to produce superior pain relief

Major Task 1: Dose Treatment Studies on Rats Exposed to GW Chemicals

Subtask 1: KVO combination Dose 1
Subtask 2: KVO combination Dose 2
Subtask 3: KVO combination Dose 3

All behavior studies were conducted in year 2.

This aim was designed to apply discoveries from Specific Aims 2 and 3 to identify KVO dose combinations as a treatment for rats that developed pain-signs following exposure to GW pesticides. Based upon our findings, 120 rats were exposed to GW chemicals (permethrin, DEET,

PB and chlorpyrifos or vehicle). Two distinct experiments were conducted to examine the effectiveness of RET DIC combinations (BEH1, BEH2). In each study KVO treatments were applied after pain-like signs had emerged. In BEH1, we tested the capacity of a 1:4 molar ratio of RET:DIC. This study followed from our observations in Specific Aims 1 and 2, that a 1:4 molar ratio (5 μM RET and 20 μM DIC) would amplify current, hyperpolarize membranes, and abrogate nociceptor action potentials. A pilot study was carried out to determine whether certain concentrations of RET or DIC would be tolerated by the rats. Free range behavior was assessed using an automated system (Accuscan Fusion) that recorded movements of rats in an activity cage. Rats were tested once per week (15 min) and treated by oral gavage (see Appendix, p. 56 for further details).

Behavior Study 1 (BEH1)

Following a 4 week exposure to GW chemicals (permethrin, DEET, PB and chlorpyrifos or vehicle), rats (n=48) developed signs of pain manifested as decreased movement and increased resting (1:4 molar ratio; figure12). Subsequently, 12 rats were treated with a combination of 2.5 mg RET and 10 mg DIC (oral gavage). Another 36 rats received either RET 2.5 only, DIC 10 only or DMSO vehicle. Twelve, non-exposed, rats also received DMSO. Two hours after gavage, free range behaviors were assessed. Only those rats receiving the combination treatment exhibited a reduction in pain-like behaviors (decreased rest times; figure 12C). Changes in rest times were relatively slight, but statistically significant. There were no significant changes in movement distance measures.

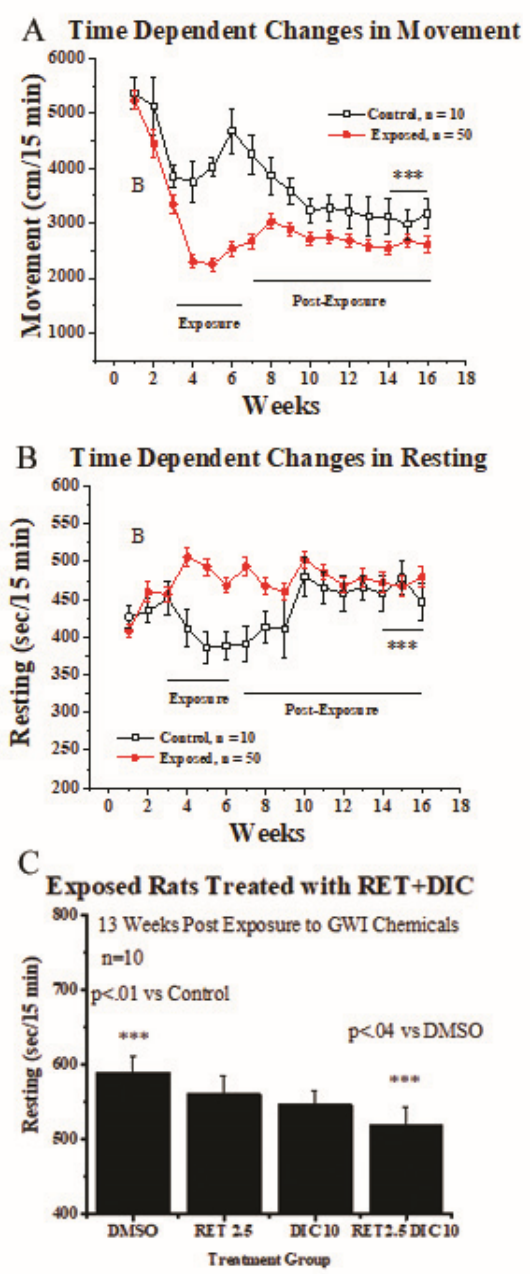

Figure 12. The Combination of RET and DIC (1:4) Reduced Pain-Like Behavior. A) Weekly progression of movement distance data before, during and following exposure to GW chemicals. **B)** Weekly progression of rest time scores before, during and following exposure to GW chemicals. **C)** The influence of single and combination treatments on rest time scores. B: baseline testing. *** significantly different by repeated measures ANOVA ($p<.02$ and $p<.002$*, rest and movement respectively). 2.5 mg/kg RET and 10 mg/kg DIC. The actual dose concentrations varied from these due to slight differences in molar weights for RET and DIC. All doses were administered by oral gavage 2 hours prior to testing.

Although this outcome was promising, repeated treatments with this combination of RET and DIC were not well tolerated. Two rats were found dead in their cages after a week of daily treatment at this level. One rat that received 10 mg/kg DIC alone also expired after a week of daily treatment. Other rats in these groups became ill and lethargic and were euthanized. The BEH1 study had to be terminated at this point. Although pilot studies at these doses were run prior to BEH1, we had not tested the pilot study rats for more than 5 days of consecutive treatments. Further pilot studies were then undertaken in order to find safe levels of DIC and RET+DIC combinations. The dosing strategies of the BEH2 study below were adjusted accordingly; weekly treatments were substituted for daily treatments as a safety precaution.

The BEH2 study was conducted identically to BEH1 with the main exceptions that treatments were started earlier in the post-exposure period and treatments were limited to one/week. Starting treatments at post 9 weeks (rather than post-13 weeks) allowed us to make serial dose combination studies. Guided by our cellular and molecular studies of Aims 2 and 3, above, we tested RET:DIC combinations reflecting the following molar ratios: 1) RET2/DIC1; 2) RET7/DIC1; and 3) RET1/DIC4). The actual mg/kg doses were governed, not only by molar dose ratios, but also by limits established by failed dosing strategies of BEH1 and subsequent pilot studies. The results of 4 dose combinations representing these molar ratios and dose level restrictions are presented in figure 13, below. Simply described, no beneficial effect of any dose combination could be identified when dose levels were restricted to concentrations below levels where significant side effects were present.

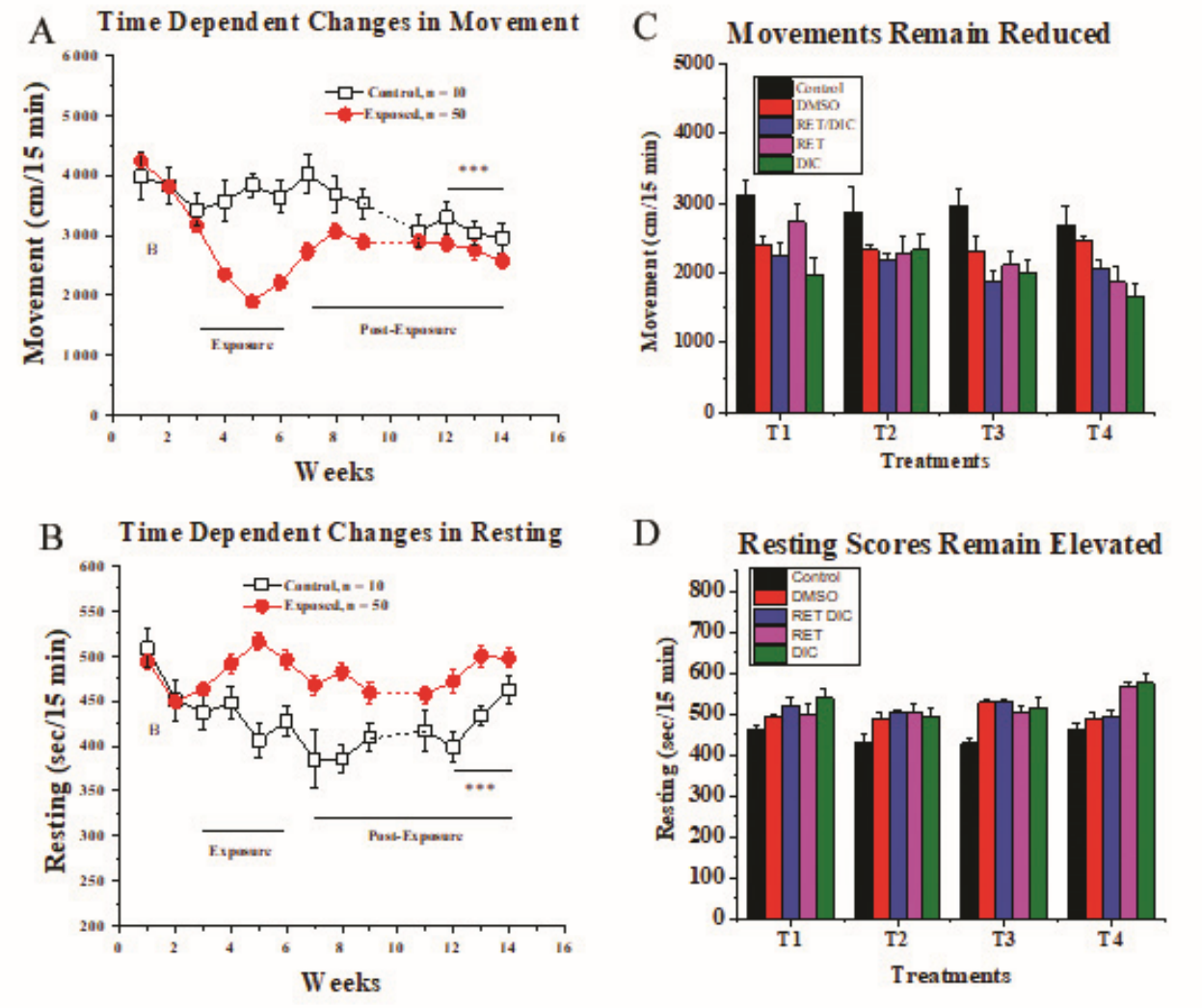

Figure 13. Multiple Dose Combinations of RET and DIC Fail to Modify Pain-Like Behaviors. **A)** Weekly progression of movement distance scores before, during and following exposure to GW chemicals. **B)** Weekly progression of rest time scores before, during and following exposure to GW chemicals. **C)** The influence of single and combination treatments on movement distance scores; **D)** The influence of single and combination treatmens on rest time scores. B: baseline testing. *** significantly different by repeated measure ANOVA ($p<.001$ and $p<.002$, rest and movement respectively). T1: 2.5 mg/kg RET and 1.25 mg/kg DIC; T2: 7 mg/kg RET and 1.25 mg/kg DIC; T3: 4.4 mg/kg RET and 0.62 mg/kg DIC; T4: 1.25 mg/kg RET; and 4 mg/kg DIC. Actual doses varied from these due to slight differences in molar weights for RET and DIC. All doses were administered by oral gavage 2 hours prior to testing.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

We applied for, and received, an extention to complete the remaining studies of Specific Aims 1 and 3. These studies were impacted by the COVID-19 pandemic. UF laboratories were closed for several months. During the 7 month extension, we will conduct cellular studies to optimized combinations of RET and MEC (Specific Aim 3). We will use these findings to conduct behavioral studies on rats that have been exposed to GW chemicals.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Two major drawbacks are associated with analgesics and palliatives. In some cases, they are insufficiently effective or non-effective for a particular type of pain. In other instances they may be effective, but have serious side effects, such as addiction, that limit or preclude their long term use.

We have demonstrated that the combination of RET/DIC or RET/MEC can greatly increase the activity of the K_v7 channel in deep tissue nociceptors. The amplification of activity should allow for a substantial reduction of doses. These effects were particularily powerful with the RET+MEC combination. The RET+DIC combination was significantly weaker than the RET+MEC combination in molecular studies, but was effective to a degree in cellular studies. In behavioral studies with RET+DIC, we found that we could not achieve an analgesic effect without using dose combinations that had severe side effects in rats. This does not mean that this combination approach to pain relief cannot work, but it does mean that it cannot work with DIC as the adjunctive drug. Successful development of experimental drugs that attack the preferential DIC binding site on $K_v7.2$ (Tatluian et al., 2001; Peretz et al., 2005), with decreased side effect risks, could be used in an effective pain relief combination treatment strategy for veterans suffering form GWI chronic pain. Hopefully, upcoming studies on the RET+MEC combination will prove safe and effective.

What was the impact on other disciplines?

Control of acute and chronic pains that afflict active duty soldiers, veterans and the general public, is a high priority field of research. Activation of K_v7 is a recognized target for drug development.

Hopefully, if KVO combination therapies prove useful in our animal model of GWI, other applications will be found for post-traumatic and disease related pain syndromes.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

We experienced more problems with side effects during the behavioral studies than we had anticipated. Therefore pilot studies will be expanded to identify safe dose concentrations to be used in the remaining studies.

Actual or anticipated problems or delays and actions or plans to resolve them

As part of the response to the viral pandemic that began in Wuhan, China, in November of 2019, the University of Florida ordered all research laboratories to close by March 30, 2020. For logistical and other reasons, our experiments ceased on March 15th. Research operations were reopened by the Dean on May 24th, 2020 under limited conditions and with restrictions imposed by guidelines issued by a special task force. However, our rat experiments could not be restarted immediately as new orders had to be placed for rats and certain time limited chemicals had to be replaced as they had expired during the shutdown. This resulted in a further delay of about 2 weeks. Damage to our water purifier systems, probably due to inactivity during the shutdown, caused further delays restarting experiments in June 2020 as we had to schedule repair service with external companies. Relatively normal operations resumed in early July, 2020.

In March 2020, due to separate issues (e.g., PPE and staffing) in the animal care facility (ACS), we were first asked to trim (50%) and later to eliminate all rats that were housed in the facility. Thirty-seven rats were euthanized prior to the March 30 shutdown.

We applied for, and received, a 7 month funded extension to complete the unfinished studies.

Changes that had a significant impact on expenditures

There were no new impacts on expenditures in year 2.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Rats did not tolerate some of the dose concentrations that were employed in Specific Aim 1; in particular, side effects were worse with dose combinations than with individual administration of RET and DIC. It is noteworthy that other laboratories have used even higher concentrations of individual doses of RET and DIC in their behavioral studies (Peretz, 2005; Khattab, et al., 2018). The difference may have been that these other labs did not use a daily dosing schedule, as we did, in the experiments of BEH1 (Khattab et al., 2018). Accordingly, we have extended pilot studies for combination effects and eliminated multiple weekly dosing.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

We have extended pilot studies for combination effects and eliminated multiple weekly dosing (see above).

Significant changes in use of biohazards and/or select agents

Nothing to report

6. Products

Publications, conference papers, and presentations

Journal publications

One manuscript was submitted to *Toxicology and Applied Pharmacology*.

Books or other non-periodical, one-time publications

Nothing to report

Other publications, conference papers, and presentations

Due to the shutdown of our laboratory and delays in restarting experiments, we were unable to put together enough data to confidently submit an abstract to the GWIRP conference that was held, in virtual form, in August of 2020. Our attempts to submit an abstract to the Society for Neuroscience conference (scheduled for October, 2020) were thwarted when the entire conference was cancelled due to COVID related restrictions.

We presented a poster to the Global Connectome virtual symposium in January 2021.

Combining K_v7 openers amplifies outward current and blocks deep tissue nociceptor discharge

Cooper, B.Y., Nutter, T.J, University of Florida College of Dentistry, USA, Dept. of Oral and Maxillofacial Surgery

Background: In order to repurpose analgesics for use in GWI (Gulf War Illness), we examined whether combinations of classic (Retigabine, RET) and secondary (Diclofenac, DIC; Meclofenamate, MEC) K_v7 channel openers (KVO) could retard deep tissue nociceptor activity.

Methods: Cells were harvested from the dorsal root ganglion of male rats (n=106). Delayed rectifier K^+ currents were isolated from other voltage sensitive currents using a K^+ isolation solution. A stepped voltage protocol was applied (-55 to -40 mV; V_h=-60 mV; 1500 msec) before and after KVO or vehicle (2 min; RET, DIC, MEC). Outward currents were assessed. Linopirdine (20 μM), was then applied for 3 minutes. The Linopirdine sensitive currents were determined by subtraction. The influence of KVOs was also assessed in current clamp studies where nociceptors were challenged by a muscarinic agonist known to evoke burst discharges. (Oxotremorine-M, OXO; 10 μM, 1 min). This is the first attempt to conduct such studies in confirmed nociceptors.

Results: KVOs differed widely with respect to their influence on nociceptors. MEC produced the highest maximal currents and broadest voltage sensitivity shifts (I_{max} = 6.3 pA/pF; p<.002 and p<.03, respectively; ED_{50}=60.7 μM; n=55). DIC and RET produced weaker maximal currents (I_{max} = 1.0 pA/pF DIC and 1.24 pA/pF RET; ED_{50}: 96.1 μM DIC and 62.0 μM RET; n=46 and 42, respectively), but voltage sensitivity shifts were observed only near current activation thresholds. Using subthreshold combinations of DIC (50 μM) and RET (5 μM), we observed that evoked currents were significantly amplified. The combination of DIC (50 μM) and RET (5 μM; n=8) outperformed individual applications of 90 μM DIC (p<.004) or 40 μM RET (p<.05), but there was little influence on voltage sensitivity. In contrast, the combination of DIC 70 μM and RET 2.5 μM (n=5) produced no significant current amplification in voltage clamp studies. In current clamp studies, both of the above combinations produced hyperpolarization (-3.5.48 +/- 1.58 mV and -6.44 +/-1.86 mV; p<.04 and .005; n=10 and 9) and significantly blocked OXO evoked action potential bursts (p<.03 and .001, respectively, n=8, 10 and 9).

Conclusions: When used in combinations, KVOs that bind to different K_v7 subunits ($K_v7.2$ vs $K_v7.3$) can amplify evoked currents through nociceptor K_v7 channels and block action potentials evoked by a cholinergic challenge. However, current clamp studies indicated that factors other than K_v7 opening might also be involved. Combinations of KVOs could be an effective strategy for treatment of deep tissue pain as experienced by veterans suffering from GWI.

We submitted, and had accepted, an abstract to the MHSRS symposium scheduled for August 2021. The abstract was scheduled for presentation but the meeting was cancelled in July 2021, due to COVID19.

Combining Classical and Non-classical K_v7 Openers to Develop Alternative Treatments for Deep Tissue Pain in a Rat Model of GWI

Cooper, B.Y., Flunker, L.K. and Nutter, T.J., University of Florida College of Dentistry, Dept. of Oral and Maxillofacial Surgery, Division of Neuroscience, Gainesville, Florida, USA.

Background: In order to repurpose analgesics for use in GWI (Gulf War Illness), we examined whether combinations of classical (Retigabine, RET) and non-classical (Diclofenac, DIC) K_v7 channel openers (KVO) could retard deep tissue nociceptor activity. RET and DIC bind to different portions of the K_v7 ion channel to affect its opening and consequent hyperpolarization of neural membranes (Peretz et al., 2005, 2007). We hypothesized that combinations of these agents would substantially improve net KVO potency while diminishing side effects of either agent. We had previously shown that a molar ratio of 1 μM RET to 4 μM DIC (1:4 RD) greatly amplified currents through isolated nociceptor K_v7 channels and suppressed nociceptor discharge to a cholinergic challenge (Cooper and Nutter, 2021). We now report behavioral effects of 1:4 RD ratio treatments in an animal model of GWI chronic pain and examine other molar ratios with reduced side effect potential.

Methods: In behavioral studies, rats were exposed to Gulf War (GW) agents known to produce a delayed pain-like syndrome (pyridostigmine, chlorpyrifos, permethrin and DEET; Flunker et al., 2017). Treatments with single and combined KVOs (1:4 RD) began 13 weeks after exposure to the GW chemicals (n=60). Free range ambulation and resting were assessed by an infrared recording system (Fusion Systems, AccuScan/Omnitech Instruments Inc). In cellular experiments, neurons were harvested from the dorsal root ganglion of male rats (n=19). The influence of KVOs were assessed in current clamp studies. Deep tissue nociceptors were identified and subsequently challenged by a muscarinic agonist known to evoke burst discharges (Oxotremorine-M, OXO; 10 μM, 1 min maximum exposure time; Cooper et al., 2018). The total number of action potentials (APs) evoked in 30 seconds were contrasted against single and combined KVO

applications (10 μM or 5 μM RET; 2.5 μM DIC; 2 min). Data were analyzed by Student's T-test (alpha set to .05).

Results: In behavioral studies, a 1:4 RD molar ratio significantly reduced resting behavior deficits at post-exposure week 13 ($p<.04$; n=10 and 10). Single KVO treatments (2.5 mg/kg RET; 10.5 mg/kg DIC) did not have any influence. However, with continued treatment, the 1:4 RD group developed considerable side effects that forced termination of the tests on post-week 14. Cellular studies were then conducted to assess the potency of alternative molar ratios on nociceptor membranes and AP discharge. In current clamp studies, RET and DIC combinations of 4:1 RD and 2:1 RD significantly hyperpolarized membranes relative to vehicle tested controls (1.93 +/- 0.67 mV vs -2.54 +/- 0.82, $p<.001$, n=7 and 7; -2.59 +/- 0.65 mV, $p<.001$, n=5 and 7; vehicle, 4:1 and 2:1 RD, respectively). When KVO combinations were presented prior to the OXO challenge, action potential bursts were also significantly reduced (89.4 +/- 19.3 APs vs 10.5 +/- 4.3 APs; $p<.009$, n=7 and 9; and 10.2 +/- 4.27 APs; $p<.02$, n=5 and 9; vehicle, 4:1 and 2:1 RD molar ratios, respectively). Combination dosing with 5 μM RET and 2.5 μM DIC were significantly superior than either agent alone ($p<.002$ and $p<.02$ vs 5 μM RET and 2.5 μM DIC, respectively). In contrast to previous studies, we did not observe any significant side effects of these combined treatments when they were administered to naïve rats tested in free range conditions (n=9). Studies on GW exposed rats are now underway.

Conclusions: When used in combination, KVOs that bind to different K_v7 subunits ($K_v7.2$ or $K_v7.3$; Peretz et al., 2005, 2007) hyperpolarize membranes and reduce nociceptor action potentials evoked by a cholinergic challenge. The combination of both agents produced significantly better outcomes than either agent tested independently. Combinations of KVOs could be an effective strategy for treatment of deep tissue pain as experienced by veterans suffering from GWI or deep penetrating wounds.

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Personnel:

Name: Brian Y. Cooper, Ph.D., College of Dentistry
Project role: Principal Investigator,
Researcher Identifier (ORCID ID): 0000-0002-7592-588X
Nearest person month worked: 12
Contribution to Project: Design, execution and analysis of physiology and behavior experiments; Preparation of Reports

Name: Thomas J. Nutter, Ph.D., College of Dentistry
Project Role: Biological Scientist IV
Researcher Identifier: none
Person Months: 12
Contribution to Project: Execution of physiology experiments

Name: Linda Flunker, MS, College of Dentistry
Role on Project: Biological Scientist III
Research Identifier: none

Person Months: 12
Contribution to Project: Preparation of solutions; Preparation of rats; ordering laboratory items; General laboratory duties

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. Special Reporting Requirements

Award Chart

GW180038: Optimizing Kv7 Channel Openers for Treatment of GWI Pain

PI: Brian Cooper, PhD, Univ. of Florida, Florida **Budget:** $1.028,939

Topic Area: Gulf War Illness Research Program 2018 **Mechanism:** Investigator Focused Research Award, Tier 2, Applied Research Program, W81XWH-18-GWIRP-IIFRA

Research Area(s): 1406-Pain **Award Status:** Funded, W81XWH-19-1-0667 9/01/2019-03/31/2022

Study Goals: Identify and Optimize FDA Approved Kv7 Channel Openers for Gulf War Illness Pain

Specific Aims: 1) Recovery from Pain Behaviors using multiple Kv7 Ion Channel Openers
2) Optimizing Molecular Synergisms of KVOs on Deep Tissue Nociceptor Kv7
3) Optimizing Functional (Cellular) Synergisms

Key Accomplishments and Outcomes:

Specific Aim 2: Molecular Tests of Conductance for Meclofenamate, Diclofenac and Retigabine
Outcome: Range of Conductance Established
Molecular Combination Testing of Retigabine and Diclofenac Completed
Outcome: Good Amplification of Molecular Activity
Molecular Combination Testing of Retigabine and Meclofenamate Completed
Outcome: Outstanding Amplification of Molecular Activity
Specific Aim 3: Cellular Combination Testing of Retigabine and Diclofenac Completed
Outcome: Partial to Complete Block of Nociceptor Activity
Cellular Combination Testing of Retigabine and Meclofenamate Ongoing
Specific Aim 1: Behavioral Testing of Retigabine and Diclofenac Completed
Outcome: Side Effects of Combinations Overwhelm Analgesic Benefits
Outcome: Analgesic Testing of Retigabine and Meclofenamate Ongoing

Publications: none to date
Patents: none to date
Funding Obtained: Start Date: 09/01/19

9. Appendices

STATEMENT OF WORK – November 7, 2018
PROPOSED START DATE April 15, 2019

Site 1: University of Florida
JHM Health Center
1600 SW Archer Rd
Gainesville, FL
32610
PI: Brian Y Cooper

Specific Aim 1: Recovery from Pain Behaviors Using Multiple K_v7 Ion Channel Openers	**Timeline**	**Site 1**	**Site 2**
Major Task 1: Dose Treatment Studies on Rats Exposed to GW Chemicals	Months		
Subtask 1: KVO combination Dose 1	12-17	Drs. Cooper and Nutter (n=60 rats)	
Subtask 2: KVO combination Dose 2	15-20	Drs. Cooper and Nutter (n=60 rats)	
Subtask 3: KVO combination Dose 3	18-23	Drs. Cooper and Nutter (n=60 rats)	
Milestone(s) Achieved: Full Recovery from Pain			
IACUC Approval: **Approved 07/2018-2021**	0	Dr. Cooper	
ACURO Approval	<2	Dr. Cooper	

Specific Aim 2 Optimizing Molecular Synergisms of KVOs on Deep Tissue Nociceptor K_v7	**Timeline**	**Site 1**	**Site 2**
Major Task 1: KVO Dose Response Testing on Nociceptor K_v7 Activity (in vitro molecular studies)	Months		
Subtask 1: Meclofenamate Dose Response	1-4	Dr. Cooper (n=25 rats)	
Subtask 2: Diclofenac Dose Response	1-4	Dr. Nutter (n=25 rats)	
Subtask 3: Celecoxib Dose Response	5-9	Dr. Cooper (n=25 rats)	
Subtask 4: Retigabine Dose Response	5-9	Dr. Nutter (n=25 rats)	
Milestone(s) Achieved: Peak Conductance Identified			
Major Task 2: KVO Synergisms on Nociceptor K_v7 Activity (in vitro molecular studies)			
Subtask 1: Retigabine and Meclofenamate	10-11	Dr. Cooper (n=15 rats)	
Subtask 2: Retigabine and Diclofenac	10	Dr. Nutter (n=15 rats)	
Subtask 3: Retigabine and Celecoxib	10-11	Dr. Cooper (n=15 rats)	
Milestone(s) Achieved: Peak Conductance Surpassed			

Specific Aim 3: Optimizing KVO Functional Synergisms	**Timeline**	**Site 1**	**Site 2**
Major Task 1: Cellular Synergisms of KVOs	Months		
Subtask 1: KVO combination Dose 1	12	Dr. Cooper (n=8 rats)	
Subtask 2: KVO combination Dose 2	13	Dr. Cooper (n=8 rats)	
Subtask 3: KVO combination Dose 3	14	Dr. Cooper (n=8 rats)	
Milestone(s) Achieved: Depolarization and Action			

Potential Reduction Optimized			

Methods

Electrophysiology

Subjects. In year 2, one hundred five (105) young adult male rats were used in physiology experiments. All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO (Animal Care and Use Review Office of the Army Medical Research and Materiel Command).

Preparation of Cells. Male Sprague-Dawley rats were anesthetized (Isoflurane) and euthanized by decapitation. The dorsal root ganglia were excised, digested in a solution containing, 2 mg/ml, type-A collagenase and 5 mg/ml Dispase II (Sigma-Aldrich). The procedure for digestion and plating of cells has been described, in detail, previously (Flunker et al., 2018). Isolated neurons were plated on 9, 35 mm Petri dishes. Cells 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. Experiments began 2 hours after plating. Only one cell was used per Petri dish, and recording experiments were completed within 10 hr after plating. Studies were conducted at room temperature (20° C).

Recording and Characterization of Skin, Muscle and Vascular Nociceptors. Whole cell recordings were made with an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 65-70% with Axopatch compensation circuitry. Whole cell resistance and capacitance were determined by the Clampex software utility. Recorded currents were sampled at 10-20 kHz and filtered at 2 kHz (Bessel filter).

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 our laboratory as well as others (Cardenas et al., 1995; Petruska et al., 2000; Petruska et al 2002; see also Xu et al., 2010; Ono et al., 2010). Characterizations were conducted in Tyrode's solution.

Once the whole cell mode was achieved, 3 voltage characterization protocols (CP1, CP2 and CP3), were used to classify medium sized neurons as type 5 muscle or type 8 vascular nociceptors (Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). The physiological signature of type 5 nociceptors included small I_H (<1.5 pA/pF; CP1), a high threshold I_A (0 mV; CP2) that exhibited a prolonged settling time (>50 msec) and a Na^+ current that was broad at its base (> 3.5 msec at 0 mV) and achieved maximum values at a voltage step to 0 mV (CP3). The physiological signature

of type 8 nociceptors included small I_H (<1.5 pA/pF; CP1), an I_A threshold of -20 mV with prolonged I_A settling time (>40 msec; CP2), and a Na^+ current that exhibited a broad base (>2.25 msec at 0 mV) and achieved maximum values at a voltage step to -10 mV (CP3). The main distinguishing feature between type 5 and type 8 cells was the 20 mV difference in the threshold of I_A. These physiological current signatures are very different from other medium sized neurons encountered in DRG recordings, and are visually obvious (Petruska et al., 2000; 2002). Cells not fitting the classification criteria of type 5 or 8 were discarded.

Voltage Clamp Studies

KVO Testing. KVOs were obtained from Sigma-Aldrich (Meclofenamate; Diclofenac) and Alomone Labs and AdooQ Biosciences (Retigabine Cl). Agents were prepared in stock solutions (50-200 mM; in DMSO or H_2O as needed). Stock solutions were kept at -20 °C. On the day of the experiment, solutions of 5-140 μM were prepared in a K^+ isolation solution for application to isolated deep tissue nociceptors (see below). Each tested cell received only one dose of KVO or vehicle. Typically, a given KVO and its vehicle were both tested on individual DRG neurons during a single day experiment.

After achieving the whole-cell mode, cells were characterized and classified at type 5 or type 8 nociceptors. Cells not fitting these criteria were rejected. Series resistance was compensated and a K^+ isolation (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) was applied, for 2 minutes, by close superfusion (1000 um).

In the presence of the K_{iso} solution, we assessed both the influence of a given KVO on K_v7 voltage sensitivity and the capacity to directly open K_v7 channels. For voltage sensitivity, and in the presence of the appropriate vehicle, a 1000 msec conditioning pulse of -70 mV was presented (V_h=-60 mV) followed immediately by a series of 5 mV steps (-55 to -40 mV; 1500 msec duration). We refer to this test as VS1. Subsequently, the KVO was applied for 2 min, at the V_h. A rapidly developing, dose dependent, outward current could be observed during KVO application.

KVO applications were made by close superfusion via a glass tube positioned approximately 1 mm from the cell. During superfusion, a series of brief pulse steps to -70 mV (10 ms) were applied to confirm that any outward current represented an increased cell membrane conductance. Immediately following superfusion of a KVO, the voltage sensitive protocol was presented a second time (VS2). To isolate the K_v7 specific VS current from other voltage sensitive K^+ currents that were present, we superfused the cell with the K_v7 specific inhibitor, linopirdine (20 μM; 3 min; Sigma-Aldrich). In the presence of linopirdine, the VS protocol was applied for a third time (VS3). All currents measured during VS protocols were leak corrected using the p/4 procedure. Prior to each presentation of the VS protocol, we checked the series resistance and corrected these adjustments as needed. Subsequently, the initial KV7 VS (VS1 and VS2), linopirdine sensitive, current was determined by subtraction.

The peak difference currents obtained were converted to conductances (G1 and G2) according to the formula: $I_L/(V_{step}-V_{rev})$. Where I_L is the measured peak of the linopirdine sensitive current; V_{step} is the applied test voltage and V_{rev} is the calculated reversal potential for K^+ (-86 mV). The influence of the KVO on voltage sensitivity was determined as the difference in conductance (G)

measured in the pre-KVO (G_1) and post-KVO test (G_2) conductances: $G_{diff}=G_2- G_1$. For statistical analysis, the G_{diff} was normalized to cell size, where cell size was determined by capacitive charging (pF).

Identical procedures were carried out using only vehicle applications for both VS1 and VS2 protocols (water (meclofenamate, retigabine), DMSO (diclofenac, celecoxib)). Statistical tests were made between the G_{diff} determined for cells tested in the presence of vehicle only contrasted with the G_{diff} determined in the presence of a KVO.

Current Clamp Studies

Membrane Excitability. Following cell characterization and classification, cells were brought into current clamp mode. Cells whose resting membrane potential did not fall between -55 and -65 mV were rejected if their RMP could not be brought into this range by injection of 10 pA. Type 8 and type 5 cells were tested by challenge with a muscarinic agonist (see Cooper et al., 2018). In our previous studies we had determined that rats exhibiting pain-like behaviors following exposure to GW neurotoxicants manifested increased depolarizations and burst discharges to a muscarinic agonist (MDBD; muscarine dependent burst discharge).

Muscarinic Dependent Burst Discharges

Following initial characterization, type 5 nociceptors were brought into current clamp mode.

After a 1 minute stabilization period, a solution containing vehicle or KVO combination was applied for 2 minutes. This was followed by a 2 minute application of the highly specific muscarinic agonist, Oxotremorine-M (OXO; 10 μM). KVOs (or vehicle) were co-applied with OXO. Membrane depolarization and action potential number were quantified off-line for statistical analysis. Comparisons were made between vehicle treated and OXO-KVO cases.

Behavioral Studies

Subjects. In year 2, one hundred thirty (130) young adult male rats were used in behavioral experiments. All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO (Animal Care and Use Review Office of the Army Medical Research and Materiel Command).

Exposure to GW Chemicals.

Over a period of 4 weeks, 98 rats were exposed to GW chemicals that we previously demonstrated could produce a complex of delayed pain-like signs (Flunker et al., 2017). These GRP E rats were exposed to the 4 GW chemicals: DEET (400 mg/kg; 50% ETOH), permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), and pyridostigmine bromide (PB; 13 mg/kg). GRP C, control, rats (n=22) were exposed only to agent vehicles using the identical procedures and volumes (ETOH, corn oil, water). During exposures, DEET and permethrin (ETOH) were applied, daily, to a 1 inch square shaved area of the upper

back between the forelimbs. Chlorpyrifos was administered by a subcutaneous injection (corn oil) once every 7 days. The dose of chlorpyrifos was intended to represent a net exposure to the potentially large and varied anticholinesterases that soldiers were exposed to in the Gulf theater (Binns et al., 2008). Chlorpyrifos was administered in a sterilized corn oil formulation that released the agent over several days (Smith et al., 2009). PB, in tap water, was administered once daily by oral gavage. The dose represented a standard military dose that was adjusted to account for faster pharmacokinetics in rodents (Birtley et al 1966; Husain et al., 1968; Aquilonius et al., 1980; Breyer-Pfaff et al., 1985). Rats were weighed once per week throughout the studies and doses were adjusted by body weight. Rats developed normally and showed none of the classic signs of organophosphate or pyrethroid toxicity. Two rats (non-exposed) were dropped from the study due to unrelated issues.

Treatment Studies

At least eight weeks after exposure to GW chemicals, GRP E rats were randomnly divided into groups of 10-12 each. Two hours prior to behavioral testing: 1) GRP R rats were treated with RET; 2) GRP D rats were treated with DIC; 3) GRP RD rats were treated with the combination of the above dosages; 4) GRP E DMSO , and unexposed GRP C rats were treated with DMSO only (oral gavage). Behaviors testing occurred 2 hours after treatments.

Assessment of Pain Behaviors. Open field testing was conducted before, during and after chemical exposures. Upon arrival, rats were acclimated to the behavioral procedures for 2 weeks. Activity levels (movement distance (cm), average movement rate (cm/sec), and rest time duration (sec)) were recorded automatically by infrared sensors in a modified activity box (15 min test period; Fusion Systems, AccuScan/Omnitech Instruments Inc.). The 35 by 40 cm test chamber was modified to prevent rearing behaviors. The chamber was cleaned after each 15 minute test period. Behavioral tests were conducted on both chemically exposed (permethrin, chlorpyrifos, DEET, PB) and vehicle treated (ETOH, corn oil, water) animals over an identical time course. Each rat was tested once per week on the behavioral tasks.

Statistics. A repeated measures ANOVA (QIMacros) was used to assess the influence of Chemical Exposure on dependent measures of movement distance (cm/15 min), average movement rate (cm/sec) and rest duration (sec/15 min). Statistical comparisons were made between vehicle exposed and neurotoxicant chemical exposed rats. Student's T test was used to assess the influence of treatments (KVOs) and also to contrast the amplitude of K_v7 currents and voltage dependent conductance shifts that were measured in neurons from KVO exposed or vehicle exposed nociceptors. The alpha level was set at .05.

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Abstracts

Combining K_v7 openers amplifies outward current and blocks deep tissue nociceptor discharge

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Background: In order to repurpose analgesics for use in GWI (Gulf War Illness), we examined whether combinations of classic (Retigabine, RET) and secondary (Diclofenac, DIC; Meclofenamate, MEC) K_v7 channel openers (KVO) could retard deep tissue nociceptor activity.

Methods: Cells were harvested from the dorsal root ganglion of male rats (n=106). Delayed rectifier K^+ currents were isolated from other voltage sensitive currents using a K^+ isolation solution. A stepped voltage protocol was applied (-55 to -40 mV; V_h=-60 mV; 1500 msec) before and after KVO or vehicle (2 min; RET, DIC, MEC). Outward currents were assessed. Linopirdine (20 μM), was then applied for 3 minutes. The Linopirdine sensitive currents were determined by subtraction. The influence of KVOs was also assessed in current clamp studies where nociceptors were challenged by a muscarinic agonist known to evoke burst discharges. (Oxotremorine-M, OXO; 10 μM, 1 min). This is the first attempt to conduct such studies in confirmed nociceptors.

Results: KVOs differed widely with respect to their influence on nociceptors. MEC produced the highest maximal currents and broadest voltage sensitivity shifts (I_{max} = 6.3 pA/pF; p<.002 and p<.03, respectively; ED_{50}=60.7 μM; n=55). DIC and RET produced weaker maximal currents (I_{max} = 1.0 pA/pF DIC and 1.24 pA/pF RET; ED_{50}: 96.1 uM DIC and 62.0 uM RET; n=46 and 42, respectively), but voltage sensitivity shifts were observed only near current activation thresholds. Using subthreshold combinations of DIC (50 μM) and RET (5 μM), we observed that evoked currents were significantly amplified. The combination of DIC (50 μM) and RET (5 μM; n=8) outperformed individual applications of 90 uM DIC (p<.004) or 40 μM RET (p<.05), but there was little influence on voltage sensitivity. In contrast, the combination of DIC 70 μM and RET 2.5 μM (n=5) produced no significant current amplification in voltage clamp studies. In current clamp studies, both of the above combinations produced hyperpolarization (-3.5.48 +/- 1.58 mV and -6.44 +/-1.86 mV; p<.04 and .005; n=10 and 9) and significantly blocked OXO evoked action potential bursts (p<.03 and .001, respectively, n=8, 10 and 9).

Conclusions: When used in combinations, KVOs that bind to different K_v7 subunits ($K_v7.2$ vs $K_v7.3$) can amplify evoked currents through nociceptor K_v7 channels and block action potentials evoked by a cholinergic challenge. However, current clamp studies indicated that factors other than K_v7 opening might also be involved. Combinations of KVOs could be an effective strategy for treatment of deep tissue pain as experienced by veterans suffering from GWI.

Combining Classical and Non-classical K_v7 Openers to Develop Alternative Treatments for Deep Tissue Pain in a Rat Model of GWI

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Background: In order to repurpose analgesics for use in GWI (Gulf War Illness), we examined whether combinations of classical (Retigabine, RET) and non-classical (Diclofenac, DIC) K_v7 channel openers (KVO) could retard deep tissue nociceptor activity. RET and DIC bind to different portions of the K_v7 ion channel to affect its opening and consequent hyperpolarization of neural membranes (Peretz et al., 2005, 2007). We hypothesized that combinations of these agents would substantially improve net KVO potency while diminishing side effects of either agent. We had previously shown that a molar ratio of 1 uM RET to 4 uM DIC (1:4 RD) greatly amplified currents through isolated nociceptor K_v7 channels and suppressed nociceptor discharge to a cholinergic challenge (Cooper and Nutter, 2021). We now report behavioral effects of 1:4 RD ratio treatments in an animal model of GWI chronic pain and examine other molar ratios with reduced side effect potential.

Methods: In behavioral studies, rats were exposed to Gulf War (GW) agents known to produce a delayed pain-like syndrome (pyridostigmine, chlorpyrifos, permethrin and DEET; Flunker et al., 2017). Treatments with single and combined KVOs (1:4 RD) began 13 weeks after exposure to the GW chemicals (n=60). Free range ambulation and resting were assessed by an infrared recording system (Fusion Systems, AccuScan/Omnitech Instruments Inc). In cellular experiments, neurons were harvested from the dorsal root ganglion of male rats (n=19). The influence of KVOs were assessed in current clamp studies. Deep tissue nociceptors were identified and subsequently challenged by a muscarinic agonist known to evoke burst discharges (Oxotremorine-M, OXO; 10 μM, 1 min maximum exposure time; Cooper et al., 2018). The total number of action potentials (APs) evoked in 30 seconds were contrasted against single and combined KVO applications (10 μM or 5 μM RET; 2.5 μM DIC; 2 min). Data were analyzed by Student's T-test (alpha set to .05).

Results: In behavioral studies, a 1:4 RD molar ratio significantly reduced resting behavior deficits at post-exposure week 13 ($p<.04$; n=10 and 10). Single KVO treatments (2.5 mg/kg RET; 10.5 mg/kg DIC) did not have any influence. However, with continued treatment, the 1:4 RD group developed considerable side effects that forced termination of the tests on post-week 14. Cellular studies were then conducted to assess the potency of alternative molar ratios on nociceptor membranes and AP discharge. In current clamp studies, RET and DIC combinations of 4:1 RD and 2:1 RD significantly hyperpolarized membranes relative to vehicle tested controls (1.93 +/- 0.67 mV vs -2.54 +/- 0.82, $p<.001$, n=7 and 7; -2.59 +/- 0.65 mV, $p<.001$, n=5 and 7; vehicle, 4:1 and 2:1 RD, respectively). When KVO combinations were presented prior to the OXO challenge, action potential bursts were also significantly reduced (89.4 +/- 19.3 APs vs 10.5 +/- 4.3 APs; $p<.009$, n=7 and 9; and 10.2 +/- 4.27 APs; $p<.02$, n=5 and 9; vehicle, 4:1 and 2:1 RD molar ratios, respectively). Combination dosing with 5 μM RET and 2.5 μM DIC were significantly superior than either agent alone ($p<.002$ and $p<.02$ vs 5 μM RET and 2.5 μM DIC, respectively). In contrast

to previous studies, we did not observe any significant side effects of these combined treatments when they were administered to naïve rats tested in free range conditions (n=9). Studies on GW exposed rats are now underway.

Conclusions: When used in combination, KVOs that bind to different K_v7 subunits ($K_v7.2$ or $K_v7.3$; Peretz et al., 2005, 2007) hyperpolarize membranes and reduce nociceptor action potentials evoked by a cholinergic challenge. The combination of both agents produced significantly better outcomes than either agent tested independently. Combinations of KVOs could be an effective strategy for treatment of deep tissue pain as experienced by veterans suffering from GWI or deep penetrating wounds.

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Development of KVO Treatment Strategies for Chronic Pain in a Rat Model of Gulf War Illness

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Abstract

We examined whether combinations of K_v7 channel openers could be effective modifiers of deep tissue nociceptor activity; and whether such combinations could then be optimized for use as safe analgesics for pain-like signs that developed in a rat model of GWI (Gulf War Illness) pain. Voltage clamp experiments were performed on subclassified nociceptors isolated from rat DRG (dorsal root ganglion). A stepped voltage protocol was applied (-55 to -40 mV; V_h=-60 mV; 1500 msec) and K_v7 evoked currents were subsequently isolated by linopirdine subtraction. Directly activated and voltage activated K^+ currents were characterized in the presence and absence of Retigabine (5-100 μM) and/or Diclofenac (50-140 μM). Retigabine produced substantial voltage dependent effects and a maximal sustained current of 1.14 pA/pF ± 0.15 (ED_{50}: 62.7 ± 13.7 μM). Diclofenac produced weak voltage dependent effects but a similar maximum sustained current of 1.01 ± 0.26 pA/pF (ED_{50}: 93.2 ± 8.99 μM). Combinations of Retigabine and Diclofenac substantially amplified resting currents but had little effect on voltage dependence. Using a cholinergic challenge test (Oxotremorine, 10 μM) associated with our GWI rat model, combinations of Retigabine (5 uM) and Diclofenac (2.5, 20 and 50 μM) substantially reduced or totally abrogated action potential discharge to the cholinergic challenge. When combinations of Retigabine and Diclofenac were used to relieve pain-signs in our rat model of GWI, only those combinations associated with serious subacute side effects could relieve pain-like behaviors.

Introduction

Gulf war illness (GWI) is a multi-symptom disease affecting ~30% of veterans of the 1991 Persian Gulf War (ODS; Operation Desert Storm). Although usually associated with cognitive symptoms, GWI clusters into 3 major syndromes that include chronic pain, confusion-ataxia as well as cognitive deficits (Haley et al., 1997). Those that suffer from chronic pain manifest an unusual variety of symptoms that defy simple explanation. These include combinations of muscle, back, joint, abdominal and headache pains (Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013; White et al., 2016; Haines et al., 2017; Maule et al., 2018; Porter et al., 2018; Dursa et al., 2019). After more than two decades, an effective treatment for GWI has not been identified.

There is substantial evidence that the symptoms of GWI arose from exposure to multiple insecticides and other neurotoxicants that were present in the Gulf theater (Binns et al., 20008; RAC2014; Naughton and Terry, 2018; Nizamutdinov et al., 2018; Michalovicz et al., 2018; Dickey et al., 2021). These agents can have direct (pyrethroids) or indirect (organophosphates) interactions with pain system ion channels (Na_v, K_v7; TRPA1; Narahashi et al., 1998; Soderlund et al., 2002; Jiang et al., 2013; Ding et al., 2017; Cooper et al., 2018). Over the last several years, our laboratory developed and refined an animal model of GWI pain (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al. 2015). We demonstrated that a 4 week exposure to permethrin, chlorpyrifos and PB (pyridostigmine bromide) produced a delayed manifestation of pain-like

ambulation deficits that persisted at least 12 weeks after exposures had ceased (Nutter et al., 2015). When the 3 agent exposure set was augmented with DEET, pain behaviors were more robust and were maintained out to 24 weeks post-exposure (Flunker et al., 2017). The influence of DEET on rat pain behaviors mirrored the unique association of DEET with pain symptoms of GW veterans (Haley and Kurt, 1997). Although DEET was shown to be a magnifying factor, only the anticholinesterases (PB and chlorpyrifos) were shown to be necessary for the development of delayed pain-like behaviors (Flunker et al., 2017).

In an effort to repurpose current and former FDA approved drugs for treatment of GWI pain, we tested the capacity of a specific K_v7 opener (KVO), Retigabine (RET), to reverse an established pattern of chronic pain behavior in rats 9-12 weeks following exposure to GWI chemicals (Cooper et al., 2018). KVOs were chosen because: 1) RET, and other KVOs, reduce evoked and spontaneous reactivity of central and peripheral neurons (Peretz et al., 2005; Abd-Elsayed et al., 2015; Du et al., 2017; Wu et al., 2017; Yu et al., 2018) and pain related behaviors in rodents (Abd-Elsayed et al., 2015: Teng et al., 2016; Wu et al., 2017; Yu et al., 2018); 2) K_v7 channels modulate muscarinic burst discharges (MDBD) that we have linked to GW pesticide exposures (Cooper et al., 2018); and 3) Retigabine and Flupirtine have a demonstrated capacity relieve some forms of chronic pain in humans (Luben et al., 1994; Worz et al., 1996; Uberall et al., 2011; Mishra et al., 2013; Uberall et al., 2013; Worz, 2014; Djouhri et al., 2019; see also Blackburn-Monro et al., 2005). In a test of the analgesic potential of RET in our model of GWI pain, we found that a daily dose of RET (7.0 mg/kg; 4 weeks), reversed established ambulation deficits that emerged 9 weeks following exposure to GW neurotoxicants. While pain-like behaviors significantly and rapidly

diminished, the improvement was transitory; and pain signs gradually returned over the four week treatment period (Cooper et al., 2018).

In addition to classical KVO's like RET and Flupirtine, certain FDA approved cyclooxygenase inhibitors (Diclofenac; Meclofenamate; Celecoxib) also have the capacity to open K_v7 channels (Peretz et al., 2005: Bruggermann et al., 2010; Frolov and Singh, 2011). Given that a classic KVO, Retigabine, had some effectiveness in our model of GWI pain, it is possible that these secondary KVOs could prove effective, as well. Moreover, combinations of classic and secondary KVOs bind to distinct subunits of the K_v7 ion channel, and when presented in combination can promote both direct and voltage dependent activation of K_v7 channels ($K_v7.2/K_v7.3$) expressed in CHO cells (Peretz et al., 2005).

In the experiments described below, we examined whether combinations of primary and secondary KVOs: 1) optimized activity of K_v7 channels expressed in deep tissue nociceptors; 2) suppressed muscarinic coupled burst discharges associated with GW neurotoxicants (Cooper et al., 2018); and 3) relieved pain-like behaviors in rats exposed to GW neurotoxicants.

Methods

Subjects

One hundred and five (105) young adult male rats were used in physiology experiments. An additional 120 male rats were used in the behavioral studies. Of the latter, 96 were exposed to the GW neurotoxicants. All rats were obtained from Envigo (Sprague-Dawley) and weighed 90-110

grams on arrival. Terminal weights of behavior study rats did not differ significantly in the chemical exposure group (434 vs 420 grams; control and exposed, respectively). All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO (Animal Care and Use Review Office of the Army Medical Research and Materiel Command). Two behavioral study rats developed unrelated health issues and were euthanized. There were no signs of acute pesticide toxicity typically associated with permethrin or chlorpyrifos during the execution of these studies.

Electrophysiology

Preparation of Cells. Male Sprague-Dawley rats were anesthetized (Isoflurane) and euthanized by decapitation (Harvard Apparatus). The dorsal root ganglia were excised, digested in a solution containing, 2 mg/ml, type-A collagenase and 5 mg/ml Dispase II (Sigma-Aldrich). The procedure for digestion and plating of cells has been described, in detail, previously (Flunker et al., 2018). Isolated neurons were plated on 9, 35 mm Petri dishes. Cells 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. Experiments began 2 hours after plating. Only one cell was used per Petri dish, and recording experiments were completed within 8 hr after plating. Studies were conducted at room temperature (20° C).

Recording and Characterization of Neurons. Whole cell recordings were made with an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 65-70% with Axopatch compensation circuitry. Whole cell resistance and capacitance were determined by the Clampex software utility. Recorded currents were sampled at 10 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 our laboratory, as well as others (Cardenas et al., 1995; Petruska et al., 2000; Petruska et al 2002; see also Xu et al., 2010; Ono et al., 2010). Characterizations were conducted in Tyrode's solution without compensation.

Once the whole cell mode was achieved, 3 voltage characterization protocols (CP1, CP2 and CP3), were used to classify medium sized neurons as type 5 muscle or type 8 vascular nociceptors (Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). The physiological signature of type 5 nociceptors included small I_H (<2 pA/pF; CP1), a high threshold I_A (0 mV; CP2) that exhibited a prolonged settling time (>50 msec) and a Na^+ current that was broad at its base (> 3.0 msec at 0 mV) and achieved maximum values at a voltage step to 0 mV; CP3). The physiological signature of type 8 nociceptors included small I_H (<2.0 pA/pF; CP1), an I_A threshold of -20 mV with prolonged I_A settling time (>40 msec; CP2), and a Na^+ current that exhibited a broad base (>2.5 msec at 0 mV) and achieved maximum values at a voltage step to -10 mV; CP3). The main

distinguishing feature between type 5 and type 8 cells was the 20 mV difference in the threshold of I_A. These physiological current signatures are very different from other medium sized neurons encountered in DRG recordings, and are visually obvious (Petruska et al., 2000; 2002). Cells not fitting the classification criteria of type 5 or 8 were discarded.

Voltage Clamp Studies. KVOs were obtained Sigma-Aldrich (Diclofenac; Retigabine), Alomone Labs and AdooQ Bioscience (Retigabine). Agents were prepared in stock solutions (50-200 mM; in DMSO or H_2O as needed). Stock solutions were kept at -20 °C. On the day of the experiment, solutions were prepared in a K^+ isolation solution for application to isolated deep tissue nociceptors (see below). Each tested cell received only one dose of KVO or vehicle.

After achieving the whole-cell mode, cells were characterized and classified as type 5 or type 8 nociceptors. Series resistance was compensated and a K^+ isolation (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 HCL) was applied, for 2 minutes, by close superfusion (1000 um).

In the presence of the K_{iso} solution, we assessed both the influence of a given KVO on K_v7 voltage sensitivity and the capacity to directly open K_v7 channels. For voltage sensitivity, and in the presence of the appropriate vehicle, a 1000 msec conditioning pulse of -70 mV was presented (V_h=-60 mV) that was followed immediately by a series of 5 mV steps (-55 to -40 mV; 1500 msec duration). We refer to this test as VS1. Subsequently, the KVO was applied for 2 min, at the V_h. A rapidly developing outward current could be observed during KVO application. During

superfusion, a series of brief pulses steps to -70 mV (10 ms) were applied to confirm that any outward current represented an increased cell membrane conductance. Immediately following superfusion of a KVO, the voltage sensitive protocol was presented a second time (VS2). To isolate the K_v7 specific VS current from other VS K^+ currents that were present, we superfused the K_v7 specific inhibitor, linopirdine (20 μM; 3 min; Sigma-Aldrich). In the presence of linopirdine, the VS protocol was applied for a third time (VS3). All currents measured during VS protocols were leak corrected using the p/4 procedure. Prior to each presentation of the VS protocol, we checked the series resistance and corrected these adjustments as needed. Subsequently, the initial K_v7 VS current was determined by subtraction as the linopirdine sensitive current at each voltage step (-55 to -40 mV):

(VS1-VS3)

The KVO modified VS current was determined by subtraction as the linopirdine sensitive current:

(VS2-VS3)

The peak difference currents obtained were converted to conductances (G1 and G2) according to the formula: $G=I_{peak}/(V_m-V_{rev})$, where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be -86 mV. The influence of the KVO on voltage sensitivity was determined as:

$G_{diff}=G2- G1$

For analysis, the G_{diff} was normalized to cell size, where cell size was determined by capacitive charging (pF).

For the influence of vehicles, identical procedures were carried out using only vehicle applications for both VS1 and VS2 protocols (water for RET; DMSO for DIC). Statistical tests were made between the G_{diff} determined for cells tested in the presence of vehicle only contrasted with the G_{diff} determined in the presence of a KVO. Statistical comparisons were made between cells grouped by voltage step and KVO dose versus vehicle tested controls.

Analyses of KVO evoked currents were conducted on the net change in the current produced by application of KVO for two minutes relative to those shifts present during application of the appropriate vehicle. Currents were normalized for cell capacitance.

Current Clamp Studies. Following initial characterization, nociceptors were brought into current clamp mode. After 2 minutes, a solution containing vehicle or KVO combination was applied for 2 minutes. This was followed by a 1 minute application of the highly specific muscarinic agonist, Oxotremorine-M (OXO, 10 μM). KVOs (or vehicle) we co-applied with OXO. The resting membrane potential was recorded continuously. Application of OXO is known to produce a rapid depolarization that is typically accompanied by a powerful burst discharge (Cooper et al., 2018). Comparisons were made between vehicle treated and OXO-KVO cases.

Behavioral Studies

Assessment of Pain Behaviors. Open field testing was conducted before, during and after chemical exposures. Upon arrival, rats were acclimated to the behavioral procedures for 2 weeks. Activity levels (movement distance (cm)) and rest time duration (sec)) were recorded automatically by infrared sensors in a modified activity box (15 min test period; Fusion Systems, AccuScan/Omnitech Instruments Inc.). The 35 by 40 cm test chamber was modified to prevent rearing behaviors. The chamber was cleaned after each 15 minute test period. Behavioral tests were conducted on both chemically exposed (permethrin, chlorpyrifos, DEET, PB) and vehicle exposed (ETOH, corn oil, water) animals over an identical time course. Each rat was tested once per week on the behavioral tasks.

Exposure Protocol. Over a period of 4 weeks, rats were exposed to GW chemicals (or vehicles) that we previously demonstrated could produce a complex of pain-like signs (Flunker et al., 2017). EXP rats were exposed to the 4 GW chemicals: DEET (400 mg/kg; 50% ETOH), permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), and pyridostigmine bromide (PB; 13 mg/kg). Non-exposed, control, rats received only agent vehicles using the identical procedures and volumes (ETOH, corn oil, water). During exposures, DEET and permethrin (ETOH) were applied, daily to a 1 inch square shaved area of the upper back between the forelimbs. Chlorpyrifos was administered by a subcutaneous injection (corn oil) once every 7 days. The dose of chlorpyrifos was intended to represent a net exposure to the potentially large and varied anticholinesterases that soldiers were exposed to in the Gulf theater (Binns et al., 2008). Chlorpyrifos was administered in a sterilized corn oil formulation that released the agent over several days (Smith et al., 2009). PB, in tap water, was administered once

daily by oral gavage. The dose represented a standard military dose that was adjusted to account for faster pharmacokinetics in rodents (Birtley et al 1966; Husain et al., 1968; Aquilonius et al., 1980; Breyer-Pfaff et al., 1985). Rats were weighed once per week throughout the studies and doses were adjusted by body weight. Rats developed normally and showed none of the classic signs of organophosphate or pyrethroid toxicity.

Treatment Protocol. At least eight weeks after exposure to GW chemicals, the toxicant exposed rats were divided into 4 groups of 12 each. Two hours prior to behavioral testing: 1) GRP R rats were treated with RET; 2) GRP D rats were treated DIC; 3) GRP RD rats were treated with the combination of the above dosages; 4) GRP DMSO, and unexposed GRP C rats were treated with DMSO only (oral gavage). Behavioral testing occurred 2 hours after treatments.

Statistics. A repeated measures ANOVA (QIMacros) was used to assess the influence of Chemical Exposure on dependent measures of movement distance (cm/15 min), and rest duration (sec/15 min). Statistical comparisons were made between vehicle exposed and neurotoxicant chemical exposed groups. Student's T test was used to assess the influence of treatments (KVOs) and also to contrast the amplitude of K_V7 currents and voltage dependent conductance shifts that were measured in neurons from KVO exposed or vehicle exposed nociceptors. The alpha level was set at .05.

Results

Voltage Clamp Studies

In the first series of experiments we examined the capacity of KVOs, diclofenac (DIC) and retigabine (RET), to alter voltage dependence and/or produce standing K^+ currents in deep tissue nociceptors. Studies were conducted in a K_{iso} solution in identified classes of deep tissue nociceptors that our laboratory has associated with GWI chemical maladaptations (Petruska et al., 2000, 2002; Cooper et al., 2018).

Diclofenac. Voltage shifts from -55 to -40 mV were applied in the presence and absence of DIC (see Methods). K_v7 currents were isolated by subtraction using linopirdine. Following cell characterization, 50 to 140 μM DIC was applied by close superfusion. At 60 μM there was a substantial influence of DIC on voltage sensitivity. However, as doses were increased, little influence on voltage dependent opening was detected (figure 1A, B, C, D). At doses at which voltage shifts were realized (60 μM), standing currents were not detected (figure 2). Above this dose, DIC proved to be a reliable activator of K_v7. At 80 μM, significant K^+ currents developed rapidly and grew steadily through 120 μM (figure 2). At 120 μM K_v7 current appeared to be maximal (1.01 ± 0.26 pA/pF). Dose-current data fits indicated an ED_{50} of 93.2 ± 8.99 μM. All standing K_v7 current was fully reversed by application of linopirdine (20 μM).

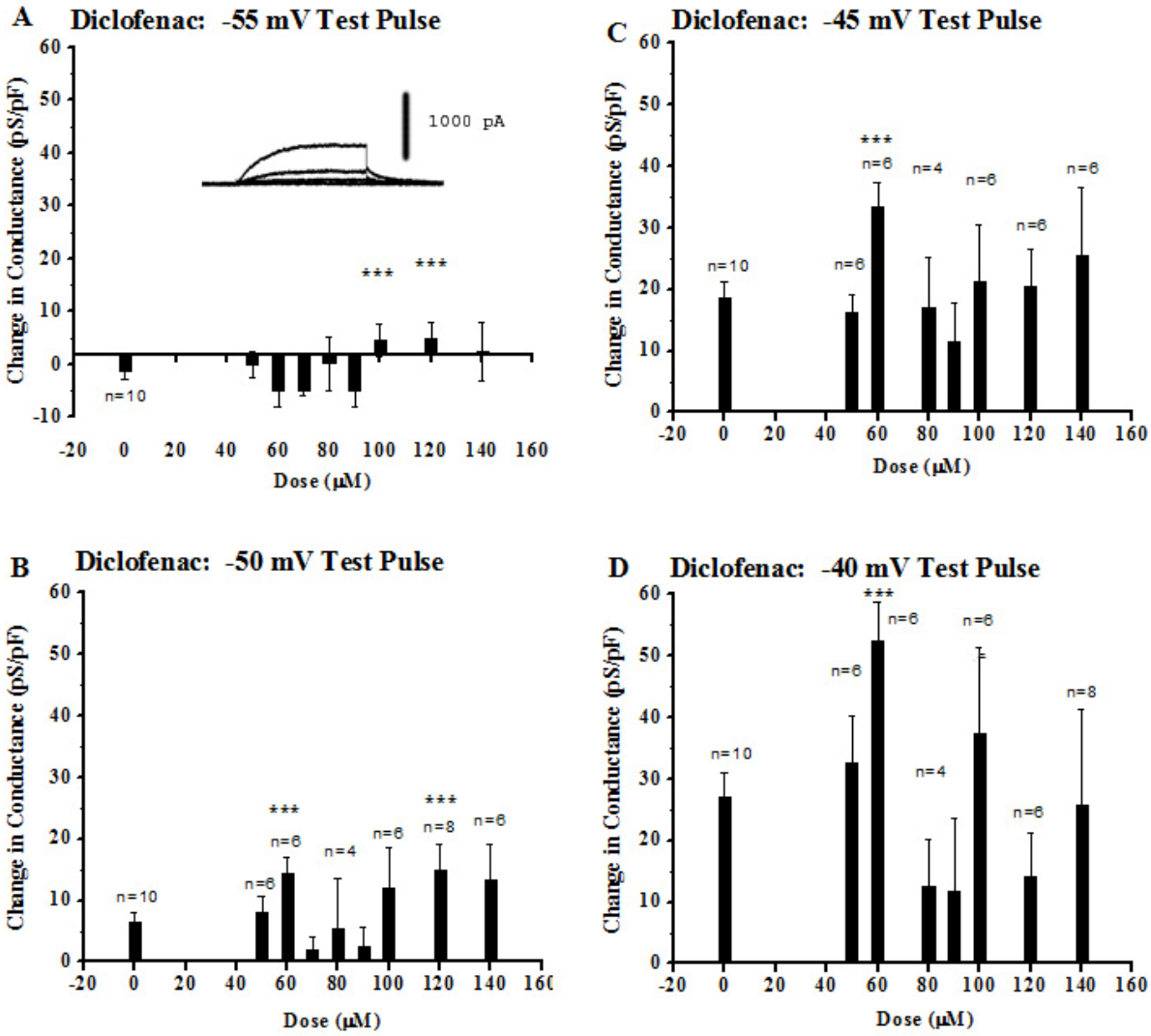

Figure 1. The Influence of Diclofenac on K_v7 Voltage Sensitivity. DIC had a narrow effect on the voltage sensitivity of K_v7. **A)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -55 mV (V_h=-60 mV). **B)**. The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -50 mV (V_h=-60 mV). **C)**. The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -45 mV (V_h=-60 mV). **D)**. The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -40 mV (V_h=-60 mV). Data collected from voltage clamp experiments in a K^+ isolation solution. Only one dose was applied per cell. Statistical comparisons were made between vehicle-vehicle and vehicle-DIC tests. Vehicle: DMSO; *** p<.05 or greater

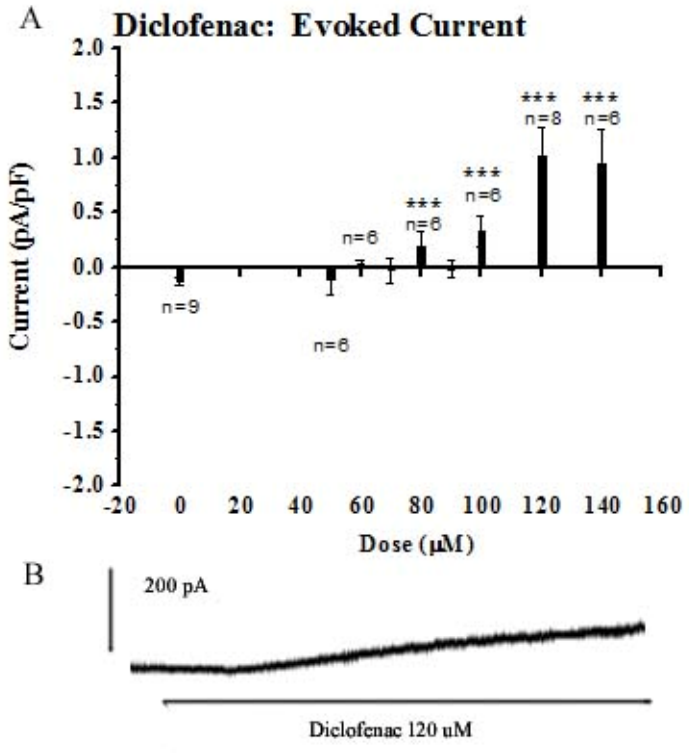

Figure 2. The Effect of Diclofenac on Resting Currents Mediated by K_v7. DIC opened K_v7 channels without a shift in applied voltage. Relatively small outward (hyperpolarizing) currents were observed. **A)** Average normalized current produced at -60 mV (V_h=-60 mV) following a 2 minute application of DIC. Evoked currents increased in proportion to the dose applied, but reached a maximum at 120 μM (1.01 ± 0.26 pA/pF). Current was measured at the end of the 2 min application of DIC. **B)** A typical current trace produced by DIC. Currents grew slowly but were stable within 2 minutes. Data collected from voltage clamp experiments conducted in a K^+ isolation solution. Each neuron was exposed to only 1 dose. Statistical comparisons were made between vehicle and DIC tests. Vehicle: DMSO; *** p<.05 or greater.

Retigabine. Using identical methods, we examined the influence of RET on voltage dependent and direct activation of K_v7. In contrast to DIC, RET amplified the voltage sensitivity of K_v7 at doses as low as 5 μM (figure 5). A persistent increase in resting current could not be demonstrated at this dose. As doses increased to 40 μM, Voltage dependent conductance shifts were retained, but fell of as resting currents tended to dominate the effect. Persistent resting currents were observed at doses of 20 to 120 μM; however some transient currents were present at 5 and 10 μM. Transient currents reached a relatively rapid peak but subsided within the 2 minute test period. An ED_{50} of 62.7 ± 13.7 μM was determined based upon currents present at the 2 min timepoint. Evoked currents reached a maximum at 100 μM (1.14 ± 0.15 pA/pF; figure 4). Resting currents produced by application of RET were completely reversed by linopirdine (20 μM). Although the influence on voltage sensitivity and ED_{50} differed markedly from DIC, maximal maintained currents evoked by RET were similar in amplitude from those evoked by DIC.

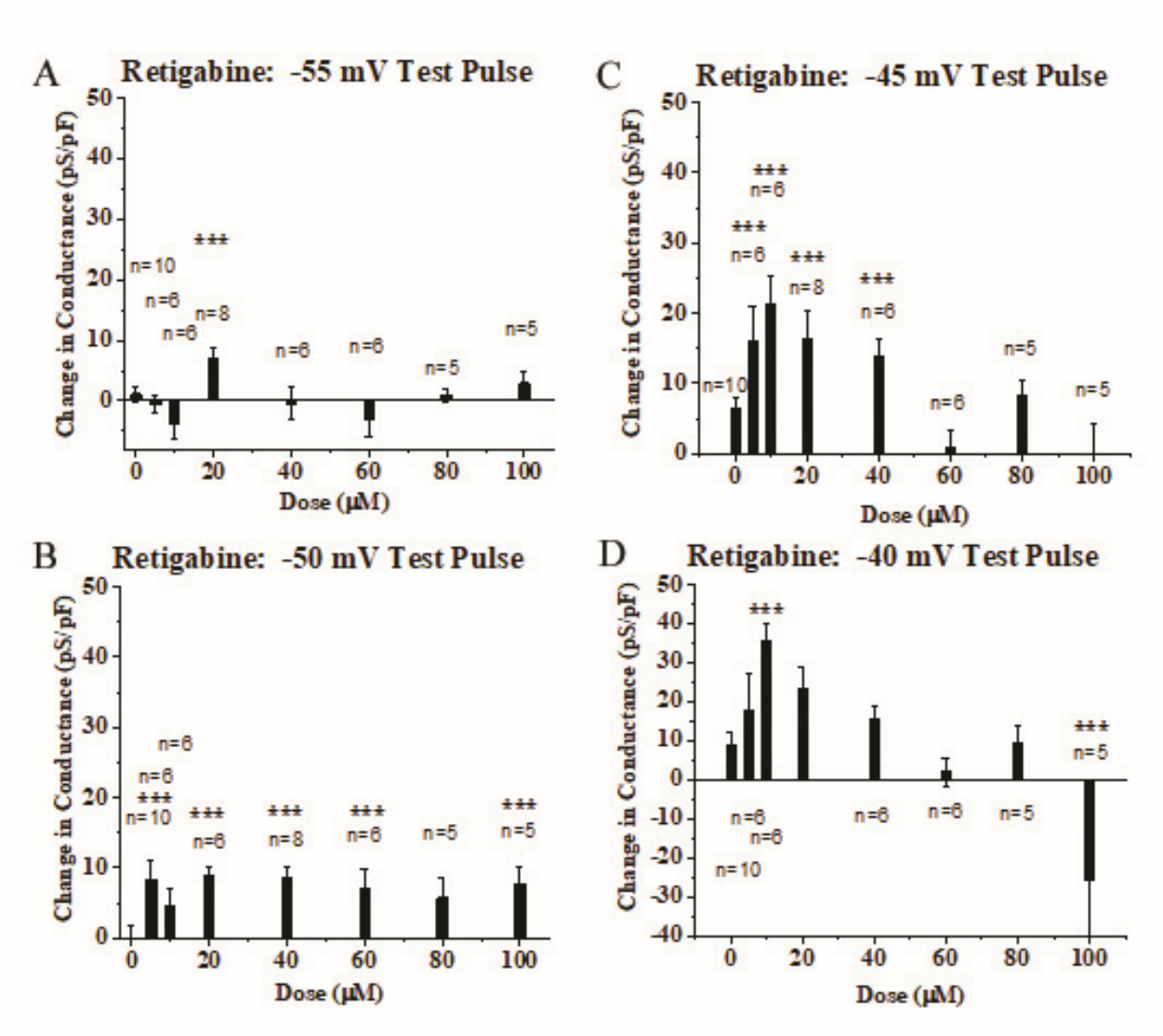

Figure 3. Retigabine Shifts K_V7 Voltage Sensitivity. A-D) The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-RET). Conductance was derived from currents evoked by a voltage step to -55, -50, -45, and -40, respectively. Data collected from voltage clamp experiments in a K^+ isolation solution. One dose was applied per cell. Statistical comparisons were made between vehicle-vehicle and vehicle-RET tests. Vehicle: Tyrode's; *** p<.05 or greater.

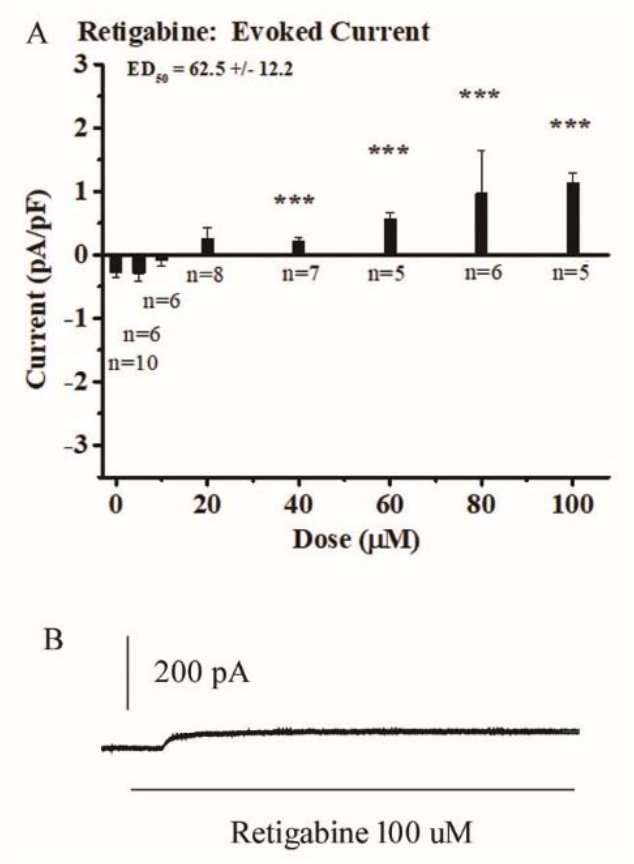

Figure 4. Resting K^+ Currents Evoked by Retigabine. A) Average normalized current produced at -60 mV (V_h=-60 mV) following a 2 minute application of RET. Evoked currents increased in proportion to the dose applied, but reached a maximum at 100 μM. **B)** A typical current trace produced by RET. Currents grew rapidly and were stable. ED_{50}=62.5±12.2 μM; *** p<.05 or greater. Vehicle: Tyrode's.

Combinations of Retigabine and Diclofenac. We examined whether combinations of RET and DIC could improve voltage sensitivity or amplitude of resting currents over those observed following single application studies. Two strategies were employed: 1) amplifying DIC response with a low dose of RET; and 2) amplifying RET responses with a low dose of DIC

Sub-threshold combinations of RET and DIC were tested using procedures identical to those described above. Several combinations were investigated, and two are presented in detail. Combining 5 μM RET with 50 μM DIC significantly improved resting current amplitudes (figure 5). The average evoked currents by 5 μM RET or 50 μM DIC (applied singly) were -22.7 + 3.7 pA and -26.7 + 20.2 respectively, the combination of 5 μM RET and 50 μM DIC generated outward currents that averaged 59.5 + 18.1 pA; an increase of over 70 pA. The combination of 5 μM RET and 50 μM DIC produce significantly more current than 70 μM DIC or 40 μM RET alone and was roughly equivalent to a dose of 80 μM DIC or 60 μM RET (see figures 2 and 4).

At this combination, we observed only sporadic improvements of voltage sensitivity relative to DIC alone (vs 70 uM DIC; not shown) or RET alone (vs 40uM or 100 uM RET; not shown). These corresponded to doses where single treatments did not improve voltage dependent shifts (figures 1 and 3). That is, when RET alone or DIC alone had the capcity to improve voltage dependent current, combinations treatments failed to improve on these.

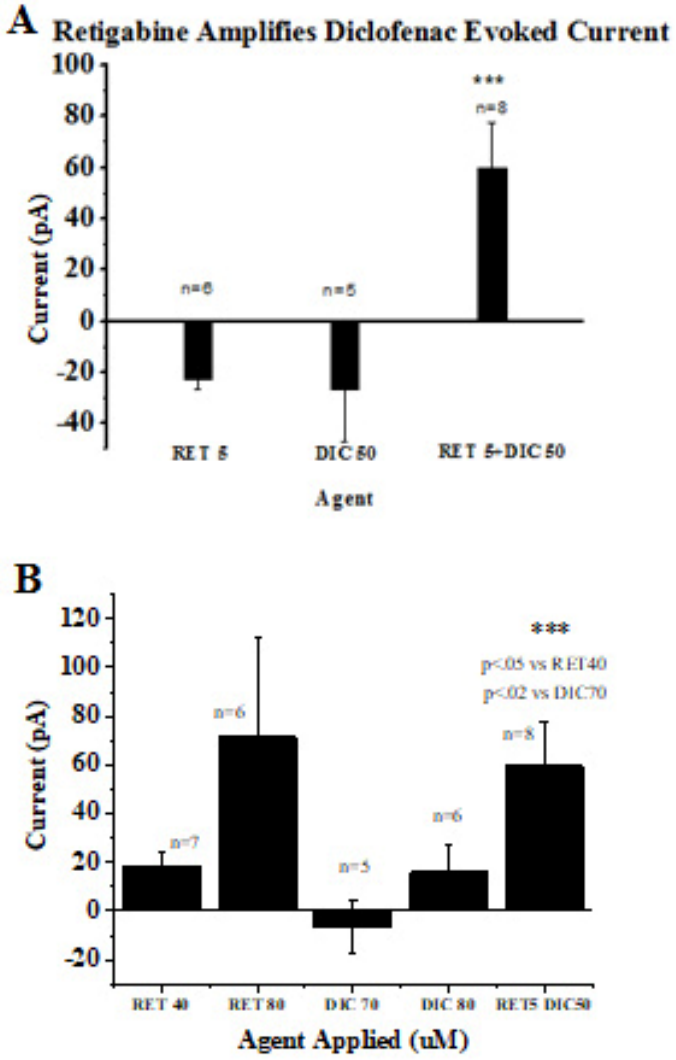

Figure 5. The combination of subthreshold doses of RET 5 μM **and DIC 50** μM **produced currents exceeding those of RET or DIC alone. A)** Currents measured at the end of the 2 min combined application greatly exceed those evoked by individual applications of sub-threshold doses (RET 5 μM or DIC 50 μM). **B)** Currents measured at the end of the 2 min combined application also exceeded those evoked by supra-threshold applications of RET 40 μM or DIC 70 μM. RET 80 μM or DIC 80 μM evoked currents that were similar to those of RET 5 μM and DIC 50 μM combined. Data were collected from voltage clamp experiments conducted in a K^+ isolation solution. Statistical comparisons were made between RET/DIC, RET and DIC tests on different cells. For statistical tests, the currents were normalized for cell size (pA/pF).

Based upon these outcomes we examined whether we could lower the DIC dose further and retain the amplifying effects of a combination with RET. Combining 5 μM RET with 20 μM DIC produced a similar pattern of outcomes at a lower dose (figure 6). The combination of 5 μM RET with 20 μM DIC generated outward currents that averaged 125.7 ± 59.7 pA. Although highly variable, the average current only nominally exceeded currents that were observed with the combination of 5 μM and 50 μM DIC. Despite the high variability, the combination of 5 μM RET and 20 μM DIC produced significantly more current than 80 μM DIC or 40 μM RET alone and was roughly equivalent to a dose of 100 μM DIC or 60 μM RET (see figures 2 and 4). In contrast to the 50 μM DIC combination, co-application of 5 μM RET with 20 μM DIC did not significantly improve voltage sensitivity beyond that which could be obtained by a single application of DIC or RET.

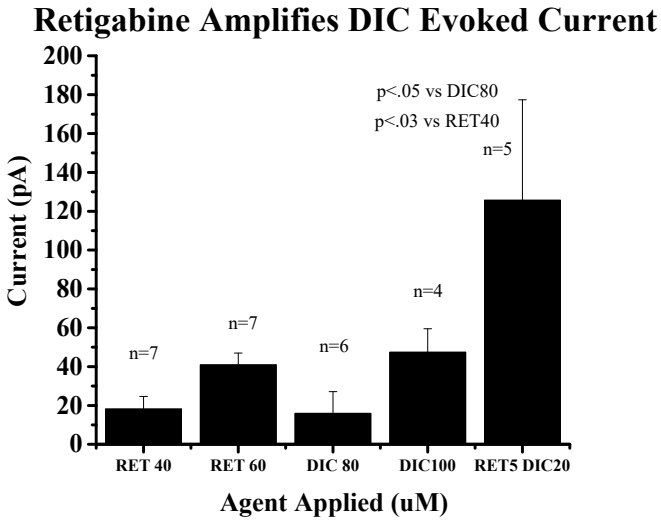

Figure 6. The combination of doses of RET 5 μM and DIC 20 μM produced currents exceeding those of RET or DIC alone. Currents measured at the end of the 2 min combined application exceeded those evoked by supra-threshold applications of RET 40 μM or DIC 80 μM. RET 60 μM or DIC 100 μM evoked currents were similar to those of RET 5 μM and DIC 20 μM combined. Data collected from voltage clamp experiments conducted in a K^+ isolation solution. Statistical comparisons were made between RET/DIC, RET and DIC tests on different cells. For statistical tests, the currents were normalized for cell size (pA/pF).

Current Clamp Studies

Retigabine and Diclofenac. We had previously reported that nociceptors, harvested from rats exhibiting pain-like signs following exposure to Gulf War chemicals, manifested heightened nociceptor burst discharges when exposed to a muscarinic agonist (MDBD; Cooper et al., 2016; Cooper et al., 2018). Because anticholinesterases play a determinant role in the development of pain signs in our model (Flunker et al., 2017), we investigated the capacity of RET/DIC combinations to modify burst discharges evoked by a cholinergic challenge. As the burst discharge data set occasionally included a 'breakthrough' case, medians are presented along with mean statistics. Graphs and statistical tests are based upon means.

Cells were characterized in voltage clamp, as types 5 or 8, and then brought into current clamp mode. After a 2 minute delay, the RMP was noted (mean initial RMP: 67.1 +/- 0.41 mV). Current was then injected to bring the RMP to -65 mV (10 pA maximum). After another 2 minute delay, individual KVOs or a KVO combination was applied by close superfusion for 2 minutes. Subsequently, the M2 specific agonist, Oxotremorine-M (10 μM), was applied (with KVOs) until action potential discharges began (maximum application time was 1 min). Several KVO combinations were examined for their capacity to produce membrane hyperpolarization and to retard AP bursting during the cholinergic challenge test. The best outcomes were obtained with the three combinations presented below.

Exposure of a deep tissue nociceptor to a single KVO produced relatively rapid hyperpolarization that remained constant through the 2 minute application period. Significant hyperpolarizations were observed with DIC at 50, and 20 μM test doses (figure 7). These were surprising findings as DIC never evoked any current, or voltage shifts at these doses in the voltage clamp studies (see figure 1 and 2 above). Further testing revealed that DIC hyperpolarizations abated at 2.5 μM (0.54 ± 1.03 mV; n=5). Significant hyperpolarization was also observed with RET at 5 and 10 μM (-2.00 ± 0.60 mV; n=6 and -1.84 ± 0.56 mV; n=6, 5 and 10 μM, respectively; figure 7). At a dose of 2.5 μM, RET produced depolarizations that were non-different from Tyrode controls. In contrast, significant standing currents were not observed at 5 μM in voltage clamp (V_h=-60 mV); however, there were voltage dependent shifts in conductance at 5-10 μM (figure 3) that could account for the hyperpolarization at this dose.

Following the application of 5 μM RET and 50 μM DIC in combination, the RMP was significantly hyperpolarized (-4.4 ± 1.4 mV; $p<.003$, n=8), and exceeded that observed with DIC alone ($p<.02$, n=6). The combination of 5 μM RET and 20 μM DIC produced significantly less hyperpolarization than 50 μM DIC (-2.83 ± 0.37; $p<.05$, n=5), but still exceeded that produced by Tyrode's in control tests ($p<.001$, n=8; see figure 7B). Similarly, the application of DIC 20 uM significantly decreased the RMP relative to Tyrode's controls (-2.47 ± 0.99, $p<.007$, n=7), but this did not differ from hyperpolarization produced in combination with RET 5 uM (figure 7).

Immediately following the 2 minute KVO exposure (or vehicle), 10 μM OXO was applied in the presence of the test KVOs. In control tests, without the presence of a KVO, OXO produced a rapid depolarization (23.3 ± 2.13 mV, n=10) and burst discharge (91.1 ±21.6 APs; median: 75.5

APs; figure 8). The combination of 5 μM RET with 50 μM DIC significantly reduced burst discharge (10.86 ± 8.94; median: 1 AP; $p<.007$, n=7) while significantly *increasing* OXO-induced depolarization (32.2 ± 2.81 mV, $p<.05$, n=8). Applied singly, DIC 50 μM was also capable reducing OXO dependent bursting (26.75 ± 19.8; median= 8 APs; n=8). This pattern indicated that, despite the voltage clamp data, the combination of RET 5 with DIC 50 μM did not increase the analgesic potency of DIC as defined by the cholinergic challenge test. The interpretation of the test was complicated by the significant increase in OXO dependent depolarizations observed with this combination.

Further experiments examined whether analgesic potency could be retained when reducing DIC concentrations. The combination of 5 μM RET with 20 μM DIC significantly reduced burst discharges (27.2 ± 20.6, median: 3.5 APs, $p<.02$, n=6) without effecting OXO induced depolarization (26.3 ± 4.6 mV). Neither 5 μM RET (89.8 + 26.8 APs; median 94.5 APs; n=6) nor 20 μM DIC alone (45.3 + 18.9; median 31 APs; n=7) reduced AP bursting (figure 8). This was an improvement over the effect pattern of RET 5/DIC 50 μM; however, the combination of RET 5/DIC 20 μM was not significantly better at reducing AP discharges than DIC 20 μM alone. The better performance achieved with a lower level of DIC may have resulted from the significantly larger OXO dependent depolarizations observed when 50 μM DIC was used as the combination dose. In that combination, hyperpolarizations were significantly greater ($p<.05$) and may have affected inactivation relief on currents supporting OXO dependent depolarization. While it is known that TRPA1 contributes prominently, the full composition of the OXO evoked plateau currents have not been explicated (Cooper et al., 2018).

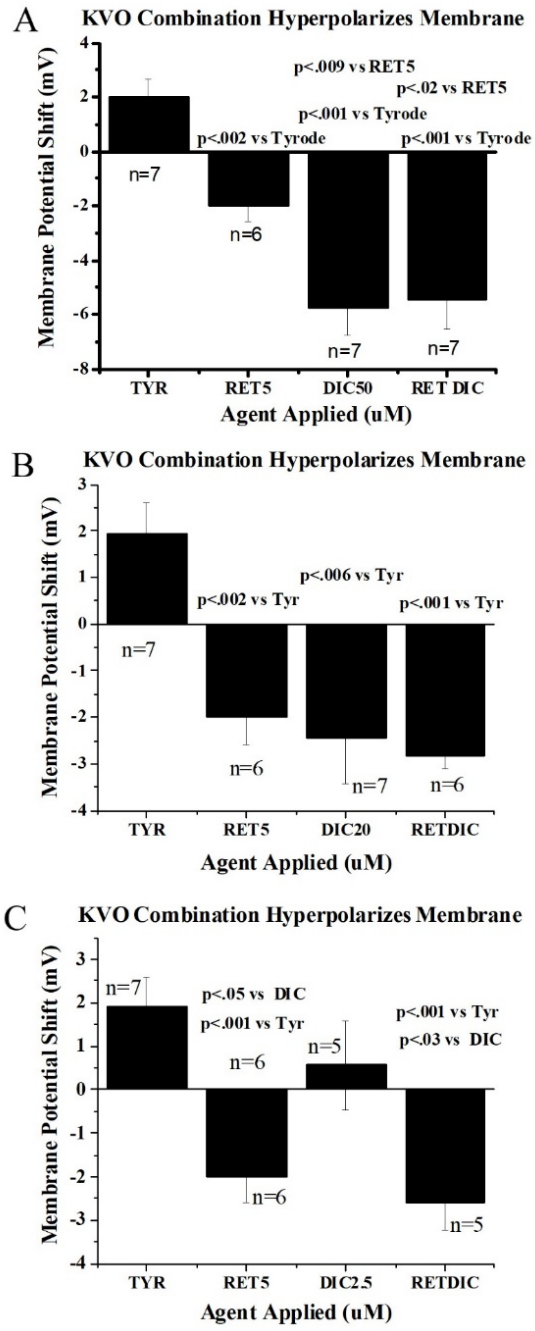

Figure 7. Combinations of RET and DIC Hyperpolarized Nociceptor Membranes. A) RET 5 μM and DIC 50 μM produce powerful shifts in the membrane potential that exceeded that of RET alone. **B)** Hyperpolarization by RET 5 μM and DIC 20 μM were significantly less than that of RET 5 and DIC 50 μM. The combination dose was not greater than either agent alone. **C)** RET 5 μM and DIC 2.5 μM produced significantly less hyperpolarization than combinations with 20 or 50 μM DIC, and significantly more than DIC 2.5 alone.

Ultimately, the best overall results were obtained with a combination dose of 5 μM RET and 2.5 μM DIC. Significant hyperpolarizations were retained with the combination (figure 7C), but neither of these doses could reduce AP discharge when presented individually. The combination dose proved to be highly effective (figure 8). Average AP bursts were significantly reduced to 10.2 ± 4.3 (n=5; median 14 APs; $p<.02$ vs OXO; figure 8). OXO-induced depolarizations were unaffected (21.3 ± 3.4 mV). Importantly, the AP reducing influence of the RET 5/DIC 2.5 μM treatment combination was significantly superior to either RET 5 or DIC 2.5 μM single applications and did not differ from those of the above combinations (RET 5/DIC 50 μM; RET 5/DIC 20 μM).

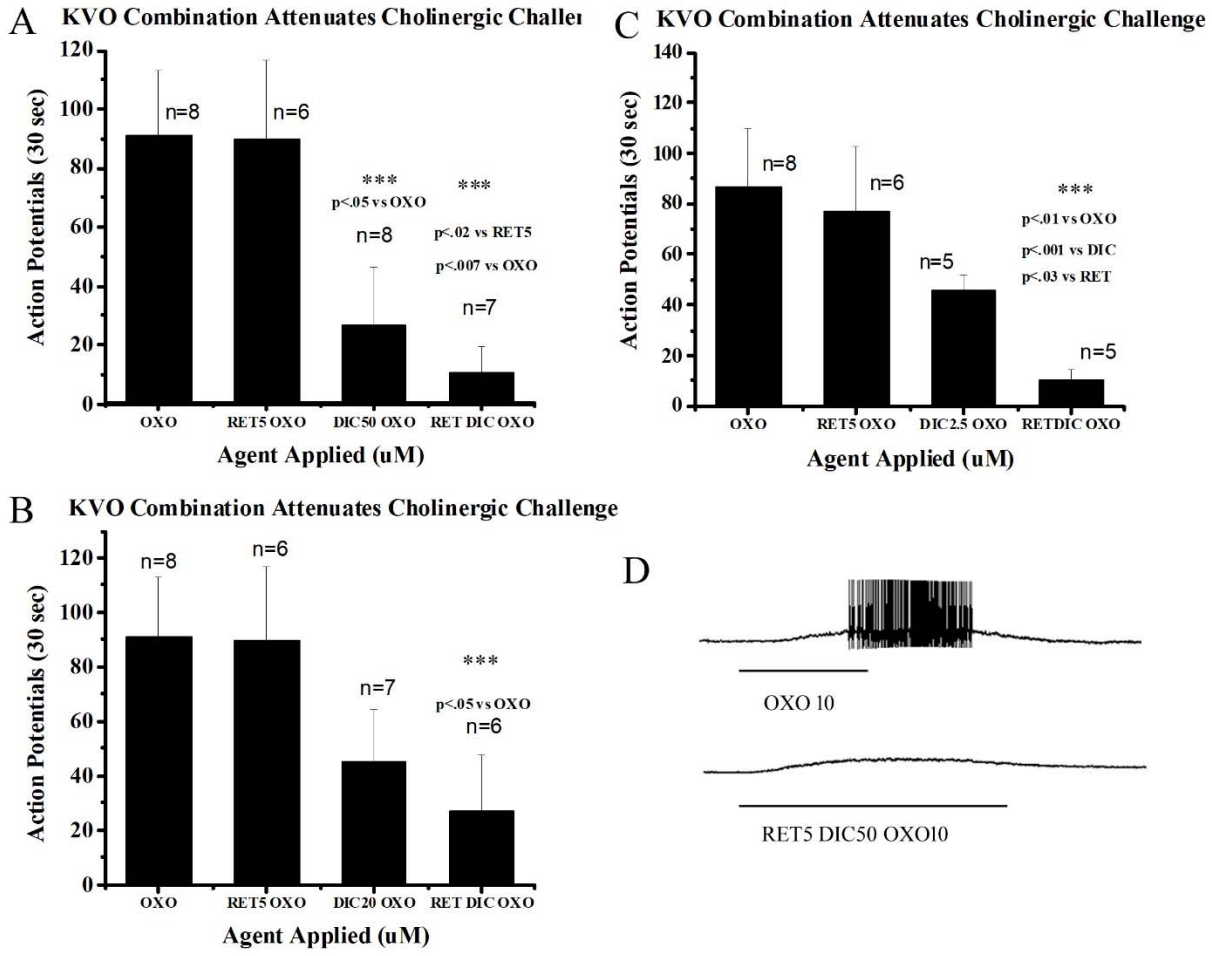

Figure 8. Combination KVOs Reduce Deep Tissue Nociceptor Discharge Consequent to a Cholinergic Challenge. A) The number of AP's evoked by 10 μM OXO were significantly reduced by RET 5 μM and DIC 50 μM. RET 5 μM had no influence. **B)** RET 5 μM and DIC 20 μM significantly reduced APs evoked by 10 μM OXO. Neither 5 μM or DIC 20 μM had any effect on AP discharge. C) RET 5 μM and DIC 2.5 μM significantly reduced APs evoked by 10 μM OXO. Neither 5 μM or DIC 2.5 μM had any effect on AP discharge **D)** Representative traces illustrate powerful burst evoked by 10 μM OXO (upper trace). AP bursting was completely abrogated by the application of RET 5 μM and DIC 50 μM (2 min pretreatment; lower trace). Both RET and DIC were present during the OXO test.

As the combination of RET 5 and DIC 2.5 proved highly successful, we made additional tests in which RET was the main agent in the combination. The combination of RET 7.5 μM and DIC 1.25 μM was also very effective. This combination both significantly hyperpolarized nociceptor membranes (-3.06 ± 0.76 mV; $p<.001$ vs Veh control) and drastically reduced action potential discharges (5.28 ± 5.12 APs; mediun of 0 APs; $p<.003$ vs Veh control). Despite the further reduction of DIC concentration, there was no statistical difference between these two combinations (5/2.5 and 7.5/1.25) with regard to membrane hyperpolarization or action potential suppression.

Behavioral Studies

GWI rats were prepared by exposure to 4 Gulf War chemicals: permethrin (topical; 2.6 mg/kg daily), chlorpyrifos (subcutaneous; 120 mg/kg; once per week in corn oil), DEET (topical; 400 mg/kg; daily and pyridostigmine bromide (oral gavage; 13 mg/kg; daily). A control group received only vehicle exposures (ETOH, water, corn oil). Chemical exposure followed a two week baseline testing period and continued for 4 weeks (see Method). Ambulation deficits appeared soon after exposure and were maintained in the post-exposure period.

We have shown previously that RET (7.0 mg/kg) could produce acute recovery from ambulatory deficits that followed exposure to the cluster of 4 GW chemicals. In that study, recovery was not retained past the first week of treatment (Cooper et al., 2018). Presently, two distinct experiments (BEH1, BEH2), were conducted to examine whether RET/DIC combinations would improve upon our previous findings using a single RET treatment. In each experiment, KVO single and combined treatments were applied after pain-like signs had emerged and were

retained for at least 8 weeks post-exposure. In BEH1, we tested the capacity of a 1:4 molar ratio of RET/DIC. This study followed from our observations, above, that a 1:4 molar ratio (5 μM RET and 20 μM DIC) would amplify current, hyperpolarize membranes, and abrogate nociceptor action potentials. Free range ambulation was assessed using an automated system (Accuscan Fusion) that recorded movements of rats in an activity cage. Rats were tested once per week (15 min) and treated by oral gavage daily.

Behavior Study 1 (BEH1). Following a 4 week exposure to GW chemicals, rats (n=48) developed signs of pain manifested as decreased movement and increased resting (figure 9B and C). Subsequently, 12 rats were treated with a combination of 2.5 mg/kg RET and 10 mg/kg DIC (oral gavage). Another 36 rats received either RET 2.5 only, DIC 10 only or DMSO vehicle. Twelve, non-exposed, rats also received DMSO. Two hours after gavage, free range behaviors were assessed. Only those rats receiving the combination treatment exhibited a reduction in pain-like behaviors (decreased rest times; figure 9A). Changes in rest times were relatively slight, but statistically significant. There were no significant changes in movement distance measures attributable to the KVO combination.

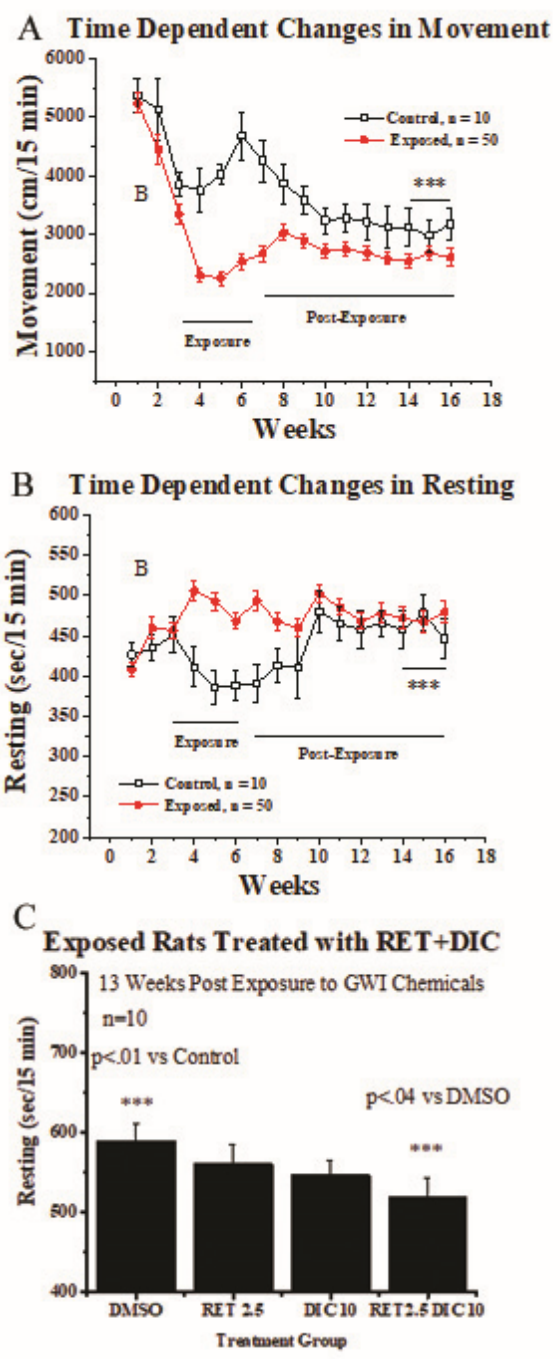

Figure 9. The Combination of RET and DIC (1:4) Reduced Pain-Like Behavior. A) Weekly progression of movement distance data before, during and following exposure to GW chemicals. **B)** Weekly progression of rest time scores before, during and following exposure to GW chemicals. **C)** The influence of single and combination treatments on rest time scores. B: baseline testing. *** significantly different by repeated measure ANOVA (p<.001* and p<.002*, rest and movement respectively). 2.5 mg/kg RET and 10 mg/kg DIC. The actual dose concentrations varied from these due to slight differences in molar weights for RET and DIC. All doses were administered by oral gavage 2 hours prior to testing.

Although this outcome was promising, repeated treatments with this combination of RET and DIC were not well tolerated. Three rats were found dead in their cages after a week of daily combined treatment. One rat that received 10 mg/kg DIC alone also expired after a week of daily treatment. Other rats in these groups became lethargic and were euthanized. Although pilot studies were conducted prior to the full BEH1 experiment, we had not tested the pilot study rats for more than 5 days of consecutive treatment. Further pilot studies were then undertaken in order to find safe levels of DIC and RET+DIC combinations. The dosing strategies of the BEH2 study below were adjusted accordingly; weekly treatments were substituted for daily treatments as a safety precaution.

Behavior Study 2 (BEH2). We tested RET/DIC combinations reflecting the following molar ratios: 1) RET2/DIC1; 2) RET7/DIC1 and 3) RET1/DIC4). The actual mg/kg doses were governed, not only by molar dose ratios, but also by concentration limits established by failed dosing strategies of BEH1 and subsequent pilot studies. The results of the 4 dose combinations representing these molar ratios and dose level restrictions are presented in figure 10, below. Simply described, no beneficial effect of any dose combination could be identified when dose levels were restricted to concentrations below levels where significant side effects were present.

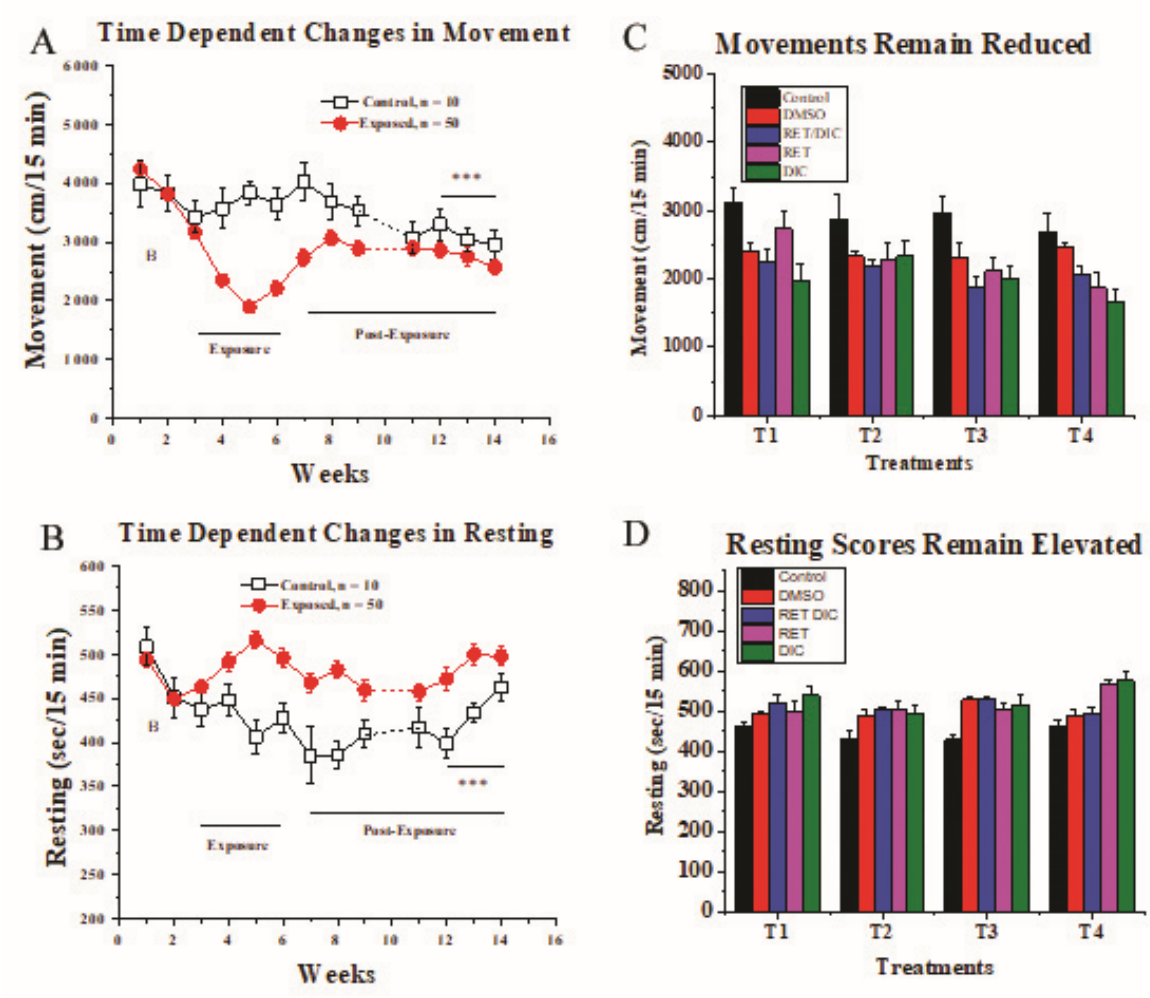

Figure 10. Multiple Dose Combinations of RET and DIC Fail to Modify Pain-Like Behaviors. **A)** Weekly progression of movement distance scores before, during and following exposure to GW chemicals. **B)** Weekly progression of rest time scores before, during and following exposure to GW chemicals. **C)** The influence of single and combination treatments on movement distance scores; D) The influence of single and combination treatmens on rest time scores. B: baseline testing. *** significantly different by repeated measure ANOVA ($p<.001$ and $p<.002$, rest and movement respectively). T1: 2.5 mg/kg RETand 1.25 mg/kg DIC; T2: 7 mg/kg RET and 1.25 mg/kg DIC; T3: 4.4 mg/kg RET and 0.62 mg/kg DIC; T4: 1.25 mg/kg RET; and 4 mg/kg DIC. Actual doses varied from these due to slight differences in molar weights for RET and DIC. All doses were administered by oral gavage 2 hours prior to testing.

Discussion

In 2005, Peretz and colleagues characterized the K_v7 modulating properties of RET, DIC and meclofenamate (Peretz et al., 2005). Expressing $K_v7.2/K_v7.3$ hetero-multimers in CHO cells, they demonstrated that combinations of RET and meclofenamate shifted voltage dependence, increased maximal current and slowed deactivation kinetics relative to RET alone. Although no data was presented, they also reported similar effects for RET and DIC combinations (Peretz et al., 2005). Our laboratory extended these investigations to determine whether such combinations had analgesic potential. Studies were conducted in the context of our laboratory history with molecular, cellular and behavioral investigations into the chronic pain of Gulf War Illness (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al. 2015).

We were able to determine, for the first time, in DRG nociceptors: 1) ED_{50}s for RET and DIC evoked K_v7 currents; 2) demonstrate that these two agents could act cooperatively to amplify these currents; and 3) that deep tissue nociceptor burst discharges, produced by a GWI relevant cholinergic challenge test, could be abrogated by KVO combination treatments. The latter studies were guided not only by reduction of bursting, but also by the intent to achieve reductions of effective doses in order to minimize side effects known to develop with chronic use of these medications in clinical applications (Singh et al., 1994; Blackburn-Monro et al., 2005; Ulubay et al., 2018; Bock and Link, 2019). In a subsequent set of experiments, we found that pain-like

behaviors of rats, resulting from an exposure to GW neurotoxicants, could not be successfully treated with KVO openers at doses that avoided serious side-effects associated with DIC.

Voltage Clamp Studies. Standing, linopirdine sensitive, K^+ currents were evoked following application of RET and/or DIC. ED_{50}s and maximal currents were determined for individual KVOs expressed in two classes of deep tissue nociceptors (RET: ED_{50} of 62.7 μM, I_{max}: 1.14 pA/pF; DIC: ED_{50} 93.2 μM; I_{max}: 1.05 pA/pF; Cooper et al., 2014). The nociceptor based ED_{50} for RET varied considerably from those reported for $K_v7.2/K_v7.3$ hetero-multimers expressed in CHO cells or Xenopus Oocytes (1-4 μM; 3.6 μM Main et al., 2000; 1.7 μM, Rundfelt and Netzer 2000; 1.6 μM, Wickendon et al., 2000; 2 μM, Peretz 2005). These values, with the exception of Main et al., reflect calculations based on $V_{.50}$ shifts rather than evoked currents and are derived from non-native heteromultimers. In fact, we did observe consistent increases in voltage dependent conductance following RET application; including at doses that did not evoke any sustained current (5-20 μM). However, one report that did base ED_{50} calculations on evoked current, obtained results similar to those reported in CHO cells (~1 μM; Tatulian et al., 2001). This test was conducted on a single rat superior cervical ganglion neuron exposed to increasing doses of RET at a different holding potential (V_h of -20 mV and -60 mV).

The considerable divergence between the above ED_{50s} and the one we obtained in nociceptors could arise from fundamentally different channel composition. It is well established that DRG neurons express not only $K_v7.2$ and $K_v7.3$, but also express $K_v7.5$ (Passmore et al., 2003; Brown and Passmore, 2009; Yu et al., 2018; Djouhri et al., 2020). This distribution pattern appears to be associated particularily with DRG nociceptive neurons. Yu and colleagues reported an 80%

overlap between the expression of K_v7.2/7.3/7.5 and TRPV1 (Yu et al., 2018). Both the Type 5 and Type 8 neurons, used in our experiments, are uniformally capsaicin sensitive and would have a high probability for expression of K_v7.5 (Petruska et al., 2000; 2002; Rau et al., 2007). Indications are that SCG neurons also express K_v7.5 but exhibit currents mainly derived from K_v7.2/K_v7.3 as opposed to K_v7.3/K_v7.5 (Hadly et al., 2003). Given that the report of Tatulian and colleagues was based on a recording from only one SCG neuron, it is difficult to come to any conclusion regarding the 'true' ED_{50} for DRG nociceptors.

Regulatory factors could also play a role in determing the sensitivity of nociceptor K_v7 channels to RET (e.g., phosphorylation status and interaction with membrane PIP_2; Erdem et al., 2017; Salzer et al., 2017). ED_{50}s for DIC were also considerably higher than that reported of K_v7.2/7.3 in CHO cells (2.6 vs 92 μM; Peretz et al., 2005), but the influence of DIC on spinal dorsal horn or cortical neurons suggested a lesser sensitivity that was more consistent with our own data (Peretz et al., 2005; Vicente-Baz and Rivera-Arcondada, 2020). Other methodological differences between our findings and those of previous studies (bath solutions; holding potentials) led us to examine whether the use of a K_{iso} solution shifted the sensitivity of K_v7 to RET activation. This was confirmed. Nevertheless, the use of an isolation solution was necessitated by interaction of DIC with several ion channels present in DRG (Lee et al., 2003; Gwanyanya et al., 2012; Nakamura and Jang, 2016).

Within this context, RET/DIC combinations powerfully influenced the production of persistent K^+ currents. Applying single ineffective doses of RET (5 μM) and DIC (20, 50 μM) in combination, produced K^+ currents that were significantly higher than those that could be produced

by much higher levels of RET or DIC alone (60 RET or 80 DIC). Because we used doses that were, by themselves, ineffective at activating K_v7, we argue that the current amplification we observed represented a cooperative effect resulting from the binding of RET and DIC at distinct subunits of the channel. RET preferably binds to $K_v7.3$ (Tatulian et al., 2001; Peretz et al., 2005) while DIC binds to $K_v7.2$ (Tatulian et al., 2001; Tatulian and Brown, 2003; Schenzer et al., 2005; Wuttke et al., 2005; Peretz et al., 2005; Du et al., 2011; Linley et al., 2012). A simple additivity effect would be produced by the independent opening of channels by the singular binding of RET and DIC to different channels. DIC was completely ineffective at doses as high as 50 μM. The importance of a cooperative effect, is that predicts a lower dose of agents that would be needed to reduce or abrogate nociceptor discharge, and thereby, lower the risk of side effects in a clinical application.

Current Clamp Studies. In 2015, we reported that vascular and muscle nociceptors, in naïve rats, exhibited a unique acetylcholine instigated burst discharge that was dependent on muscarinic receptor activation. MDBD (muscarinic dependent burst discharge) was shown to be initiated by mAChRs (muscarinic acetylcholine receptors), modulated by K_v7, and reliant on a TRPA1 mediated depolarization component (Nutter et al., 2015; Cooper et al., 2016; Cooper et al., 2018). Follow-up studies demonstrated that nociceptors, harvested from rats exhibiting GW agent-induced ambulatory pain behaviors, exhibited exaggerated muscarinic depolarization, and that the development of this exaggerated depolarization was dependent upon the inclusion of PB in the neurtoxicant exposure protocol (Cooper et al., 2018). These findings were consistent with studies that revealed alterations in the expression of muscarinic receptors (Nostrandt et al., 1997; Liu et al., 1999; Huff et al., 2001; Abou-Donia et al., 2003; Zhang et al., 2002; Padilla et al., 2005; Pung

et al., 2006; Proskocil et al., 2010) and TRPA1 activity (Ding et al., 2017) following exposure to some of these same Gulf War chemicals. Based upon these findings, we elected to use a cholinergic challenge test to determine whether RET and DIC combinations could retard nociceptor discharge. As predicted by their capacity to greatly amplify standing K^+ currents in voltage clamp studies, several combinations proved to be highly effective at hyperpolarizing nociceptor membranes and reducing or abrogating nociceptor discharge to OXO.

Of the four RET/DIC combinations characterized above, the best overall outcome was obtained by the combination of RET 5 μM and DIC 2.5 μM. This combination significantly suppressed AP discharge, and was significantly better than either RET 5 μM or DIC 2.5 μM alone. Other combinations were arguably more effective than 5 RET+2.5 DIC at membrane hyperpolarization (i.e., RET 5 μM and DIC 50 μM) but they did not produce significantly greater suppression of AP discharge in the cholinergic challenge test.

High concentrations of DIC were to be avoided as they could increase the potential for interactions with non-K_v7 channels, and thereby confound interpretations of any positive outcome. This was expecially a concern when DIC alone was shown to suppress AP discharge (50 μM test; figure 8; see Paz et al., 2018). DIC is known to interact with other important nociceptor ion channels independent of its influence on K_v7. The potential that these secondary effects contributed to our observations appears to be low. Although voltage gated Na^+ channels (Na_v), TTXs and TTXr, expressed in rat DRG, are inhibited by DIC (Gwanyanya et al., 2012; Nakamura and Jang, 2016), the reported IC_{50} for TTXr is in excess of 1000 μM (Nakamura and Jang, 2016; but see Lee et al., 2003, 96 μM). DIC exerts a much greater influence on TTXs channels (I_{50} = 14

μM; Lee et al., 2003; see also Acosta et al. 2007), but TTXr Na_v would be the most important channel for AP formation in our classified deep tissue nociceptor population (Nutter et al., 2013). Given these considerations, we could rule out the possibility that DIC interactions with TTXr or TTXs Na_v contributed to DIC effectiveness in the cholinergic challenge test at the 2.5 μM concentration. In any case, minimalizing DIC concentrations in a combination therapy was an important priority. With its extensive clinical history, DIC is known for its cardiac, hepatic and renal side effects, in addition to those typically associated with the prolonged use of NSAIDs (Bort et al., 1999; Hickey et al., 2001; Trelle et al., 2011; see also Singh et al., 1994; Ulubay et al., 2018).

Behavioral Studies. In two distinct behavioral experiments, we examined whether RET/DIC combination treatments, that were highly effective in amplifying current and reducing nociceptor discharge, could be used to reduce pain-like behaviors in a rat model of GWI chronic pain. We used molar ratios that were established in voltage clamp and current clamp studies to translate dose strategies for behavioral experiments. As we had previously demonstrated that 7.0 mg/kg RET had a positive, but transient, effect in our GWI model (Cooper et al., 2018), we used that dose as an anchor point about which to design dose ratio combination treatments. Five different combinations, based upon molar ratios, were tested in two distinct studies. Only combinations associated with with severe side effects were shown to reduce pain behaviors significantly. We were able to detect the analgesic properties because the side effects were only manifested when repeated exposures to the combinations were extended a week or more. With repeated treatments at high doses of DIC only (> 7 days; 10 mg/kg), rats became relatively immobile and were euthanized. Three rats, receiving the combination treatment for more than 7 days (2.5 mg/kg RET and 10 mg/kg DIC), were found dead in their cages. Rats receiving RET alone never exhibited

any observable side effects. Published studies using combinations of RET and DIC to manage electroshock seizures, in mice, did not reported any side effects; however in these studies, only a single dose exposure of RET 4 mg/k and 10 mg/kg DIC was used (Khattab et al., 2018).

Summary and Conclusions

This is the first investigation of the influence of KVO combinations on noccieptor excitability. The data provided proof of the concept that cooperative action at a common receptor can enhance performance of that receptor and that applications to pain control are potentially advantageous. Clearly, we found that the combination of RET and DIC was not ideal for pain management. The doses required to suppress nociceptor activity, in vitro, could not be reduced sufficiently to avoid very significant side effects, in vivo. The side effects we observed were likely to have arisen from DIC; possibley via inhibition of cyclooxygenase and TTXs Na_v. Despite this untoward outcome, the principle of cooperative action was verified. Development of alternative agents with activity at $K_v7.2$, but lacking cyclooxygenase and TTXs interactions are indicated (Peretz et al., 2007, 2010; Zhang et al., 2013; Du and Gamper, 2013; Du et al., 2017; Yekkirala et al., 2017; Eid and Gurney, 2018).

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