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TITLE: Optimizing Kv7 Channel Openers for Treatment of GWI Pain

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14. ABSTRACT Our laboratory developed a rat model of the chronic pain of GWI. After identification of several molecular maladaptations in pain sensing neurons (nociceptors) after exposure to GW neurotoxicants, we initiated studies to identify effective treatments to reverse established pain-signs. Kv7 openers (KVOs) have known analgesic/palliative capacities that could serve as treatments for the chronic pain associated with GWI. We previously reported that the classic KVO, Retigabine (RET), could temporarily reverse signs of GWI pain in rats. This project examined whether NSAID KVOs (Diclofenac (DIC) and Meclofenamate (MEC)), could act synergistically with RET to improve analgesic potency. We determined that combinations of RET/DIC, RET/MEC and MEC/DIC synergized to improve both molecular and cellular events in nociceptors. In our animal model of GWI chronic pain, RET/MEC reversed pain-signs in rats 14 weeks after exposure to GW neurotoxicants without the side effects observed with RET/DIC. Chronic treatment with RET/MEC should be examined to determine whether side effects would emerge.					
15. SUBJECT TERMS Pain, Nociceptor, Retigabine, Meclofenamate, Diclofenac, Gulf War Illness, Treatment					
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Introduction

GW (Gulf War Illness) is a multisymptom disease with a substantial chronic pain component. Our laboratory developed an animal model of GW based upon exposure to pesticides and PB (pyridostigmine bromide). Through a series of studies we identified several ion channel maladaptations associated with deep tissue nociceptors harvested from rats exposed to those GW chemicals (permethrin, chlorpyrifos, DEET, and PB). K_v7 is a nociceptor ion channel that, when activated, diminishes 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). Certain FDA approved NSAIDS (Diclofenac, Meclofenamate, Celecoxib) also open the K_v7 ion channel but bind at diverse locations. This project proposed experiments to determine whether combinations of Retigabine, Diclofenac, Meclofenamate, and Celecoxib could produce synergistic amplification of K_v7 activity that would result in improved reduction of nociceptor discharge, reversal of pain-like signs in an animal model, and significantly lower doses required to produce these effects.

2. Keywords: pain, nociceptor, K_v7 , Retigabine, Diclofena, Meclofenamate pesticides, Gulf War Illness, Treatment

3. Accomplishments

Major Goals:

To determine the influence of:

Retigabine + Meclofenamate on voltage dependent opening of K_v7

Retigabine + Diclofenac on voltage dependent opening of K_v7

Meclofenamate + Diclofenac on voltage dependent opening of K_v7

Retigabine + Meclofenamate on nociceptor reactivity

Retigabine + Diclofenac on nociceptor reactivity

Meclofenamate + Diclofenac on nociceptor reactivity

Retigabine + Meclofenamate on pain-like behaviors

Retigabine + Diclofenac on pain-like behaviors

Meclofenamate + Diclofenac on pain-like behaviors

Retigabine + Celecoxib on voltage dependent opening of K_v7

Retigabine + Celecoxib on nociceptor reactivity

Retigabine + Celecoxib on pain-like behaviors

What was accomplished under these goals?

A Summary of Accomplishments:

KVOs can be effective analgesics, but over a long course of treatment, adverse side effects can be an issue. We examined whether we could lower the analgesic dose required (thus reducing side effect risks) 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 combinations, 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, we demonstrated that sub-threshold doses of RET (5 μ M) greatly enhanced the effect of sub- and near threshold doses of DIC (2.5, 20 and 50 μ M) and MEC (30 μ M). 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, and were candidates for analgesic testing.

In further proof of this concept, we demonstrated that combinations of RET/DIC, RET/MEC and MEC/DIC could substantially reduce deep tissue nociceptor action potential activity that was evoked by an acetylcholine agonist (Oxotremorine-M; OXO) for muscarinic receptors that were previously shown to be modified by GW chemicals.

Using the KVO molar ratios determined in the cellular studies, we conducted behavioral experiments on rats exhibiting pain-like behaviors following exposure to GW chemicals (permethrin, chlorpyrifos, DEET and pyridostigmine bromide). 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 at other molar ratios of RET and DIC were ineffective in additional behavioral studies using DIC as the adjunct.

In behavioral studies employing combinations of RET/MEC (5 mg/kg RET; 0.5 mg/kg MEC; 10:1 dose ratio), we obtained evidence that this combination could also normalize pain-like behaviors at low doses; however, we did not attempt daily treatments that could have detected a build-up of deleterious side effects observed with DIC.

It was concluded that dose combinations of KVOs targeting multiple Kv7 binding sites, was a good approach for reversing GWI pain; however, the use of DIC, even at low doses, might result in serious side effects. Of the two candidates, MEC had superior potential as an adjunct and was safe for single treatment combinations with RET. Further studies are required to determine whether the RET/MEC combination is safe for extended treatments as well as effective. Development of synthetics that bind at DIC and MEC preferred sites are recommended to avoid toxic side effects.

In the detailed presentations below, we provided a summary statement for each Task and Sub-Task.

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 have shown that combination KVO treatments can achieve all three of these outcomes.

Subtask 2: Diclofenac Dose Response

Timeline: Months 1-4

100% completed

Using the protocol described in 'Methods' (Appendix, p. 63) 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 .

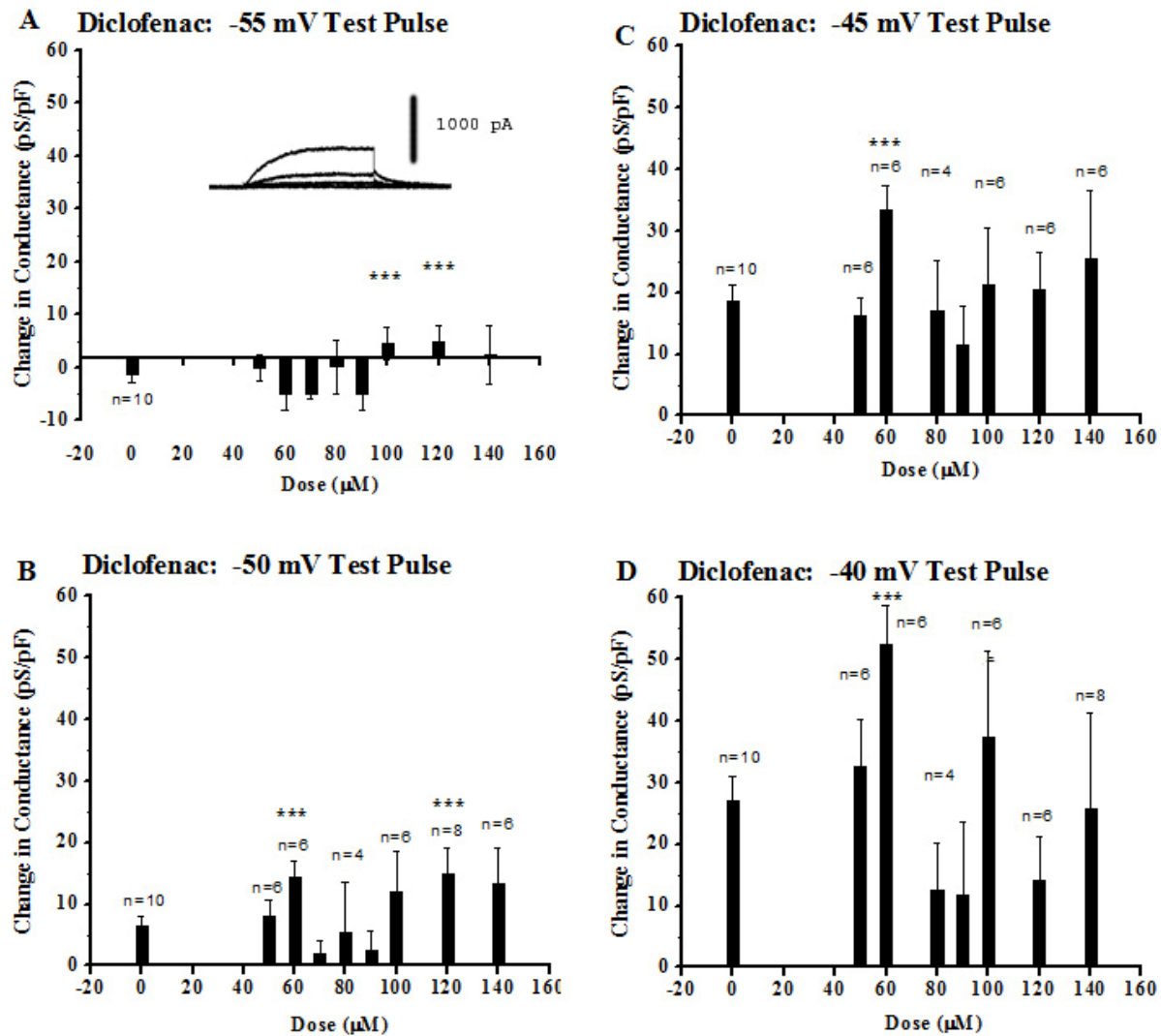


Figure 1. The Influence of Diclofenac on Kv7 Voltage Sensitivity. DIC had a narrow effect on the voltage sensitivity of Kv7. **A)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived 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

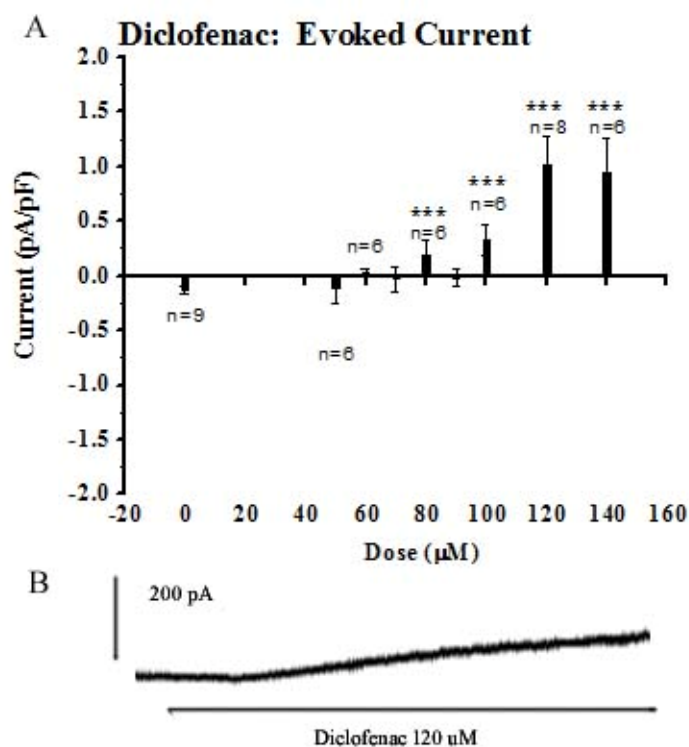


Figure 2. The Effect of Diclofenac on Resting Currents Mediated by Kv7. DIC opened Kv7 channels without a shift in applied voltage. Relatively small outward (hyperpolarizing) currents were observed. **A)** Average normalized current produced at -60 mV ($V_h = -60$ mV) following a 2 minute application of DIC. Evoked currents increased in proportion to the dose applied, but reached a maximum at 120 μM. 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

20% completed

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

There was little evidence that Celecoxib (CEL) could influence the voltage dependence of Kv7. Although CEL was an effective opener of Kv7 channels at 20-40 μM, it was clear there was a strong non-specific effect on other voltage sensitive K^+ channels. This non-Kv7 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
100% completed

Using the protocol described in ‘Methods’ (Appendix, p. 63) 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 the 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 10 to 100 μ M were applied in separate studies. When the dose of MEC reached 70 μ M, we observed a significant increase of K_v7 conductance at all test voltages (figure 3). Some significant increases were observed at doses as low as 40 μ M. As MEC doses increased through 100 μ M, 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.

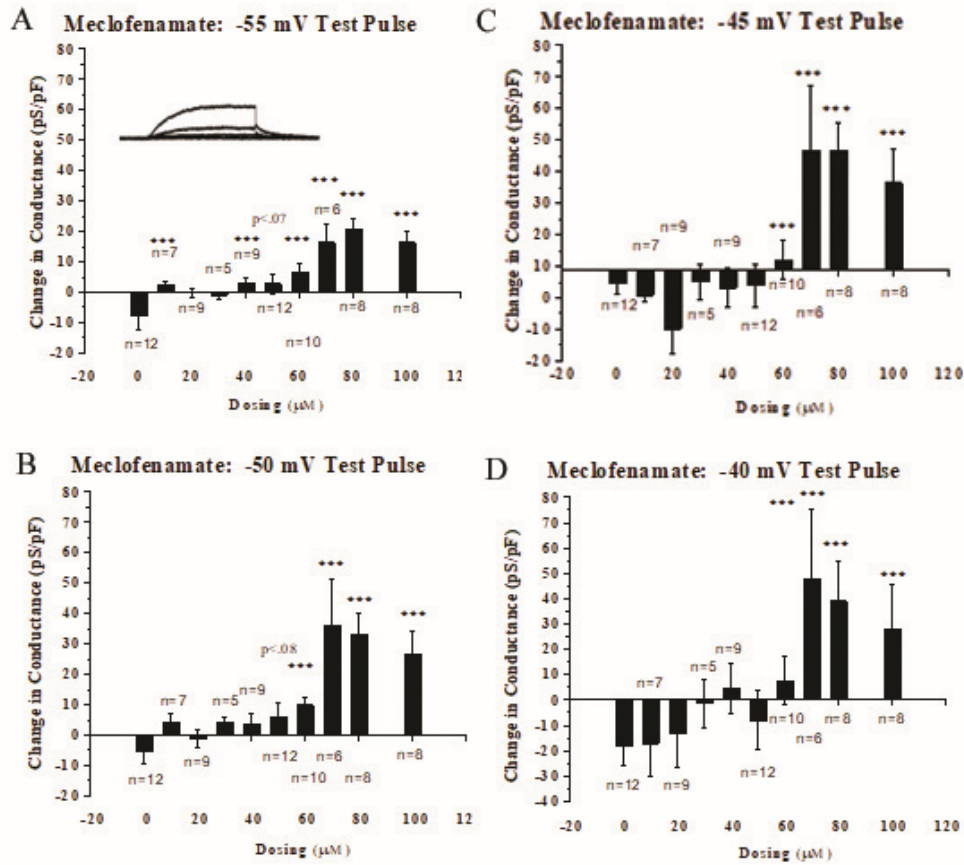


Figure 3. The influence of Meclofenamate on K_v7 voltage dependent conductance. The dose dependent change in normalized conductance of deep tissue nociceptors following exposure to MEC or vehicle (vehicle-vehicle or vehicle-MEC). **A)** Conductance derived from currents evoked by a voltage step to -55 mV ($V_h = -60$ mV). **B)** Conductance derived from currents evoked by a voltage step to -50 mV ($V_h = -60$ mV). **C)** Conductance derived from currents evoked by a voltage step to -45 mV ($V_h = -60$ mV). **D)** Conductance 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 activator of K_v7 . At doses as low as 30 μM , a weak K_v7 current could be detected. Current amplitude grew substantially with increasing concentration. A maximum outward current was reached at 80 μM (6.3 $\pm$ 1.2 pA/pF; figure 4). The ED_{50} (effective dose for 50% activation) for MEC was determined to be 62.2 $\pm$ 3.2 μM .

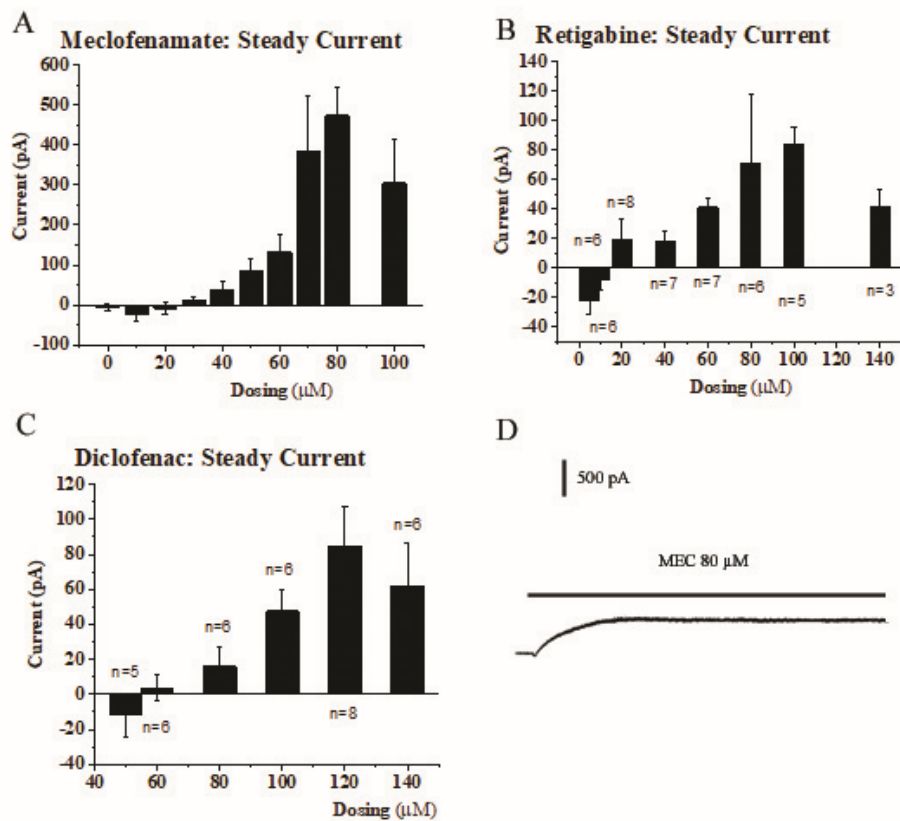


Figure 4. The influence of Meclofenamate, Retigabine and Diclofenac on resting currents. MEC opened K_v7 channels independent of any shift in applied voltage. Relatively large outward (hyperpolarizing) currents were observed. **A)** Steady current produced at the holding potential ($V_h = -60$ mV) following a 2 minute application of MEC. Evoked currents increased in proportion to the dose applied, but reached a maximum at 80 μM . **B and C)** Steady currents produced by Retigabine and Diclofenac, respectively (or comparison). In 'C' note the test at 10 μM that is partially obscured. Note differences in scales. **D)** A representative trace shows the rapid increase of K^+ current with MEC (2 min application). Data collected from voltage clamp experiments in a K^+ isolation solution. Current was measured at the end of the 2 min application. The number of cells contributing to each bar is indicated as 'n='. Each cell was exposed to only one concentration.

In summary, MEC strongly opened K_v7 channels. Threshold doses for direct activation were lower (30 vs 50 μ M), and maximal currents were much greater with MEC compared to DIC (6 vs 1 pA/pF). Direct activation began at 30 μ M, and voltage dependent shifts in conductance were present. 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

100% completed

Shifts in Voltage Sensitivity. RET amplified the voltage sensitivity of K_v7 at doses as low as 5 μ M (figure 5). No directly activated current could be demonstrated at this dose. Voltage effects were observed above the threshold of 5 μ M, 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 μ M, evoked currents steadily increased reaching a peak of 80 pA (1.14 +/- .15 pA/pF; figure 4B). An ED₅₀ of 64.3 +/- 12.4 μ M was determined by fit to a sigmoidal curve. Maximal maintained currents, evoked by RET, were similar in amplitude to those evoked by DIC.

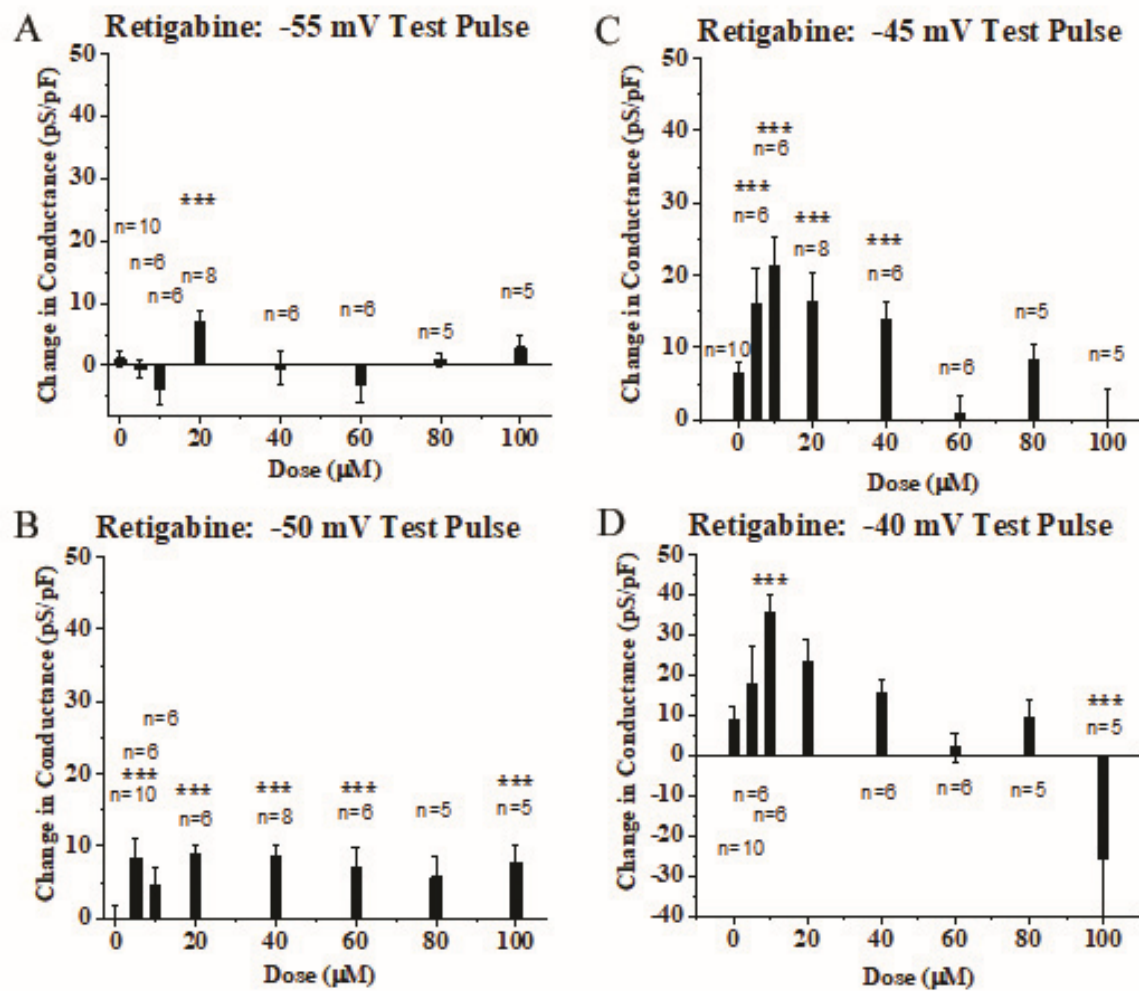


Figure 5. Retigabine Shifts Kv7 Voltage Sensitivity. A-D) The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-RET). Conductance was derived from currents evoked by a voltage step to -55, -50, -45, and -40, respectively. 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.

Summary (major task 1, subtask 1-4, Specific Aim 2)

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). The characterization of the basal influence of these KVOs on voltage dependence and current allowed us to assess how various combinations improved performance.

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

Subtask 1: Retigabine, Meclofenamate and Diclofenac

Timeline: Months 10-11
100% Completed

Subtask 2: Retigabine and Celecoxib

Timeline: Months 10-11
0% Completed

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 phase of the project, we demonstrated that combinations of RET with MEC or DIC did substantially amplify currents through nociceptor K_v7 ion channels. This finding supported the hypothesis that combination therapies could be effective analgesics in GW veterans. The lowering of required doses 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

100% Completed

We were able to show that combinations of RET and MEC could substantially increased K_v7 resting currents. Several combinations were examined but only one is presented below. This data can be found in the Appendix p. 85)

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 RET (5 μ M) and MEC (20 μ M), there was little

evidence of any amplification of evoked current (not shown). Nor did combination of RET 2.5 μ M with 70 μ M MEC show any significant amplification. When the MEC concentration was raised to 30 μ M (barely above threshold), very substantial current amplification was observed with RET 5 μ M. As shown in figure 6, subthreshold and near threshold combination of 5 μ M RET and 30 μ M MEC produced outward currents in excess of 250 pA. That greatly exceeded the maximum current evoked by any dose of RET or DIC (<100 pA; see figure 4). As shown in figure 6, the combination of RET 5 μ M and MEC 30 μ M produced currents significantly greater than 80 μ M RET, or 60 μ M MEC alone, and were roughly equivalent to 100 μ M RET or 70 μ M MEC. We did not observe any shifts in the voltage dependent conductance at any of the combinations tested.

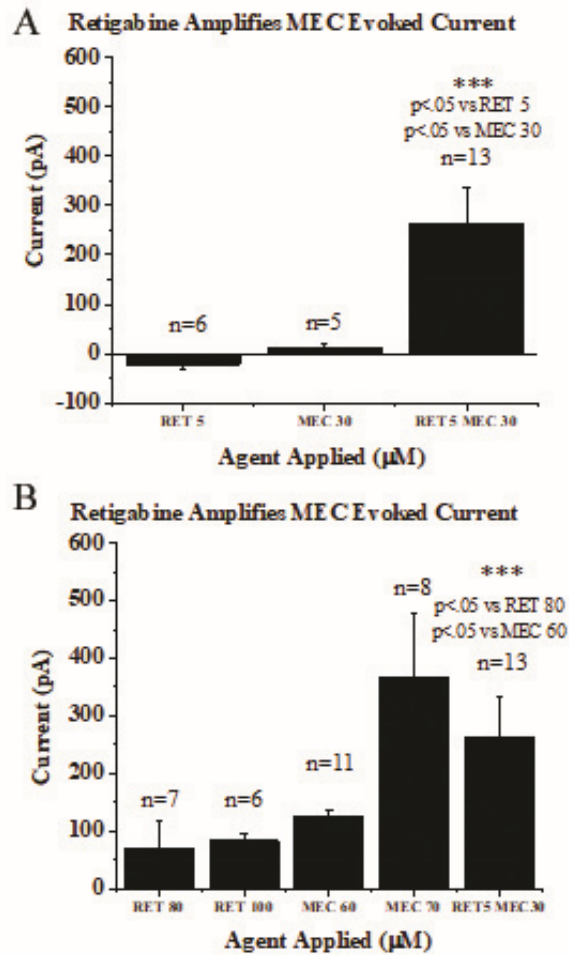


Figure 6. The combination of doses of RET 5 μM and MEC 30 μM produced currents exceeding those of RET or MEC alone. **A)** Currents measured at the end of the 2 min combined application greatly exceeded those evoked by individual applications (RET 5 μM or MEC 30 μM). **B)** Currents measured at the end of the 2 min combined application significantly exceeded those evoked by supra-threshold applications of RET 80 μM or MEC 60 μM . RET 100 μM or MEC 70 μM evoked currents that were similar to those of RET 5 μM and MEC 30 μM 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). RET data from Flunker et al., 2022, but experiments were conducted in the same time period.

Summary (major task 2, subtask 1, specific aim 2)

The combinations of RET/MEC exhibited excellent potential for analgesic capacity. This was evidenced by the substantial amplification of K_v7 activity at very low doses of the two KVOs. Although evoked current was greatly amplified, the voltage dependence was unaffected.

Major Task 2, Subtask 2: Retigabine and Diclofenac

Timeline: Months 12-14

100% completed

We were able to demonstrate that a combination of RET with DIC could strongly amplify resting K_v7 currents, and therefore was a good candidate for analgesic treatments.

Sub-threshold combinations of RET and DIC (see figure 4) were tested using procedures identical to those described above. Several combinations were investigated (see p. 84), and three are presented in detail. Combining 5 μ M RET with 50 μ M DIC significantly improved resting current amplitudes (figure 7). The average evoked currents by 5 μ M RET or 50 μ M DIC (applied singly) were -22.7 ± 3.7 pA and -26.7 ± 20.2 respectively, the combination of 5 μ M RET and 50 μ M DIC generated outward currents that averaged 59.5 ± 18.1 pA; an increase of over 70 pA. The combination of 5 μ M RET and 50 μ M DIC produce significantly more current than 70 μ M DIC or 40 μ M RET alone and was roughly equivalent to a dose of 80 μ M DIC or 60 μ M RET (figure 7). However, co-application of 5 μ M RET with 50 μ M DIC did not significantly improve voltage sensitivity beyond that which could be obtained by single application of DIC or RET (not shown).

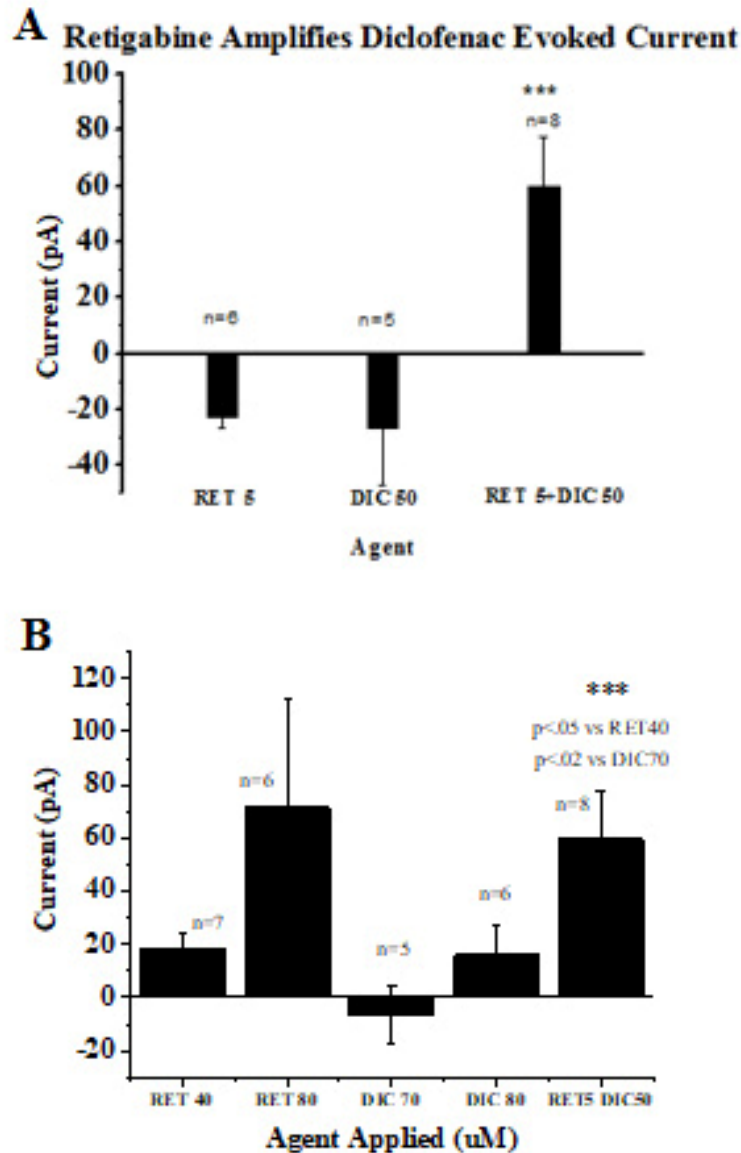


Figure 7. The combination of subthreshold doses of RET 5 uM and DIC 50 uM produced currents exceeding those of RET or DIC alone. **A)** Currents measured at the end of the 2 min combined application greatly exceed those evoked by individual applications of sub-threshold doses (RET 5 uM or DIC 50 uM). **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). *** $p < 0.05$ 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 μ M RET with 20 μ M DIC produced a similar pattern of outcomes at a lower dose (figure 8). The combination of 5 μ M RET with 20 μ M 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 μ M and 50 μ M DIC. Despite the high variability, the combination of 5 μ M RET and 20 μ M DIC produced significantly more current than 80 μ M DIC or 40 μ M RET alone and was roughly equivalent to a dose of 100 μ M DIC or 60 μ M RET.

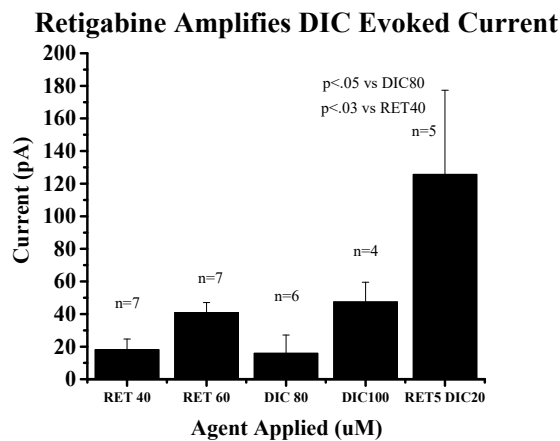


Figure 8. The combination of doses of RET 5 μ M and DIC 20 μ M produced currents exceeding those of RET or DIC alone. Currents measured at the end of the 2 min combined application exceeded those evoked by supra-threshold applications of RET 40 μ M or DIC 80 μ M. RET 60 μ M or DIC 100 μ M evoked currents were similar to those of RET 5 μ M and DIC 20 μ M 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).

Summary (major task 2, subtask 2, specific aim 2)

The combination of RET/DIC was very effective on resting current, but did not amplify voltage dependent conductance. Based upon comparative resting currents achieved by the best combination (125 pA), it did not perform as well as RET/MEC. (250 pA).

Major Task 2, Subtask 3: Meclofenamate and Diclofenac

Timeline: Months 10-11

100% Completed

The combination of MEC with DIC proved to be very effective means for amplification of both resting current activation and voltage dependent conductance. The influence on voltage dependent conductance set this combination apart from other combinations that were tested.

Studies were conducted as described above. Many combinations were tested (see p. 87), with the best results obtained with subthreshold levels of DIC and minimally supra-threshold levels of MEC (see figure 4). As shown in figure 9, the combination of MEC 40 μ M with DIC 40 μ M evoked large currents in deep tissue nociceptors (363 ± 109 pA). This combination exceeded the maximum current that could be evoked by DIC alone (120 μ M) or 60 μ M MEC. Good results were also obtained by the combination of 5 μ M MEC with 40 μ M DIC. Although both of these concentrations were considerably below threshold, when they were presented in combination, sustained outward currents could be produced (figure 9A).

The combination of DIC with MEC (DIC 40/MEC 40) was the lone instance in which a potentiation of voltage dependent conductance was observed with any of the RET/DIC or RET/MEC combinations (figure 10A). Although trends were visible, no significant potentiation was found at DIC40/MEC 5, DIC40/MEC20, or DIC30/MEC40. Voltage dependent increases in conductance, when combined with potentiated resting current amplification should substantially increase the analgesic potential of DIC/MEC combinations.

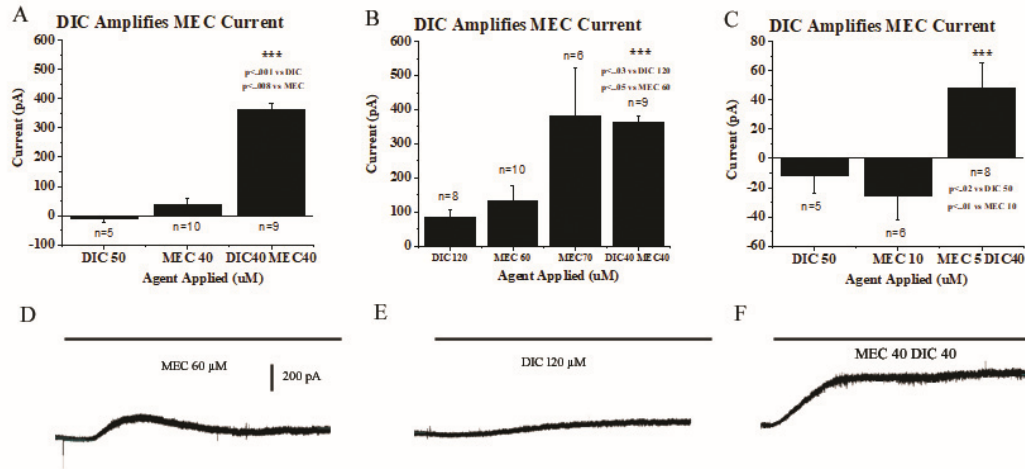


Figure 9. Combinations of DIC and MEC produced currents exceeding those of DIC or MEC alone. **A)** Currents measured at the end of the 2 min combined application greatly exceed those evoked by individual applications of sub- and near- threshold doses (DIC 40 μM, MEC 40 μM, respectively). **B)** Currents measured at the end of the 2 min combined application exceeded those evoked by applications of DIC 120 μM or MEC 60 μM. **C)** Application of MEC 5 μM and DIC 40 μM exceeded those evoked by subthreshold applications of DIC 50 μM or MEC 10 μM. We did not conduct tests of DIC at 40 μM or MEC 5 μM specifically, but these were well below thresholds for current activation. **D, E and F)** Representative traces of MEC 60 μM, DIC 120 μM or DIC40 μM /MEC40 μM. Note that for MEC60 μM, the peak current was substantially higher. This was not uncommon for low dose applications. Data were collected from voltage clamp experiments conducted in a K⁺ isolation solution. Statistical comparisons were made between DIC/MEC, DIC and MEC tests on different nociceptive cells. For statistical tests, the currents were normalized for cell size (pA/pF).

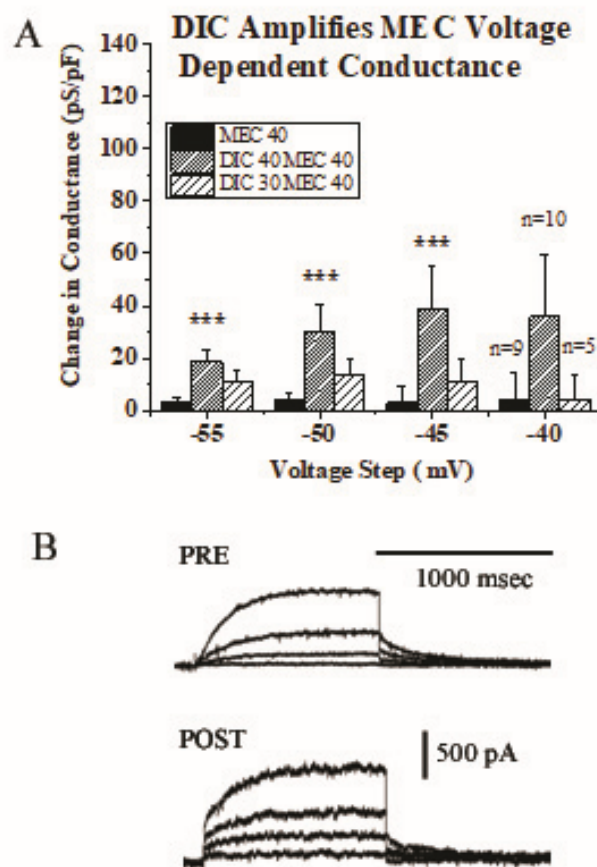


Figure 10. K_v7 conductance enhanced by a DIC/MEC combination. **A)** DIC 40/MEC 40 showed significant voltage dependent effects while DIC30/MEC 40 did not (voltage steps as in figure 1). Other combinations with MEC40 also failed. **B)** Representative voltage traces prior to and following DIC40/MEC40 (same cell; leak corrected traces shown). Data were collected from voltage clamp experiments conducted in a K^+ isolation solution. Statistical comparisons were made between DIC/MEC and MEC tests on different nociceptive cells.

Summary (major task 2, subtask 3, specific aim 2)

The combinations of MEC/DIC exhibited superior potential for analgesic capacity. This was evidenced by the massive amplification of K_v7 activity at low doses of the two KVOs. Although evoked current was greatly amplified by both RET/DIC and RET/MEC combinations, the voltage dependence was only potentiated with the MEC/DIC combination.

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 follows a complex series of intracellular events that include the activation of TRPA1, the closing of K_v7, activation of Nav1.8, Nav1.9, 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 determined whether KVO combinations were able to block muscarinic depolarization and action potential discharges. Good results were obtained.

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
100% completed

Subtask 2: KVO combination Dose 2

Timeline: Months 25-29
100% completed

Subtask 3: KVO combination Dose 3

Timeline: Months 25-31
100% complete

We found that combinations of KVOs were effective at reducing action potential bursting in deep tissue nociceptors.

In our acute culture preparation, 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). As a result, AP activation would be reduced or blocked and pain perception would be reduced. We examined whether our combination KVO strategy could hyperpolarize membranes (make RMP more negative) and block APs when nociceptors were subjected to a cholinergic challenge test.

Subtask 1, Combination 1: RET and DIC

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 11). 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 11). 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 11A and B).

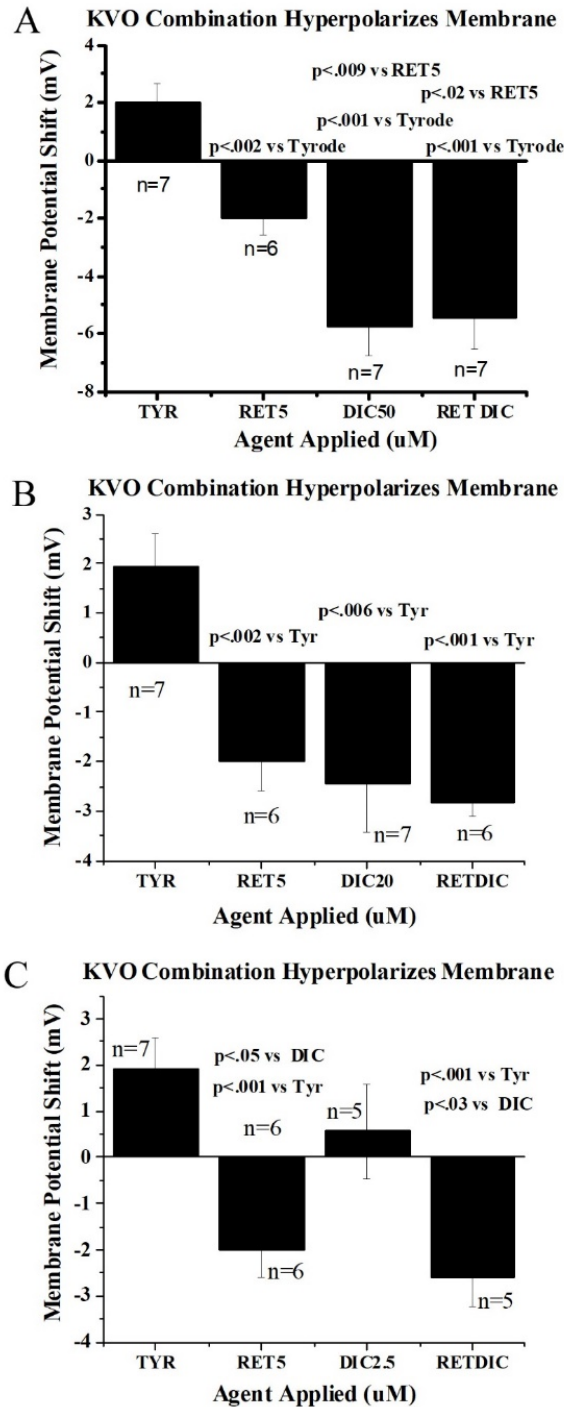


Figure 11. Combinations of RET and DIC Hyperpolarized Nociceptor Membranes. **A)** RET 5uM and DIC 50 uM produce powerful shifts in the membrane potential that exceeded that of RET alone. **B)** Hyperpolarization by RET 5 uM and DIC 20 uM were significantly less than that of RET 5 and DIC 50 uM. The 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.

Cells exposed to combination KVOs were then exposed to OXO (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 deep tissue 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 μ M 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 12A). The combination of 5 μ M RET with 50 μ M 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 μ M 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 μ M 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 μ M RET with 20 μ M 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 μ M RET (89.8 ± 26.8 APs; median 94.5 APs; $n=6$) nor 20 μ M DIC alone (45.3 ± 18.9 ; median 31 APs; $n=7$) reduced AP bursting (figure 12B). This was an improvement over the effect pattern of RET 5/DIC 50 μ M; however, the combination of RET 5/DIC 20 μ M was not significantly better at reducing AP discharges than DIC 20 μ M alone. The better performance achieved with a lower level of DIC may have resulted from the significantly larger OXO dependent

depolarizations observed when 50 μ M 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 μ M RET and 2.5 μ M DIC. Significant hyperpolarizations were retained with this combination (figure 11C), but neither of these doses could reduce AP discharge when presented individually. The combination dose also proved to be effective when challenged with OXO (figure 12C). 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 μ M treatment combination was significantly superior to either RET 5 or DIC 2.5 μ M single applications and did not differ from those of the above combinations (RET 5/DIC 50 μ M; RET 5/DIC 20 μ M).

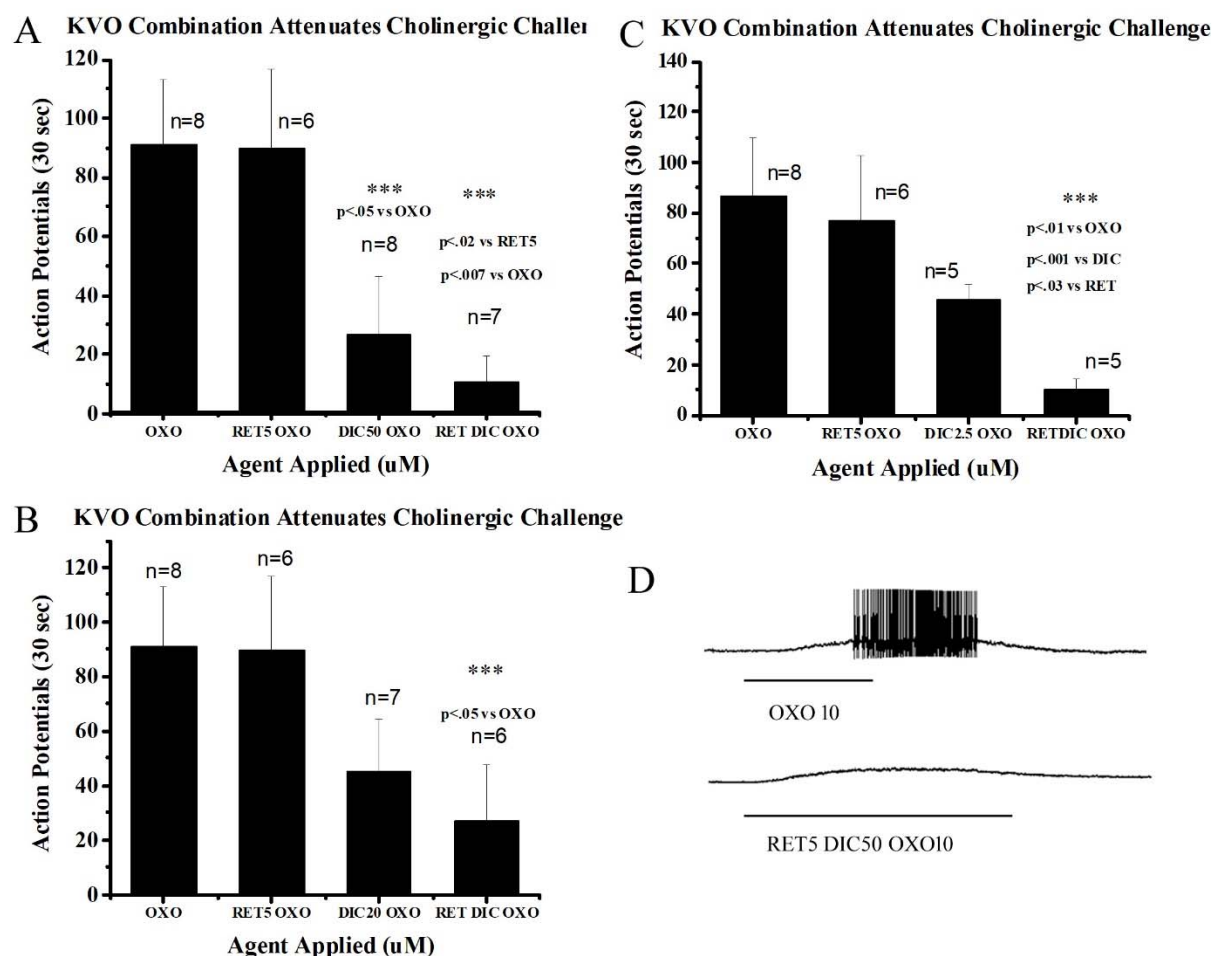


Figure 12. Combination KVOs Reduce Deep Tissue Nociceptor Discharge Consequent to a Cholinergic Challenge. **A)** The number of AP's evoked by 10 uM OXO were significantly reduced by RET 5 uM and DIC 50 uM. RET 5 uM had no influence. **B)** RET 5 uM and DIC 20 uM significantly reduced APs evoked by 10 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 μ M and DIC 1.25 μ M 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.

Summary (major task1, subtask 1, specific aim 3)

Combinations of RET and DIC hyperpolarized membranes and suppressed nociceptor action potential discharge. Due to the combination strategy, we were able to lower DIC to only 2.5 μM .

Subtask 2, Combination 2: RET and MEC

Using methods identical to those above, we determined that the combination of RET and MEC could produce synergized K_v7 activation to hyperpolarize membranes and suppress AP discharges at low dose concentrations.

One minute following current injection, the cell was exposed to either RET or MEC alone or a RET/MEC combination. Immediately afterward, OXO (10 μM), containing the test KVO(s), was superfused for a maximum of 1 minute.

At a dose of 0.5 μM , MEC application resulted in a weak depolarization ($n=4$) or hyperpolarization ($n=7$) resulting in a net depolarization of (1.58 ± 1.06 mV; figure 7A). There was no influence of OXO on evoked APs at MEC 0.5 μM (figure 7B). Accordingly, we chose 0.5 MEC to test potential synergisms with RET.

The combination of RET (5 μM) with MEC (0.5 μM) did not improve hyperpolarization over that which could be produced from RET alone (figure 13A), but significantly surpassed that of vehicle and MEC 0.5 μM . Neither

RET 5 μ M, nor .05 μ M MEC alone were able to significantly reduce AP discharges during OXO testing, but strong trends were observed (figure 13B). The RET MEC combination of 5 μ M and 0.5 μ M significantly reduced OXO dependent nociceptor APs relative to vehicle or individual KVOs (figure 13B).

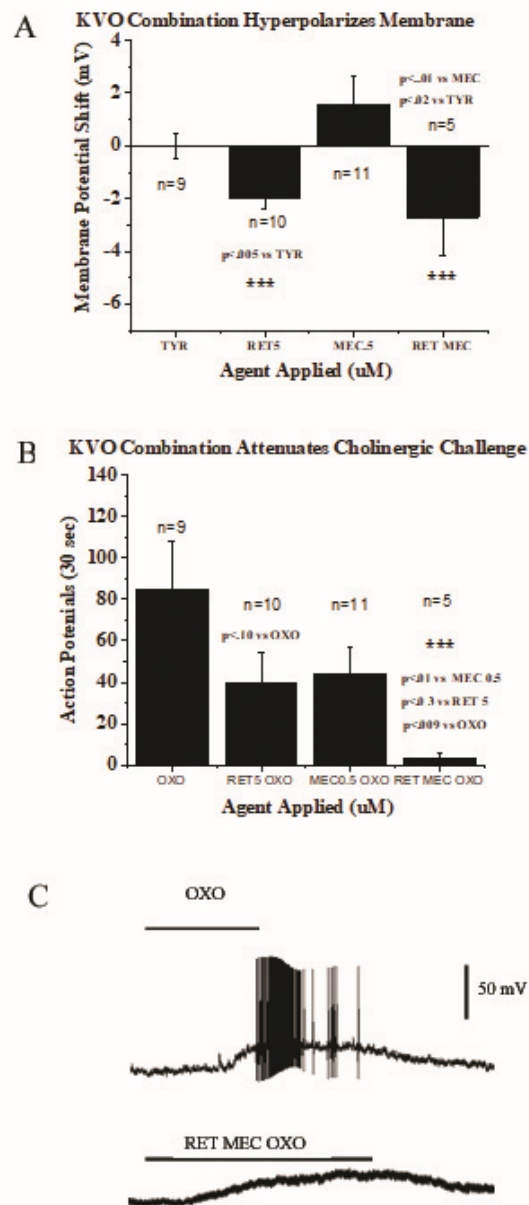


Figure 13. Combinations of RET and MEC hyperpolarized membranes and suppressed AP discharges. **A)** RET 5 μ M and MEC 0.50 μ M shifted the membrane potential to more negative levels. Shifts were larger for the KVO combination relative to MEC 0.5 alone. **B)** Action potential generation was significantly suppressed by the same RET/MEC combination. APs produced by OXO in the presence of RET or MEC trended lower but failed to reach significance (p<.10 and p<.14 for RET 5 μ M and MEC 0.5 μ M, respectively). **C)** Representative traces from a test of OXO 10 μ M (upper) and RET5/MEC0.5/OXO 10 μ M (lower). Both traces were shortened to preserve detail.

Summary (major task 1, subtask 2, specific aim 3)

The combination of RET and MEC was highly effective against nociceptor activity at very low doses.

Subtask 3, Combination 3: MEC and DIC

Nociceptive neurons were isolated as previously described and all testing procedures were identical to those above. Combinations of MEC/DIC were adjusted to avoid the powerful *depolarizing* actions of MEC at concentrations exceeding 5 μ M, as well as to strive for the lowest effective concentrations (see attached manuscript; Cooper et al., submitted, p. 154).

Of the two MEC combinations with DIC that were tested, the best results were obtained with MEC 0.5 and DIC 2.5 μ M. Within 2 min after application, nociceptor membranes were significantly hyperpolarized by MEC/DIC ($-2.02 \pm .58$ mV; figure 14A). These were similar to hyperpolarizations achieved by RET/MEC or RET/DIC combinations, described above, that were shown to suppress AP discharge. When challenged with the OXO burst discharge test, the MEC/DIC combination was also highly effective. Evoked APs were strongly suppressed (7.66 ± 5.4). AP reduction by MEC/DIC significantly exceeded the effectiveness of MEC 0.5 or DIC 2.5 μ M alone (figure 14B). Statistical contrasts between APs evoked during MEC 0.5/DIC 2.5/OXO versus RET 5/MEC 0.5/OXO testing were not significant.

Summary (major task 1, subtask 3, specific aim 3)

The combination of MEC 0.5 and DIC 2.5 μ M suppressed AP discharges evoked by the cholinergic stimulus. However, the effectiveness of MEC/DIC did not exceed optimized performance with RET/MEC or RET/DIC. Given that we had prior success with RET on behavior tests, we began behavior treatment evaluations of MEC using RET/MEC combinations. Dosing ratios that were proven effective in current clamp studies were anchored by those previous behavioral tests with RET (Flunker et al., 2017). As shown below, the RET/MEC combination performed better as an analgesic than RET/DIC.

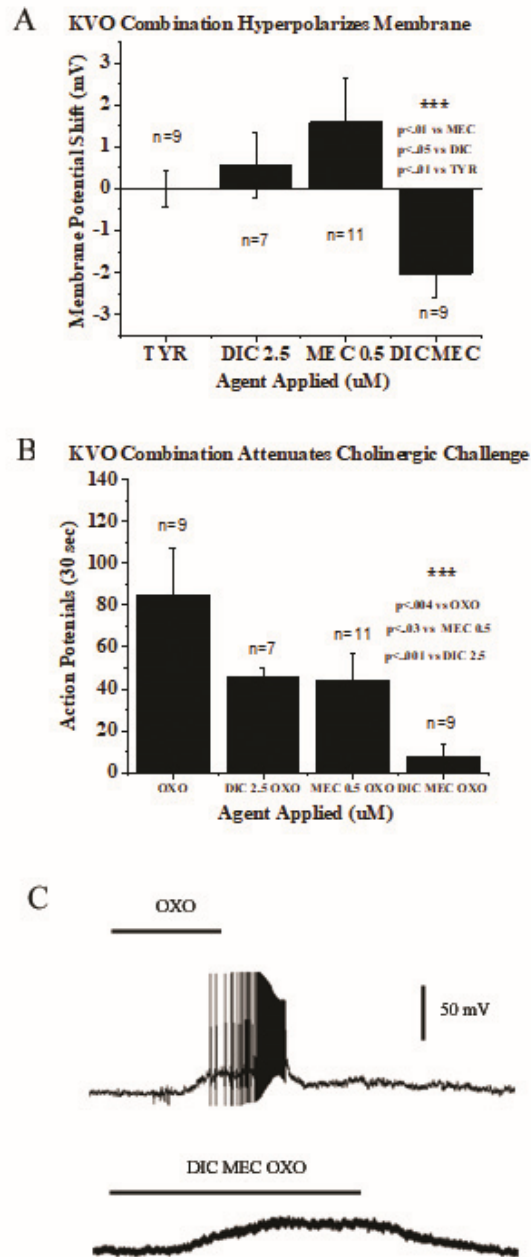


Figure 14. Combinations of DIC and MEC hyperpolarized membranes and suppressed AP discharges. **A)** MEC 0.5 and DIC 2.5 μM shifted the membrane potential to more negative levels. Shifts were larger for the KVO combination relative to MEC 0.5 or DIC 2.5 μM alone. **B)** Action potential generation was significantly suppressed by the same DIC/MEC combination. APs produced by OXO in the presence of DIC or MEC trended lower but failed to reach significance ($p<.12$ and $p<.14$ for DIC 2.5 μM and MEC 0.5 μM , respectively). **C)** Representative traces from a test of OXO 10 μM (upper) and DIC2.5/MEC0.5/OXO 10 μM (lower). Both traces were shortened to preserve detail.

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 (BEH1)

Months 14-18

100% Completed

Subtask 2: KVO combination Dose 2 (BEH2)

Months 20-24

100% Completed

Subtask 3: KVO combination Dose 3 (BEH3)

Months 25-29

100% Completed

These studies were the most impacted by COVID lab closures, as rats that were midway through the post GW chemical exposure testing had to be euthanized due to the enforced laboratory shut down at UF.

At the conclusion of these studies, we provided evidence that KVO combinations could reverse pain signs associated with GW chemical exposures. However, not all the evidence was fully supportive and serious side effects were encountered with the RET/DIC combination.

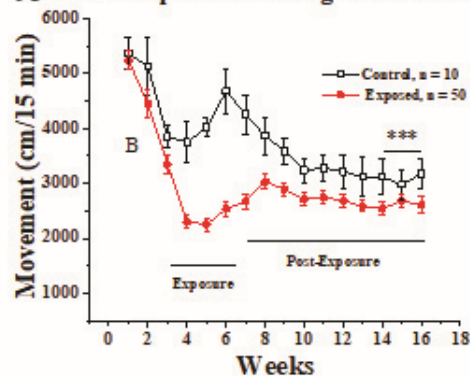
Two distinct experiments were conducted to examine the effectiveness of RET/DIC RET/MEC and MEC/DIC combinations (BEH1, BEH2). In each study KVO treatments were applied after pain-like signs had emerged. In BEH1, we tested the capacity of a 1:4 molar ratio of RET:DIC. This study followed from our observations in Specific Aims 1 and 2, that a 1:4 molar ratio (5 μ M RET and 20 μ M 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. 63 for further details).

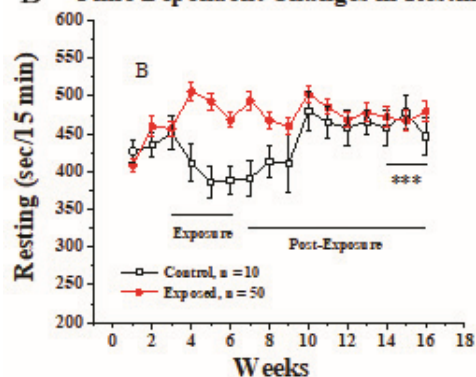
Behavior Study 1 (BEH1)

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; figure 12). 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 15C). Changes in rest times were relatively slight, but statistically significant. There were no significant changes in movement distance measures.

A Time Dependent Changes in Movement



B Time Dependent Changes in Resting



C Exposed Rats Treated with RET+DIC

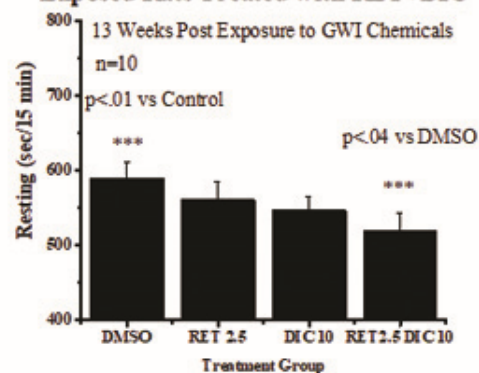


Figure 15. The Combination of RET and DIC (1:4) Reduced Pain-Like Behavior. A) Weekly progression of movement distance data before, during and following exposure to GW chemicals. B) Weekly progression of rest time scores before, during and following exposure to GW chemicals. C) The influence of single and combination treatments on rest time scores. B: baseline testing. *** significantly different by repeated measures ANOVA ($p < 0.02$ and $p < 0.002$, 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.

Behavior Study 2 (BEH2)

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 16, 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 had developed over time.

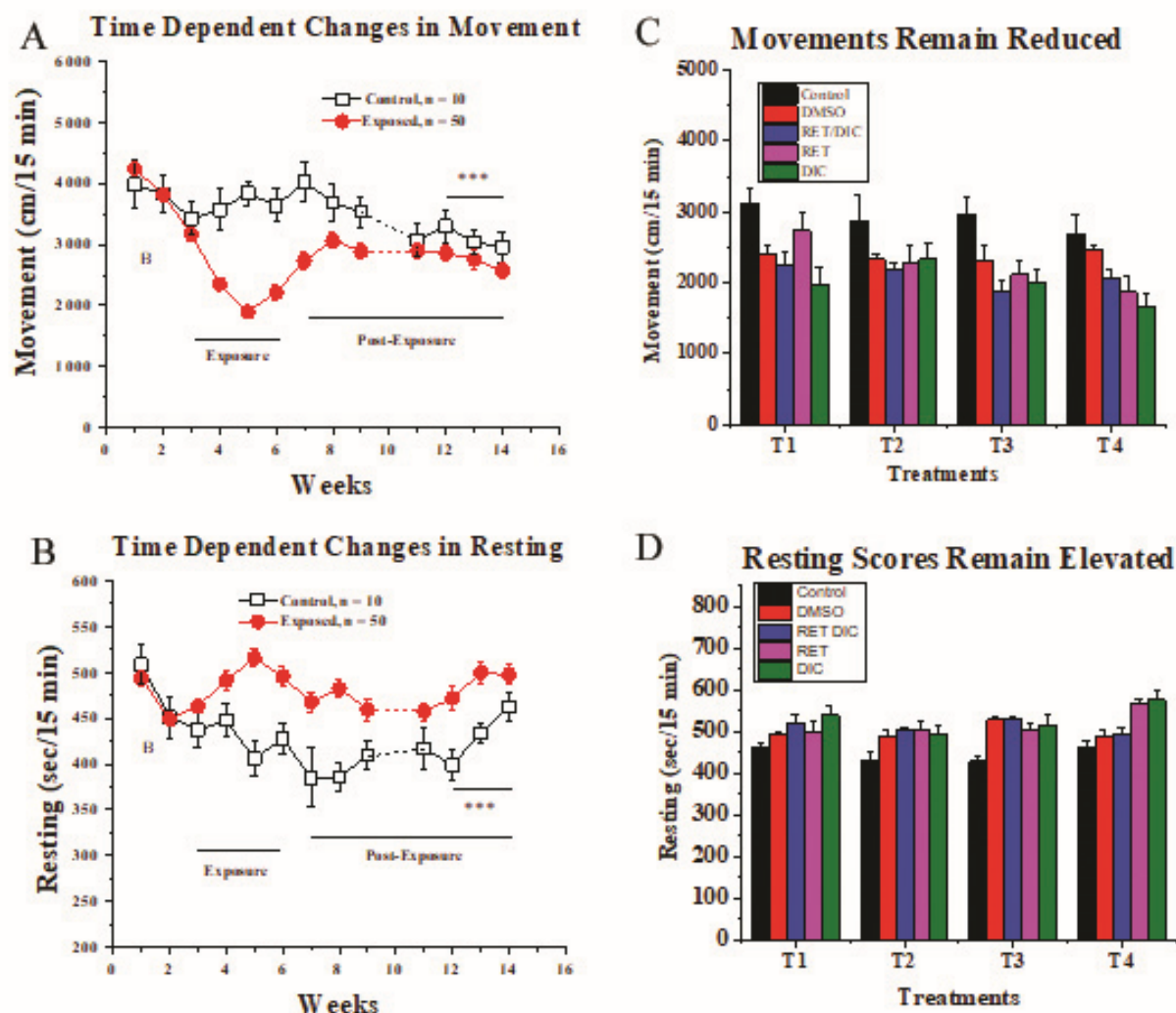


Figure 16. Multiple Dose Combinations of RET and DIC Fail to Modify Pain-Like Behaviors. **A)** Weekly progression of movement distance scores before, during and following exposure to GW chemicals. **B)** Weekly progression of rest time scores before, during and following exposure to GW chemicals. **C)** The influence of single and combination treatments on movement distance scores; **D)** The influence of single and combination treatments 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.

Behavior Study 3 (BEH3)

Studies conducted with combinations of RET and MEC produced outcomes consistent with the hypothesis that combination treatments could produce analgesia in rats with established GWI chronic pain-like signs.

Following a 4 week exposure to GW chemicals (Permethrin, DEET, PB and Chlorpyrifos or vehicle; see Methods, p. 61), rats (n=32) developed pain-like signs manifested as decreased Movement Distance, average Movement Rate and increased Resting Duration (figure 17). Eight additional rats served as unexposed controls (GRP CON). Twelve weeks following neurotoxicant exposure, the 32 exposed rats were randomly divided in 4 groups (GRP EXP, GRP RETMEC, GRP RET, GRP MEC). Treatments began in Week 17. Each group of 8 rats received a KVO treatment or water vehicle (oral gavage). Two different dose combinations were administered in weeks 17 and 18 and one was repeated in week 19 (post-exposure weeks 13, 14 and 15). Both treatments were based upon the 10:1 ratio of RET to MEC that was shown to be highly effective in current clamp studies (the actual mg/kg doses varied slightly due to small differences in the molecular weight of RET and MEC).

Since we had previously shown successful, though transient, reversal of pain-like signs at a RET dose of 7 mg/kg (Cooper et al., 2018), we used this dose as an anchor point around which to design the KVO combination treatments (Week 17, Treatment 1: RET 7 mg/kg, MEC 0.7 mg/kg; Week 18, Treatment 2 and Week 19, Treatment 3: RET 5 mg/kg, MEC 0.5 mg/kg. With these combinations, we hoped to improve the analgesic performance of RET, as demonstrated in Cooper et al., (2018), and sustain its effectiveness through repeated treatments. Groups of 8 rats received single and combination treatments of KVOs as indicated by GRP names, above.

In order to confirm the presence of pain-like signs prior to Treatment testing, we subjected free range activity scores to a repeated measures ANOVA on GRP CON and GRP EXP (n=8) across the time period of weeks 13-16 (post-exposure weeks 9-12). Significant main effects of neurotoxicant exposure (EXP) were confirmed for

activity measures of Movement Distance and Rate ($F=4.3$, $p<.04$ and $F= 13.64$, $p<.001$, respectively); indicating, that pain-signs were present and persistent prior to treatment testing (figure 17). Analysis of the Rest Duration times failed to reveal a significant effect for weeks 13-16 (data not shown). Accordingly, Rest Durations were not analyzed for subsequent Treatment effects.

KVO treatment studies (Treatment 1: 7 mg/kg RET, 0.7 mg/kg MEC) were initiated in Week 17. ANOVA conducted on the 5 GRPS of Treatment 1 (CON, EXP, RETMEC, RET, MEC) revealed a significant main effect for Movement Rate, with Movement Distance barely failing the Main effect test ($F=3.02$, $p<.03$ and $F=2.5$, $p<.06$ and, respectively). Follow-up tests (Fisher LSD) revealed a significant effect of exposure (GRP CON vs GRP EXP), for Movement Rate, but not Movement Distance (figure 17C and D; Week 17). In any case, Treatment RECMC did not improve Movement Rate or Distance relative to GRP EXP. Given that Treatment 1 tended to decrease Movement Distance and Rate relative to GRP EXP, we lowered the base dose of RET to 5 mg/kg (0.5 mg/kg MEC) for Treatment 2 studies.

In Treatment 2, groups of 8 rats were administered, 5 mg/kg RET and/or 0.5 mg/kg MEC singly and in combination. GRP EXP and CON received only water. All treatments were administered by oral gavage, and behavior testing was conducted 2 hrs after gavage. As before, we conducted a one-way ANOVA on GRP CON, EXP, RETMEC, RET, and MEC. Significant main effects of Treatments were realized for Movement Rate ($F=4.47$, $p<.005$) and approached significance for Movement Distance ($F=2.02$, $p<.11$). We chose to examine GRP differences in both analyses, given our *a priori* plan to examine individual groups.

Post-hoc testing (Fisher LSD) indicated partial support for the hypothesis that combination treatments would produce improved analgesic outcomes in our GWI model. In the Movement Rate analysis, we confirmed a significant pain effect manifested as decreased Rate for GRP EXP relative to GRP CON (figure 17D, Week 18).

Importantly, those rats treated with RETMEC demonstrated improved Movement Rate performance relative to GRP EXP. That is, the average Movement Rate increased to levels that were significantly better than GRP EXP, and improved to the point that they were not significantly different from the unexposed GRP CON (figure 17D, Week 18). GRP RETMEC was also significantly improved relative to GRP MEC (7.7 ± 0.67 vs 6.39 ± 0.39 cm/sec; figure 17D). However, GRP RET also exhibited some analgesic effects. Specifically, GRP RET also manifested improved performance relative to GRP EXP. Although improvements trended better for GRP RETMEC relative to GRP RET (7.7 ± 0.67 vs 7.13 ± 0.27 cm/sec) a difference could not be supported statistically.

A priori analysis of the Movement Distance data lent further support to a potential RET/MEC synergism. Tests on specific GRPs confirmed both a significant effect of pain, (CON vs EXP) and also a significant effect of GRP RETMEC vs GRP EXP (figure 17C, Week 18). That is, Movement Distance scores increased for RET/MEC treated rats relative to GRP EXP and were non-different from those of unexposed GRP CON. None of the other Treatment contrasts were significantly different from, water treated, GRP EXP.

In Treatment 3, we attempted to replicate and/or clarify the RET 5/ MEC 0.5 mg/kg outcome. Treatments were carried out in an identical manner. Unfortunately, we were unable to confirm a significant pain effect in week 19 (GRP EXP vs GRP CON; figure 17C and D); as a result, the treatment data was difficult to interpret. Additional testing in Week 20 also failed to confirm that a significant pain effect was still present. Behavioral tests ceased at this point, and we were unable to conduct further Treatment tests that would be meaningful.

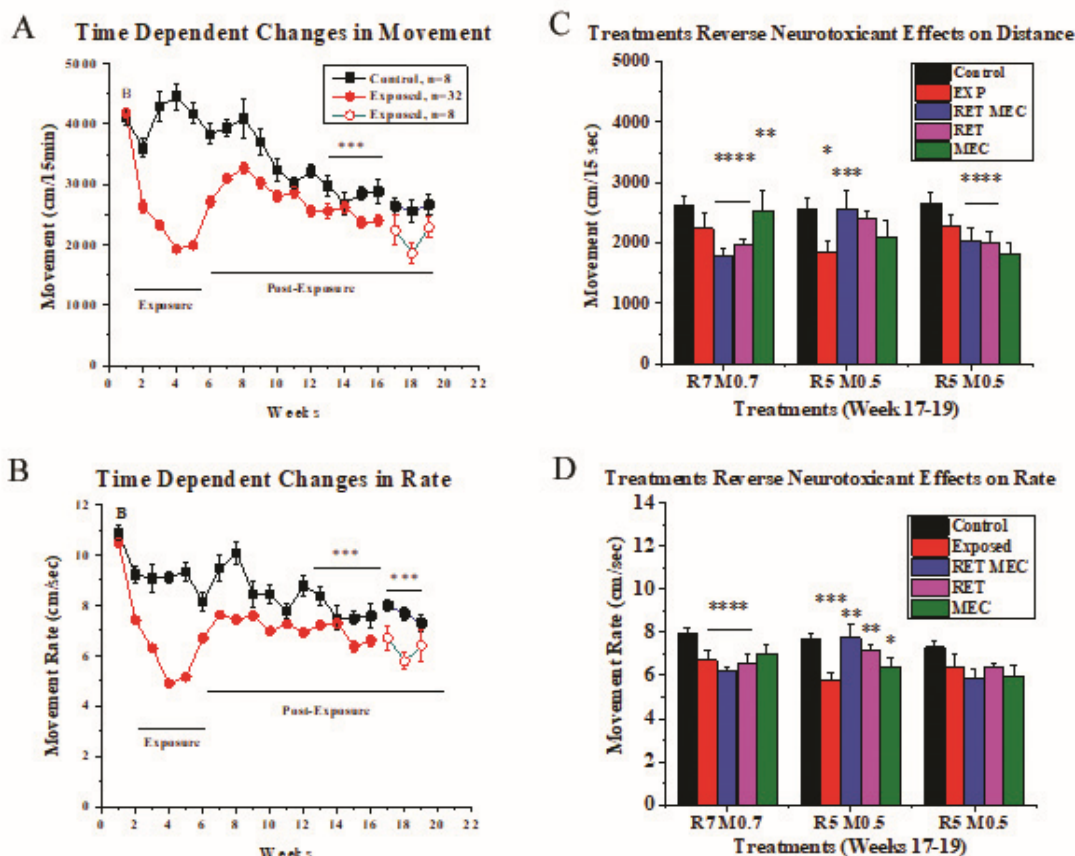


Fig. 17. The combination of RET and MEC reduced pain-like behaviors. A) and B) Weekly progression of Movement Distance and Rate data before, during and following exposure to GW chemicals. Repeated measures ANOVA verified significant pain-like changes for rats in the last 4 weeks prior to treatment testing (***). C) The influence of Treatment 1, 2 and 3 on Movement Distance scores. In Treatment 2 (Week 18), RETMEC treated rats significantly improved over water treated GRP EXP. D) The influence of Treatment 1, 2 and 3 on Movement Rate scores. In Treatment 2 (week 18) RETMEC treated rats significantly improved over water treated GRP EXP and MEC treated rats but did not differ from GRP RET. GRP RET also improved relative to GRP EXP but not relative to GRP MEC. B: baseline testing. **** significantly different from GRP C. *** significantly different from GRP EXP. ** significantly different from GRP RETMEC; * significantly different from GRP CON. $P < .05$, Fisher LSD.

Summary (major task 1, subtask 1, 2 and 3, specific aim 1)

Combining KVOs can produce an analgesic outcome in a model of GWI chronic pain. The RET/MEC combination (5 mg/kg and 0.5 mg/kg, respectively) restored normal function 14 weeks after exposure to GWI chemicals. On the negative side, a perfect outcome pattern was not obtained and there was still some reason to doubt that the observed result was purely due to combination treatments. Nevertheless, on the two measures in

the BEH3 studies (Movement Distance and Movement Rate) only the RET/MEC treated groups manifested the desired treatment outcome.

Adding to the negative, repeated treatment with the RET/DIC combinations produced serious side effects, including some fatalities. In BEH1, relatively high doses of DIC were used and repeated daily. We did not use daily treatments in BEH3 and cannot assert at this time, whether daily treatments of RET/MEC would have caused serious side effects. However, the dose of MEC was very low (0.5 mg/kg) compared to the problematic dose of DIC (10 mg/kg).

Final Summary and Conclusions

Combination therapies with KVOs can greatly improve activation of K_v7 ion channels in nociceptors, greatly reduce concentrations required for maintained activation, and improve voltage dependent conductances. All of these are consistent with improved analgesic potency. We were able to demonstrate an analgesic effect using a RET/MEC treatment. Whether MEC and DIC are the optimal adjunctive agonists for RET is questionable, as unwanted side-effects on TRPA1 and TTXs Na_v, are problematic (see attached manuscript, Cooper et al., submitted; p 154). The development of other KVO synthetics, that are designed to bind to diverse K_v7 binding sites, but lack significant influence on Na_v and TRPA1, could fulfill the promise of improved analgesics for GWI or other chronic pain conditions where K_v7 expressing nociceptors may play a role in the symptoms of chronic pain.

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?

Not applicable

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. These effects were particularly powerful with the RET/MEC and MEC/DIC combinations. The RET/DIC combination was weaker than the RET/MEC combination in molecular studies, but performed just as well in cellular studies. The amplification of K_v7 activity allowed for a substantial reduction of doses in analgesic applications. 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. In behavioral tests with RET/MEC, good behavioral results were obtained, but studies were

incomplete, as we could not test daily dose regimens. We did not have time to test a MEC/DIC combination therapy, in part, because the pain effect was not prolonged in the rats of BEH3. Further development of KVOs with improved properties is a promising direction for development of analgesics.

Successful development of experimental drugs that attack the preferential MEC binding site on K_v7.2, with decreased side effect risks, could be used in an effective pain relief combination treatment strategy for veterans suffering from GWI chronic pain.

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 analgesic drug development. This work has demonstrated that KVO combination therapies have promise in many applications, particularly for deep tissue pain.

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

Changes in approach and reasons for change

We experienced more problems with side effects during the behavioral studies than we had anticipated. We had originally planned to test Celecoxib as a KVO, but we found that this agent had broad effects on K^+ channels other than K_v7 . Accordingly we halted these studies.

We had planned to test the KVO treatment test on consecutive days. But during the BEH1 studies we found that this produced serious side effect that terminated the study. Accordingly we shifted to a one day per week treatment plan.

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 or during the extension.

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). We did not experience significant side effects during BEH2 or BEH3 testing; however, we did not attempt daily treatments in either of these studies.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

We 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*. It was accepted and published in Jan. 2022 (see attached, p. 76).

A second manuscript was submitted to *Toxicology and Applied Pharmacology*, in May, 2022. It is currently under review (see attached, p. 122).

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 later submitted an abstract to the MHSRS conference in August 2021. This conference was cancelled a month prior to the scheduled meeting.

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; ED₅₀=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; ED₅₀: 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 μ M and RET 2.5 μ M (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 μ M RET to 4 μ M 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 μ M, 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 μ M or 5 μ M RET; 2.5 μ M 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 $\pm$ 0.67 mV vs -2.54 $\pm$ 0.82, $p < .001$, $n = 7$ and 7 ; -2.59 $\pm$ 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 $\pm$ 19.3 APs vs 10.5 $\pm$ 4.3 APs; $p < .009$, $n = 7$ and 9 ; and 10.2 $\pm$ 4.27 APs; $p < .02$, $n = 5$ and 9 ; vehicle, 4:1 and 2:1 RD molar ratios, respectively). Combination dosing with 5 μ M RET and 2.5 μ M DIC were significantly superior than either agent alone ($p < .002$ and $p < .02$ vs 5 μ M RET and 2.5 μ M 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: 31

Contribution to Project: Design, execution and analysis of physiology and behavior experiments;
Preparation of Reports

Funding Support: This grant and University of Florida College of Dentistry

Name: Thomas J. Nutter, Ph.D., College of Dentistry

Project Role: Biological Scientist IV

Researcher Identifier: none

Person Months: 31

Contribution to Project: Execution of physiology experiments

Funding Support: This grant

Name: Linda Flunker, MS, College of Dentistry

Role on Project: Biological Scientist III

Research Identifier: none

Person Months: 31

Contribution to Project: Preparation of solutions; Preparation of rats; ordering laboratory items; General laboratory duties; Behavioral Testing

Funding Support: This grant

Name: Clarissa Bowers, BS

Role on Project: Student Volunteer (uncompensated)

Research Identifier: none

Person Months: 10

Contribution to Project: Execution of physiology experiments; Behavioral testing

Funding Support: Student volunteer

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

Optimizing Kv7 Channel Openers for Treatment of GWI Pain

GW180038

W81XWH-19-1-0657

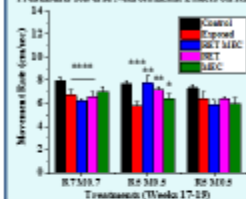
PI: Brian Y. Cooper, PhD Org: University of Florida Award Amount: \$1,122,172.00



Study/Product Aim(s)
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

Approach: Molecular and cellular studies on deep tissue nociceptors to determine analgesic potential of combined therapies (synergism) using Kv7 ion channel openers (KVOs) to hyperpolarize nociceptor membranes. Behavioral testing in a rat model of Gulf War illness chronic pain.

Treatments Reverse Neurotoxicant Effects on Rate



Graph (middle group) shows analgesic effect of combined KVO treatment RET MEC on rats exhibiting pain-like signs in a model of GWI chronic pain 14 weeks after exposure to GW toxicants.

Timeline and Cost

Activities	CY	19	20	21	22
Dose Response of KVOs					
KVO Synergisms (Molecular)					
KVO Synergisms (Cellular)					
KVO Synergisms (Behavioral)					
Estimated Budget (\$K)		\$28.75	\$358.3	483.8	250.8

Updated: 06/15/2022

Goals/Milestones

CY19 Goal – Dose Response MEC DIC CEL (Molecular)
 50% complete
CY20 Goals – Dose Response MEC DIC RET; RET DIC synergisms
 100% completed Dose Responses (Molecular)
 100% completed RET DIC synergisms observed (Molecular)
CY21 Goals – Optimized Synergism on Nociceptor Cells (RET DIC; RET MEC); MEC DIC Molecular Synergism; Behavioral Testing for RET DIC; RET MEC
 100% Completed; RET DIC Side Effect Displace Analgesia
 75% Completed RET MEC Behavior
CY22 Goal – Behavior Testing for RET MEC and MEC DIC; Optimized MEC DIC; Cellular Synergisms
 100% Completed RET MEC; Analgesic Effect for RET MEC
 50% Completed MEC DIC

Budget Expenditure to Date

Projected Expenditure: 1,122,172
 Actual Expenditure: 1,121,680

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

[illegible]

Specific Aim 2 Optimizing Molecular Synergisms of KVOs on Deep Tissue Nociceptor K _v 7	Timeline	Site 1	Site 2
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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

Subjects. Young adult male rats (n=408) were used in physiology and 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).

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 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 M Ω) by a Sutter P1000 (Sutter Instruments, Novato, CA). The pipette solution contained (in mM): 120 KCl, 5 Na₂-ATP, 0.4 Na₂-GTP, 5 EGTA, 2.25 CaCl₂, 5 MgCl₂, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm. 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 H₂O as needed). Stock solutions were kept at -20 °C. On the day of the experiment, solutions of 5-140 μ M 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 MgCl₂, 0.2 CaCl₂, 1 CsCl₂, 2 4-amino pyridine, 10 glucose, 10 HEPES, (adjusted to pH 7.4 with KOH) was applied, for 2 minutes, by close superfusion (1000 μ m).

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 μ M; 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 (G_1 and G_2) according to the formula: $I_L/(V_{\text{step}}-V_{\text{rev}})$. 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: $G_{\text{diff}}=G_2- G_1$. 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 μ M). 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

Exposure to GW Chemicals.

Over a period of 4 weeks, 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.

Treatment Studies

At least eight weeks after exposure to GW chemicals, GRP E rats were randomly divided into groups of 8-12 rats. Depending on the goal of BEH1, BEH2 and BEH3, rats were treated with DIC, RET, MEC, DIC+RET, MEC+RET, water or DMSO. Treatments were given two hours prior to behavioral testing.

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). In BEH1 and BEH2 statistical comparisons on groups were made between vehicle exposed and neurotoxicant chemical exposed rats. In BEH3, a one way ANOVA was used to assess the influence of treatments

on measures of movement distance and rate. The Fisher LSD test was used to make a priori tests on individual groups. For physiology experiments, 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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Data Tables

VOLTAGE CLAMP STUDIES: CONTROLS

CONTROLS		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
VEHICLE: DMSO		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pA/pF)	at 2 min post	NEGATIVE SHIFT (pA)
0	2.73	2.23	23.68	19.28		-14.9	-14.9	-0.1795	-0.1795	171
0	-6.46	6.81	10.44	9.09		-7.6	-7.6	-0.1118	-0.1118	137
0	-2.86	5.11	17.80	38.05		2.75	2.75	0.0387	0.0387	83
0	-1.89	15.82	23.98	21.58		-11.4	-11.4	-0.1500	-0.1500	124
0	7.36	7.61	13.07	21.54		-16.5	-16.5	-0.2292	-0.2292	111
0	-4.21	-2.77	4.32	10.14		-11.7	-11.7	-0.1290	-0.1290	102
0	0.58	2.95	14.51	33.57		-20.3	-20.3	-0.2207	-0.2207	71
0	-8.07	4.72	15.23	36.69		-0.5	-0.5	-0.0083	-0.0083	134
0	-7.05	5.22	1.88	7.31		-15.5	-15.6	-0.1761	-0.1773	122
MEAN	-2.21	5.30	13.88	21.91		-25.60	-25.61	-0.1295	-0.1297	136.80
STD DEVIATION	5.05	4.98	7.61	11.93		40.95	40.95	0.0913	0.0913	68.07
N	9.00	9.00	9.00	9.00		9.00	9.00	9.0000	9.0000	9.00
STD ERROR	1.68	1.66	2.54	3.98		12.35	12.35	0.0304	0.0304	21.53

CONTROLS		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
VEHICLE: WATER		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pA/pF)	at 2 min post	NEGATIVE SHIFT (pA)
0	-0.39	5.69	5.88	10.48		-8.5	-8.5	-0.1024	-0.1024	*
0	-2.77	4.48	18.88	36.00		-18.3	-18.3	-0.1968	-0.1968	*
0	6.36	-3.32	11.93	6.78		8	8	0.1111	0.1111	*
0	3.76	-8.23	2.34	14.89		-26	-26	-0.3377	-0.3377	161
0	1.17	-1.89	4.75	4.12		-35	-35	-0.3571	-0.3571	70
0	-1.73	8.63	8.28	5.15		-50	-50	-0.4950	-0.4950	332
						-31	-31	-0.4306	-0.4306	184
0	5.08	-3.86	1.14	1.27		-61	-61	-0.8026	-0.8026	179
0	3.09	3.61	1.99	-3.51		-7.3	-7.3	-0.1043	-0.1043	64
0	-6.23	-4.89	6.42	8.77		-12.8	-12.8	-0.1471	-0.1471	63
MEAN	0.92	0.02	6.85	9.33		-24.19	-24.19	-0.2863	-0.2863	150.43
STD DEVIATION	4.08	0.01	0.01	0.01		0.01	0.01	0.0108	0.0108	0.01
N	9.00	9.00	9.00	9.00		9.00	9.00	9.0000	9.0000	9.00
STD ERROR	1.36	0.00	0.00	0.00		0.00	0.00	0.0036	0.0036	0.00

VOLTAGE CLAMP STUDIES: MECLOFENAMATE

MECLOFENAMATE	CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
	Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
10	-2.90	4.17	2.44	17.83	0	-67	0.00000	-0.67000	92
10	4.72	13.55	3.18	19.35	61	24	0.74390	0.29268	211
10	2.33	9.37	-3.55	-52.91	3	-67	0.03614	-0.80723	110
10	2.93	0.63	6.37		142	-6	1.61364	-0.06818	372
10	2.87	1.54	5.69	-4.59	69	-49	0.76667	-0.54444	137
10	4.44	8.68	-0.91	-31.52	52	14	0.65000	0.17500	153
MEAN	2.40	6.32	2.20	-10.37	54.50	-25.17	0.64	-0.27	179.17
STD DEVIATION	2.76	5.04	3.83	31.50	52.03	40.96	0.59	0.46	103.00
N	6.00	6.00	6.00	5.00	7.00	8.00	9.00	10.00	11.00
STD ERROR	1.13	2.06	1.56	14.09	19.67	14.48	0.20	0.15	31.06
MECLOFENAMATE	CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
	Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
20	LOST				-1.7	-1.7	-0.0233	-0.0233	*
20	-0.94	0.43	4.05	-3.33	-15	-15	-0.1667	-0.1667	*
20	3.29	2.77	0.58	3.17	17	-41	0.2073	-0.5000	189
20	-10.32	4.25	-2.86	10.55	36	5	0.4557	0.0633	118
20	1.36	8.13	14.82	45.93	-17.6	-17.6	-0.2588	-0.2588	80
20	2.67	3.48	20.41	25.16	4.7	-10.5	0.0618	-0.1382	189
20	1.50	2.65	-6.07	-18.91	50	33	0.5814	0.3837	130
20	6.54	-2.74	-32.26	-59.30	130	84	1.7333	1.1200	203
20	-3.86	-17.11	-49.60	-77.45	21	-65	0.1963	-0.6075	154
20	-2.58	-13.95	-38.29	-46.06	2.4	-57	0.0267	-0.6333	85
MEAN	-0.26	-1.34	-9.91	-13.36	25.39	-9.34	0.32	-0.08	143.50
STD DEVIATION	4.91	8.59	24.45	40.72	44.98	46.35	0.60	0.56	47.93
N	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	8.00
STD ERROR	1.64	2.86	8.15	13.57	14.99	15.45	0.20	0.19	16.94

MECLOFENAMATE		CHANGE IN CONDUCTANCE (pS/pF)			CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT	
DOSE (uM)		Voltage (mV)			PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
		-55	-50	-45						
30		2.43	10.15	18.46	54.9	36.6	0.6778	0.4519	89.1	
30		-1.15	0.73	12.51	54	11.3	0.6429	0.3767	68.7	
30		-3.92	7.52	12.27	20.8	2.4	0.3200	0.0800	45	
30		-1.90	3.09	-7.34	13.1	-13.1	0.1456	-0.4367	69	
30		-1.20	-0.80	-10.43	65.6	18.9	0.7904	0.6300	164	
MEAN		-1.15	4.14	5.09	41.68	11.22	0.5153	0.2204	87.16	
STD DEVIATION		2.29	4.60	13.05	23.19	18.52	0.2707	0.4175	45.71	
N		5.00	5.00	5.00	5.00	5.00	5.0000	5.0000	5.00	
STD ERROR		1.03	2.06	5.83	9.80	10.37	8.28	0.1211	0.1867	20.44
MECLOFENAMATE		CHANGE IN CONDUCTANCE (pS/pF)			CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT	
DOSE (uM)		Voltage (mV)			PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
		-55	-50	-45						
40		-6.21	3.06	25.25	2	2	0.0260	0.0260	*	
40		-0.32	0.66	8.91	15.6	7.3	0.1835	0.0859	280	
40		lost			11.7	11.7	0.1125	0.1125		
40		3.34	15.16	26.68	164	122	1.8427	1.3708	340	
40		4.39	9.92	6.81	134.6	5.8	1.8694	0.0806	305	
40		11.15	-13.07	-28.12	142.8	-6.4	2.1000	-0.0941	176	
40		5.81	10.59	12.38	32.7	0.3	0.4088	0.0038	95	
40		-1.10	-12.40	-21.72	68.1	3.7	0.7021	0.0381	280	
40		2.68	12.45	3.06	90	56	1.0843	0.6747	210	
40		9.36	7.67	-5.17	216	194	2.3478	2.1087	216	
MEAN		3.23	3.78	3.12	87.75	39.64	1.0677	0.4407	237.75	
STD DEVIATION		5.35	10.37	18.84	73.98	66.85	0.9003	0.7361	79.27	
N		9.00	9.00	9.00	10.00	10.00	10.0000	10.0000	8.00	
STD ERROR		1.78	3.46	6.28	23.39	21.14	0.2847	0.2328	28.02	

MECLOFENAMATE		CHANGE IN CONDUCTANCE (pS/pF)			CURRENT EVOKED AT -60 MV				LINOPIRIDINE SHIFT	
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
70	28.98	78.63	88.51	78.75	903.6	903.6	14.5742	14.5742	862	
70	10.14	11.92	13.35	31.69	185.2	144.7	2.4368	1.9039	289	
70	40.59	87.56	125.01	147.24	877.7	722.4	13.1000	10.7821	774	
70	8.12	24.28	37.70	55.61	258.8	210.9	3.1951	2.6037	467	
70	-0.87	0.90	-4.25	-49.79	114.7	33.9	1.2333	0.3645	138	
70	10.10	12.55	20.68	23.35	369.9	276.5	4.8039	3.5909	307	
MEAN	16.18	35.97	46.83	47.81	451.65	382.00	6.5572	5.6366	472.83	
STD DEVIATION	15.42	37.35	49.69	65.25	350.48	348.09	5.7755	5.6827	288.30	
N	6.00	6.00	6.00	6.00	6.00	6.00	6.0000	6.0000	6.00	
STD ERROR	6.29	15.25	20.29	26.64	143.08	142.11	2.3578	2.3200	117.70	
MECLOFENAMATE		CHANGE IN CONDUCTANCE (pS/pF)			CURRENT EVOKED AT -60 MV				LINOPIRIDINE SHIFT	
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
80	31.30	51.87	70.11	77.74	542	542	8.0896	8.0896	618	
80	10.97	14.05	10.66	-28.82	133.7	133.7	1.9100	1.9100	186	
80	26.11	35.59	52.35	49.47	669.6	614.9	7.7860	7.1500	662	
80	38.45	65.68	89.54	106.24	1063.2	796.8	17.1484	12.8516	913	
80	20.56	42.70	50.34	55.61	541.7	526.4	6.5265	6.3422	678	
80	11.22	12.92	32.88	10.11	329.3	329.3	3.0211	3.0211	495	
80	13.56	15.15	17.36	-4.01	404.1	404.1	4.8107	4.8107	416	
80	14.71	26.07	48.81	45.62	462.6	446.8	6.3370	6.1205	469	
MEAN	20.86	33.00	46.47	38.99	518.28	474.25	6.9537	6.2870	554.63	
STD DEVIATION	10.20	19.47	26.16	44.30	272.74	197.77	4.6595	3.3620	215.54	
N	8.00	8.00	8.00	8.00	8.00	8.00	8.0000	8.0000	8.00	
STD ERROR	3.61	6.88	9.25	15.66	96.43	69.92	1.6474	1.1887	76.20	
MECLOFENAMATE		CHANGE IN CONDUCTANCE (pS/pF)			CURRENT EVOKED AT -60 MV				LINOPIRIDINE SHIFT	
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
100	16.83	25.10	34.41	20.48	293.3	279.5	3.5337	3.3675	437	
100	5.94	8.21	12.75	10.25	83	83	1.1857	1.1857	118	
100	36.72	71.47	102.85	139.83	913.4	894.8	11.7103	11.4718	1051	
100	23.13	37.09	45.36	39.33	768.1	745.5	7.7586	7.5303	1088	
100	0.88	0.10	5.75	-18.81	59.5	29.6	0.7346	0.3654	427	
100	13.41	24.60	27.05	1.92	366.8	366.8	4.8263	4.8263	421	
100	9.44	23.73	18.73	-4.44	500.2	483.7	5.3213	5.1457	549	
100	23.21	38.32	44.43	37.00	599.1	596.6	6.5120	6.4848	814	
MEAN	16.20	28.58	36.42	28.19	447.93	434.94	5.1978	5.0472	613.13	
STD DEVIATION	11.42	21.66	30.38	49.30	306.90	305.52	3.5784	3.5701	340.05	
N	8.00	8.00	8.00	8.00	8.00	8.00	8.0000	8.0000	8.00	
STD ERROR	4.04	7.66	10.74	17.43	108.50	108.02	1.2652	1.2622	120.23	

DICLOFENAC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
50	5.08	3.08	6.84	17.51		-26.1	-26.1	-0.2193	-0.2193	268
50	-4.21	7.13	19.92	43.28		-51.3	-51.3	-0.5897	-0.5897	357
50	6.55	12.13	17.54	40.11		14.8	6.4	0.1987	0.0859	177
50	-5.39	3.00	13.74	11.77		-5	-5	-0.0549	-0.0549	198
50	-2.49	15.57	23.03	50.28		12	16	0.1446	0.1928	170
MEAN	0.09	8.18	16.22	32.59		-5.60	12.00	0.1041	-0.1171	275.33
STD DEVIATION	5.52	5.57	6.24	16.92		28.27	26.99	0.3182	0.3061	123.43
N	5.00	5.00	5.00	5.00		5.00	5.00	5.0000	5.0000	5.00
STD ERROR	2.47	2.49	2.79	7.57		11.54	12.07	0.1423	0.1369	50.39
DICLOFENAC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
60	4.30	15.64	37.84	60.44		-26.6	-26.6	-0.3500	-0.3500	79
60	-10.97	16.69	36.51	38.91		16.9	16.9	0.2600	0.2600	92
60	5.64	11.22	34.95	50.97		1.1	1.1	0.0130	0.0130	146
60	-12.03	10.24	23.71	70.55		11	11	0.1358	0.1358	277
60	-11.39	24.84	46.84	62.55		-19	-19	-0.2754	-0.2754	149
60	-4.60	6.88	19.84	30.71		33	28	0.3267	0.2772	368
60		0.00	0.00	0.00		17.7	13.4	0.2476	0.1874	103.8
MEAN	-4.85	12.21	28.52	44.87		-6.99	3.54	0.0511	0.0354	187.73
STD DEVIATION	8.07	7.89	15.49	24.16		38.89	19.80	0.2690	0.2542	107.96
N	6.00	7.00	7.00	7.00		7.00	7.00	7.0000	7.0000	7.00
STD ERROR	4.02	2.85	4.73	5.79		13.75	7.48	0.1017	0.0961	38.17

VOLTAGE CLAMP STUDIES: DICLOFENAC

DICLOFENAC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
50	5.08	3.08	6.84	17.51	-26.1	-26.1	-0.2193	-0.2193	268	
50	-4.21	7.13	19.92	43.28	-51.3	-51.3	-0.5897	-0.5897	357	
50	6.55	12.13	17.54	40.11	14.8	6.4	0.1987	0.0859	177	
50	-5.39	3.00	13.74	11.77	-5	-5	-0.0549	-0.0549	198	
50	-2.49	15.57	23.03	50.28	12	16	0.1446	0.1928	170	
MEAN	0.09	8.18	16.22	32.59	5.60	-12.00	-0.1041	-0.1171	275.33	
STD DEVIATION	5.52	5.57	6.24	16.92	28.27	26.99	0.3182	0.3061	123.43	
N	5.00	5.00	5.00	5.00	5.00	5.00	5.0000	5.0000	5.00	
STD ERROR	2.47	2.49	2.79	7.57	11.54	12.07	0.1423	0.1369	50.39	
DICLOFENAC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
60	4.30	15.64	37.84	60.44	-26.6	-26.6	-0.3500	-0.3500	79	
60	-10.97	16.69	36.51	38.91	16.9	16.9	0.2600	0.2600	92	
60	5.64	11.22	34.95	50.97	1.1	1.1	0.0130	0.0130	146	
60	-12.03	10.24	23.71	70.55	11	11	0.1358	0.1358	277	
60	-11.39	24.84	46.84	62.55	-19	-19	-0.2754	-0.2754	149	
60	-4.60	6.88	19.84	30.71	33	28	0.3267	0.2772	368	
60		0.00	0.00	0.00	17.7	13.4	0.2476	0.1874	103.8	
MEAN	-4.85	12.21	28.52	44.87	-6.99	3.54	0.0511	0.0354	187.73	
STD DEVIATION	8.07	7.89	15.49	24.16	38.89	19.80	0.2690	0.2542	107.96	
N	6.00	7.00	7.00	7.00	7.00	7.00	7.0000	7.0000	7.00	
STD ERROR	4.02	2.85	4.73	5.79	13.75	7.48	0.1017	0.0961	38.17	

DICLOFENAC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
80	0.03	3.47	22.17	40.32		26.5	7.5	0.4077	0.1154	178
80	0.23	34.11	61.24	34.00		15	15	0.2542	0.2542	111
80						19.4	19.4	0.2622	0.2622	206
80	-1.35	9.40	7.47	-0.06		0.24	0.24	0.0022	0.0022	167
80	-0.05	-9.18	-8.52	-8.69		-13.5	-13.5	-0.1688	-0.1688	144
80	-21.18	-28.54	5.65	26.87		78.7	66.5	0.9962	0.8418	140
MEAN	-4.46	1.85	17.60	18.49		20.42	15.86	0.2717	0.2178	189.50
STD DEVIATION	9.37	23.16	26.71	21.62		30.83	27.40	0.3848	0.3461	108.87
N	5.00	5.00	5.00	5.00		6.00	6.00	6.0000	6.0000	6.00
STD ERROR	4.19	10.36	11.94	9.67		10.90	11.19	0.1360	0.1413	38.49

DICLOFENAC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
100	1.76	24.23	42.94	79.30		14.3	14.3	0.1882	0.1882	186
100	7.17	4.16	13.68	14.40		69.15	69.15	0.9220	0.9220	57
100	8.47	14.16	23.60	23.67		45.6	45.6	0.5700	0.5700	139
100	10.94	15.94	21.04	23.04		60.6	60.6	0.5509	0.5509	235
100	0.44	-4.95	-10.24	-31.23		1	-2	0.0104	-0.0208	187
MEAN	5.75	10.71	18.20	21.84		27.45	37.53	0.3960	0.4420	230.57
STD DEVIATION	4.48	11.29	19.22	39.30		31.19	30.41	0.3438	0.3583	145.01
N	5.00	5.00	5.00	5.00		5.00	5.00	5.0000	5.0000	5.00
STD ERROR	2.01	5.05	8.60	17.57		11.79	13.60	0.1404	0.1603	54.81

DICLOFENAC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
120	-3.55	5.33	4.45	-10.20		47	47	0.4896	0.4896	414
120	19.10	25.52	35.89	23.54		118	118	1.6164	1.6164	264
120	2.43	14.75	23.17	19.30		52	52	0.7123	0.7123	273
120	5.05	6.41	2.54	-7.52		21	21	0.2692	0.2692	164
120	2.34	13.58	18.82	15.76		73.5	73.5	0.7350	0.7350	632
120	11.12	26.98	44.42	43.65		119	119	1.4875	1.4875	274
120	-7.20	-1.85	-0.22	-4.49		31	31	0.4079	0.4079	132
120	11.20	29.20	35.12	32.60		214	214	2.3778	2.3778	497
MEAN	5.06	14.99	20.52	14.08		84.44	84.44	1.0120	1.0120	331.25
STD DEVIATION	8.54	11.40	17.08	19.78		63.88	63.88	0.7380	0.7380	170.56
N	8.00	8.00	8.00	8.00		8.00	8.00	8.0000	8.0000	8.00
STD ERROR	3.02	4.03	6.04	6.99		22.58	22.58	0.2609	0.2609	60.30
DICLOFENAC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
140	28.70	27.95	55.12	73.29		86.3	86.3	1.1355	1.1355	233
140	-2.33	14.95	20.77	20.36		68.5	68.5	0.7874	0.7874	183
140	-0.11	31.09	55.86	57.56		146	137	1.9730	1.8514	444
140	1.61	8.98	27.24	35.27		12.3	12.3	0.1454	0.1454	229
140	-10.08	-8.34	-8.29	-31.24		3.1	1.7	0.0395	0.0217	422
MEAN	3.56	14.93	30.14	31.05		76.87	61.16	0.9822	0.7883	320.67
STD DEVIATION	14.75	15.87	26.73	40.31		61.95	55.59	0.8150	0.7503	117.51
N	5.00	5.00	5.00	5.00		5.00	5.00	5.0000	5.0000	5.00
STD ERROR	6.60	7.10	11.96	18.03		25.29	24.86	0.3327	0.3355	47.97

VOLTAGE CLAMP STUDIES: RETIGABINE

RETIGABINE		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
5	-2.54	-3.98	14.00	20.86		-0.6	-66	-0.0083	-0.9167	316
5	2.62	6.53	16.90	14.44		5.2	-20.8	0.0591	-0.2364	263
5	1.42	7.38	9.86	-2.31		22.6	-11.7	0.2260	-0.1170	264
5	2.02	15.22	3.63	-7.31		15	-16.5	0.1923	-0.2115	114
5	-1.07	15.07	39.18	54.79		7.3	-11	0.1044	-0.1574	165
5	-5.81	10.00	12.79	27.39		6	-10	0.0769	-0.1282	266
MEAN	-0.56	8.37	16.06	17.98		9.25	-22.67	0.1084	-0.2945	231.33
STD DEVIATION	3.24	7.09	12.19	22.43		8.23	21.62	0.0871	0.3083	75.64
N	6.00	6.00	6.00	6.00		6.00	6.00	6.0000	6.0000	6.00
STD ERROR	1.32	2.89	4.98	9.16		3.36	8.82	0.0356	0.1259	30.88
RETIGABINE		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
10	-6.32	-2.57	38.04	57.89		28.6	7.8	0.3763	0.1026	275
10	6.49	12.25	23.67	35.36		28.5	19.2	0.4014	0.2704	203
10	-1.21	2.74	17.95	34.62		1	-11.4	0.0114	-0.1295	163
10	-4.05	0.50	7.10	30.54		63	-11.6	0.9265	-0.1706	490
10	-13.83	4.84	20.56	26.85		17	-35	0.2024	-0.4167	536
10	-3.52	9.89	20.03	27.80		11	-15	0.1310	-0.1786	290
MEAN	-3.74	4.61	21.22	35.51		24.85	-7.67	0.3415	-0.0871	326.17
STD DEVIATION	6.63	5.63	10.01	11.50		21.47	18.93	0.3224	0.2407	152.70
N	6.00	6.00	6.00	6.00		6.00	6.00	6.0000	6.0000	6.00
STD ERROR	2.71	2.30	4.09	4.69		8.77	7.73	0.1316	0.0982	62.34

RETIGABINE		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
20	17.13	13.29	17.94	41.35		71	67	0.8875	0.8375	206
20	3.94	7.69	-1.39	-4.25		103	69	1.3733	0.9200	366
20	9.39	3.36	8.14	12.81		28	11	0.3256	0.1279	206
20	2.78	10.10	22.56	20.10		18	-41	0.1818	-0.4141	345
20	5.86	2.02	18.96	30.96		55	28	0.6548	0.3333	233
20	2.46	14.41	33.12	32.51		78	40	0.9750	0.5000	568
20	8.15	11.96	9.33	16.47		32	15	0.4267	0.2000	144
20	6.47	9.93	22.54	38.67		6	-35	0.0857	-0.5000	119
MEAN	7.02	9.09	16.40	23.58		48.88	19.25	0.61	0.25	273.38
STD DEVIATION	7.02	9.09	16.40	23.58		48.88	19.25	0.61	0.25	273.38
N	8.00	8.00	8.00	8.00		8.00	8.00	8.00	8.00	8.00
STD ERROR	2.48	3.22	5.80	8.34		17.28	6.81	0.22	0.09	96.65

RETIGABINE		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)		Voltage (mV)								
	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
80	lost					311	297	4.5072	4.3043	438
80	-1.48	9.84	12.81	9.70		17	5.5	0.1828	0.0591	316
80	4.87	-2.10	11.71	-4.17		104	76	1.1685	0.8539	351
80	-0.88	12.76	9.70	6.11		39	32	0.4382	0.3596	312
80	2.14	8.48	7.44	25.09		10	-6	0.1087	-0.0652	332
80	-0.25	-0.43	0.57	10.08		29	23	0.3671	0.2911	168
MEAN	0.88	5.71	8.44	9.36		85.00	71.25	1.1288	0.9671	319.50
STD DEVIATION	2.62	6.58	4.86	10.51		115.70	114.14	1.6975	1.6653	87.42
N	5.00	5.00	5.00	5.00		6.00	6.00	6.0000	6.0000	6.00
STD ERROR	1.17	2.94	2.17	4.70		47.24	46.60	0.6930	0.6799	35.69
RETIGABINE		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)		Voltage (mV)								
	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
100	3.11	7.79	8.40	10.52		81	81	1.0062	1.0062	
100	7.67	15.60	7.38	-4.46		66	53	1.1681	0.9381	148
100	6.47	0.23	-12.44	-29.81		109	107	1.4932	1.4658	254
100	-0.39	5.44	-9.41	-86.50		116	111	1.6111	1.5417	205
100	-2.41	9.61	5.62	-19.61		65.9	65.9	0.7322	0.7322	159
MEAN	2.89	7.73	-0.09	-25.97		87.58	83.58	1.2022	1.1368	191.50
STD DEVIATION	4.32	5.63	10.00	37.12		23.69	25.27	0.3579	0.3508	48.43
N	5.00	5.00	5.00	5.00		5.00	5.00	5.0000	5.0000	4.00
STD ERROR	1.93	2.52	4.47	16.60		10.60	11.30	0.1600	0.1569	24.22

RETIGABINE AND DICLOFENAC

RETIGABINE + DIC	CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT	
	DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
RET 2.5 DIC 70		-9.01	5.31	12.38	33.57	-23.2	-23.2	-0.2795	-0.2795	193
RET 2.5 DIC 70		5.18	16.06	17.43	25.14	50	45.8	0.6579	0.6026	168
RET 2.5 DIC 70		9.84	-0.64	27.17	28.03	44.6	44.6	0.5575	0.5575	169
RET 2.5 DIC 70						34	26	0.4533	0.3467	
RET 2.5 DIC 70						67	67	0.9178	0.9178	
MEAN	2.00	6.91	18.99	28.91	34.48	32.04	0.4614	0.4290	176.67	
STD DEVIATION	9.82	8.46	7.52	4.28	34.38	34.12	0.4487	0.4456	14.15	
N	3.00	3.00	3.00	3.00	5.00	5.00	5.0000	5.0000	3.00	
STD ERROR	5.67	4.89	4.34	2.47	15.38	15.26	0.2006	0.1993	8.17	
RET 5 DIC 20	21.04	-2.73	-11.99	1.36	283.00	277.00	4.1435	4.0556	603	
RET 5 DIC 20	-1.31	4.52	23.32	4.64	159.00	156.00	2.6500	2.6000	323	
RET 5 DIC 20	-0.20	9.99	-6.00	-39.11	44.60	36.00	0.4612	0.3723	314	
RET 5 DIC 20	16.30	8.21	75.83	80.23	0.60	-13.40	0.0094	-0.2094	173	
RET 5 DIC 20					173.00	173.00	2.2468	2.2468		
MEAN	8.96	5.00	20.29	11.78	132.04	125.72	1.90	1.81	353.25	
STD DEVIATION	11.39	5.63	40.11	49.78	111.93	115.59	1.69	1.73	180.11	
N	4.00	4.00	4.00	4.00	5.00	5.00	5.00	5.00	4.00	
STD ERROR	5.70	2.82	20.06	24.89	50.06	51.69	0.75	0.77	90.06	

RETIGABINE + DIC	CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT	
	DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
RET 5 DIC 50	-10.83	4.35	10.21	17.05	91	88	0.9891	0.9565	355	
RET 5 DIC 50	1.80	-0.05	26.91	21.94	161	160	1.5333	1.5238	309	
RET 5 DIC 50	-8.43	8.88	12.76	16.09	31	21.6	0.3333	0.2323	316	
RET 5 DIC 50	5.83	12.98	33.27	37.23	16	15	0.1633	0.1531	279	
RET 5 DIC 50	-4.62	18.00	16.36	10.32	35	27	0.4321	0.3333	*	
RET 5 DIC 50	2.69	0.36	12.85	0.56	75	75	0.7895	0.7895	277	
RET 5 DIC 50	2.94	4.60	11.69	15.50	20	10	0.1869	0.0935	234	
RET 5 DIC 50	5.12	15.79	19.17	23.63	79	79	1.0822	1.0822	195	
MEAN	-0.69	8.11	17.90	17.79	63.50	59.45	0.6887	0.6455	280.71	
STD DEVIATION	6.38	6.91	8.20	10.63	48.77	51.35	0.4912	0.5203	53.40	
N	8.00	8.00	8.00	8.00	8.00	8.00	8.0000	8.0000	7.00	
STD ERROR	2.26	2.44	2.90	3.76	17.24	18.15	0.1737	0.1840	20.18	

RETIGABINE AND MECLOFENAMATE

[illegible]

RETIGABINE + MEC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
RET 2.5 MEC 50	1.87	0.25	-5.21	-37.01	168	3	2.5846	0.0462	148	
RET 2.5 MEC 50	4.59	18.17	25.37	39.02	956	761	13.2778	10.5694	977	
RET 2.5 MEC 50	1.31	1.72	-2.44	-7.45	197	152	1.9505	1.5050	233	
RET 2.5 MEC 50	2.87	-2.78	-18.77	-39.00	-23	-3	-0.2805	-0.0366	233	
RET 2.5 MEC 50	3.26	-0.10	-23.52	-48.63	235	173	2.7011	1.9885	552	
RET 2.5 MEC 50	1.52	5.95	-0.80	-5.33	159	159	1.7473	1.7473	269	
MEAN	2.57	3.87	-4.23	-16.40	282.00	207.50	3.6635	2.6366	402.00	
STD DEVIATION	1.25	7.57	17.18	32.40	342.01	282.53	4.8309	3.9819	313.83	
N	6.00	6.00	6.00	6.00	6.00	6.00	6.0000	6.0000	6.00	
STD ERROR	0.51	3.09	7.01	13.23	139.63	115.34	1.9722	1.6256	128.12	
RETIGABINE + MEC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
RET 2.5 MEC 70	4.79	3.24	-15.89	-34.26	223.4	181.3	3.3848	2.7470	331	
RET 2.5 MEC 70	-6.54	-21.36	-37.72	-65.62	40.3	40.3	0.5676	0.5676	169	
RET 2.5 MEC 70	28.14	57.00	89.34	113.89	561	474	10.3889	8.7778	555	
RET 5 MEC 70	52.98	115.84	154.95	186.16	2305	2300	26.4943	26.4368	2271	
RET 5 MEC 70	6.28	10.68	8.07	-9.97	95	91	1.6379	1.5690	150	
RET 5 MEC 70	13.09	12.04	10.85	5.67	198	136	2.8696	1.9710	228	
RET 5 MEC 70	0.43	9.77	5.93	-10.40	73	27	1.2167	0.4500	323	
RET 5 MEC 70	14.85	14.23	19.21	-1.56	557	492	6.8765	6.0741	710	
MEAN	17.53	32.51	39.80	33.98	645.60	609.20	7.8190	7.3002	736.40	
STD DEVIATION	20.63	46.61	64.56	85.33	947.74	962.28	10.6765	10.9074	884.44	
N	5.00	5.00	5.00	5.00	5.00	5.00	5.0000	5.0000	5.00	
STD ERROR	9.23	20.84	28.87	38.16	423.84	430.35	4.7747	4.8779	395.53	
RETIGABINE + MEC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)	
RET 2.5 MEC 30	4.56	1.49	-8.29	-25.35	68	41	0.8500	0.5125	130	
RET 2.5 MEC 30	11.88	30.18	53.29	50.79	332	270	5.0303	4.0909	534	
RET 2.5 MEC 30	1.05	-5.62	-16.87	-43.71	107	46	1.2892	0.5542	223	
RET 2.5 MEC 30	7.26	-2.62	-15.05	-49.60	22	9	0.2500	0.1023	126	
RET 2.5 MEC 30	6.49	8.03	11.87	-8.36	137	137	1.5055	1.5055	216	
RET 2.5 MEC 30	6.32	12.78	15.67	5.13	12	-48	0.1600	-0.6400	107	
RET 2.5 MEC 30	7.29	12.24	9.94	13.41	9	3	0.1667	0.0556	160	
RET 2.5 MEC 30	12.74	16.71	24.89	29.31	197	122	3.3966	2.1034	212	
MEAN	7.20	9.15	9.43	-3.55	110.50	72.50	1.5810	1.0355	213.50	
STD DEVIATION	3.75	11.58	23.34	35.11	111.42	100.49	1.7570	1.5043	137.14	
N	8.00	8.00	8.00	8.00	8.00	8.00	8.0000	8.0000	8.00	
STD ERROR	1.33	4.09	8.25	12.41	39.39	35.53	0.6212	0.5319	48.49	

MECLOFENAMATE AND DICLOFENAC

MEC + DIC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
MEC 30 DIC 50	13.44	10.37	5.93	-6.14		69.3	65	0.7700	0.7222	289
MEC 30 DIC 50	1.04	3.93	0.34	-38.13		129	102	1.9846	1.5692	168
MEC 30 DIC 50	4.80	-2.61	-3.46	-26.48		20	0	0.2381	0.0000	125
MEC 30 DIC 50	7.58	3.92	7.26	-1.90		106	-15	1.3250	-0.1875	230
MEAN	6.72	3.90	2.52	-18.16		81.08	38.00	1.0794	0.5260	203.00
STD DEVIATION	5.22	5.30	4.99	17.10		47.56	55.01	0.7491	0.7985	71.73
N	4.00	4.00	4.00	4.00		4.00	4.00	4.0000	4.0000	4.00
STD ERROR	2.61	2.65	2.49	8.55		23.78	27.50	0.3745	0.3992	35.86
MEC 40 DIC40	19.82	14.01	4.74	-18.78		288	268	4.1739	3.8841	296
MEC 40 DIC40	2.67	5.74	1.07	-29.88		183	124	2.4400	1.6533	297
MEC 40 DIC40	15.14	20.78	34.54	36.35		454	397	6.0533	5.2933	742
MEC 40 DIC40	49.61	89.61	131.41	182.35		1160	1070	20.0000	18.4483	1078
MEC 40 DIC40	8.52	12.21	2.65	-9.63		77	35	0.9506	0.4321	182
MEC 40 DIC40	13.75	40.48	37.40	27.41		449	436	5.3452	5.1905	620
MEC 40 DIC40	16.51	19.24	28.80	1.18		450	336	6.6176	4.9412	401
MEC 40 DIC40	43.73	80.88	132.04	169.15		593	593	11.4038	11.4038	745
MEC 40 DIC40	3.69	4.49	4.74	-17.49		156	8	1.7143	0.0800	264
MEC 40 DIC40	15.14	18.06	13.54	17.36		405	373	5.1923	4.7821	765
MEAN	18.86	30.55	39.09	35.80		423.33	363.00	6.52210	5.70296	513.89
STD DEVIATION	15.72	30.56	50.64	76.78		324.28	327.82	5.95224	5.86367	298.91
N	10.00	10.00	10.00	10.00		9.00	9.00	9.00000	9.00000	9.00
STD ERROR	3.62	5.53	8.10	12.83		23.51	20.05	2.67330	2.35513	29.72

MEC + DIC	CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRIDINE SHIFT	
	DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
MEC 30 DIC 30		0.54	-9.99	-32.96	-70.14	-29	-29	-0.40278	-0.40278	111
MEC 30 DIC 30		4.64	1.72	8.63	-16.61	10	-34	0.11236	-0.38202	110
MEC 30 DIC 30		5.44	0.87	-12.84	-30.19	108	46	1.35000	0.57500	192
MEAN		3.54	-2.47	-12.39	-38.98	29.67	-5.67	0.35	-0.07	137.67
STD DEVIATION		2.63	6.53	20.80	27.83	70.59	44.81	0.90	0.56	47.06
N		3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
STD ERROR		1.52	3.77	12.01	16.07	40.75	25.87	0.52	0.32	27.17
MEC40 DIC 30		16.23	18.90	9.50	-10.05	278	259	4.2121	3.9242	328
MEC40 DIC 30		25.51	33.04	42.17	24.44	369	369	5.5909	5.5909	627
MEC40 DIC 30		2.53	6.50	9.61	28.19	47	28	0.7231	0.4308	252
MEC40 DIC 30		6.75	5.30	-7.35	-16.84	83	39	0.9651	0.4535	114
MEC40 DIC 30		4.01	5.80	1.23	-3.49	371	270	4.0769	2.9670	618
MEAN		11.01	13.91	11.03	4.45	229.60	193.00	3.1136	2.6733	387.80
STD DEVIATION		9.70	12.10	18.76	20.55	155.41	151.82	2.1565	2.2428	227.59
N		5.00	5.00	5.00	5.00	5.00	5.00	5.0000	5.0000	5.00
STD ERROR		4.34	5.41	8.39	9.19	69.50	67.90	0.9644	1.0030	101.78

MEC + DIC	CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRIDINE SHIFT	
	DOSE (uM)	-55	-50	-45	-40	PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
MEC 20 DIC 40		10.06	6.17	23.85	37.77	115	94	1.9492	1.5932	141
MEC 20 DIC 40		1.95	-6.38	-29.24	-75.60	171	127	2.1111	1.5679	246
MEC 20 DIC 40		-0.90	-5.58	-5.45	4.43	1	-13	0.0090	-0.1171	137
MEAN		3.70	-1.93	-3.61	-11.14	95.67	69.33	1.36	1.01	174.67
STD DEVIATION		5.69	7.02	26.59	58.27	86.63	73.19	1.17	0.98	61.81
N		3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
STD ERROR		3.28	4.06	15.35	33.64	50.02	42.25	0.68	0.57	35.69
MEC 5 DIC 40		-1.85	-2.88	-0.57	-29.53	10	10	0.1220	0.1220	109
MEC 5 DIC 40		-0.32	0.23	-7.56	-45.59	-12	-12	-0.1690	-0.1690	113
MEC 5 DIC 40		-8.77	-19.72	-69.24	-135.52	24	24	0.2927	0.2927	80
MEC 5 DIC 40		2.93	15.94	22.11	-3.52	64	60	0.8421	0.7895	98
MEC 5 DIC 40		4.68	4.51	3.43	6.05	139	139	2.0145	2.0145	287
MEC 5 DIC 40		2.92	6.85	12.09	23.53	112	91	1.3333	1.0833	90
MEC 5 DIC 40		1.59	4.12	21.81	14.45	32	30	0.4384	0.4110	75
MEC 5 DIC 40						43	43	0.5059	0.5059	124
MEAN		0.17	1.29	-2.56	-24.31	51.50	48.13	0.6725	0.6312	122.00
STD DEVIATION		4.50	10.98	31.43	54.76	51.32	48.17	0.7076	0.6784	68.71
N		7.00	7.00	7.00	7.00	8.00	8.00	8.0000	8.0000	8.00
STD ERROR		1.70	4.15	11.88	20.70	18.14	17.03	0.2502	0.2398	24.29

MEC + DIC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
MEC 2.5 DIC 20	1.53	9.79	-0.56	-4.18		97	89	1.2436	1.1410	100
MEC 2.5 DIC 20	2.83	4.36	18.80	40.54		37	37	0.5692	0.5692	136
MEC 2.5 DIC 20	-2.17	1.39	-2.60	-15.95		67	67	0.7283	0.7283	278
MEC 2.5 DIC 20	0.65	-4.20	-14.73	-25.54		3	1	0.0375	0.0125	91
MEC 2.5 DIC 20	2.03	7.88	8.90	-6.97		24	24	0.2474	0.2474	107
MEC 2.5 DIC 20	0.75	8.15	16.22	30.77		-5.8	-5.8	-0.0753	-0.0753	83
MEC 2.5 DIC 20	0.38	1.93	-0.39	-12.18		19	5	0.1881	0.0495	124
MEAN	0.86	4.18	3.66	0.93		34.46	31.03	0.4198	0.3818	131.29
STD DEVIATION	1.59	4.90	11.73	24.85		36.37	35.77	0.4602	0.4482	67.24
N	7.00	7.00	7.00	7.00		7.00	7.00	7.0000	7.0000	7.00
STD ERROR	0.60	1.85	4.43	9.39		13.75	13.52	0.1739	0.1694	25.41
MEC + DIC		CHANGE IN CONDUCTANCE (pS/pF)				CURRENT EVOKED AT -60 MV				LINOPIRDINE SHIFT
		Voltage (mV)								
DOSE (uM)	-55	-50	-45	-40		PEAK (pA)	at 2 min post	PEAK (pa/pf)	at 2 min post	NEGATIVE SHIFT (pA)
MEC 2.5 DIC 5	0.43	-1.66	-8.98	-36.21		-13	-13	-0.1585	-0.1585	146
MEC 2.5 DIC 5	-0.73	-10.19	-27.58	-69.33		-12	-12	-0.1429	-0.1429	225
MEC 2.5 DIC 5	6.70	8.21	-9.73	-39.66		-23	-23	-0.2527	-0.2527	65
MEC 2.5 DIC 5	-3.52	-0.22	-4.65	-32.84		4.3	-15	0.0566	-0.1974	147
MEC 2.5 DIC 5	3.61	0.74	-6.57	-26.63		27	25	0.2673	0.2475	53
MEC 2.5 DIC 5	4.66	3.75	-5.83	-45.62		12	9	0.1622	0.1216	46
MEC 2.5 DIC 5	1.94	6.03	10.69	22.88		2	-4	0.0308	-0.0615	44
MEAN	1.87	0.95	-7.52	-32.49		-0.39	-4.71	-0.0053	-0.0634	103.71
STD DEVIATION	3.47	6.04	11.21	27.97		17.01	16.50	0.1876	0.1826	69.93
N	7.00	7.00	7.00	7.00		7.00	7.00	7.0000	7.0000	7.00
STD ERROR	1.31	2.28	4.24	10.57		6.43	6.24	0.0709	0.0690	26.43

CURRENT CLAMP STUDIES: OXOTREMORINE CONTROLS

OXOTREMORINE M		DICLOFENAC CONTROLS	
DOSING (uM)	VEHICLE: DMSO RMP SHIFT (mV)	OXO RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
10	-1.3	16.6	195
10	LOST	17.7	101
10	1.8	24.5	54
10	2.1	31.2	79
10	3	37.3	72
10	4.5	16.6	0
10	1.1	23.2	162
10	2.3	23	66
MEAN	1.929	23.76	91.13
STD DEVIATION	1.782	7.36	61.80
N	7.000	8.00	8.00
STD ERROR	0.674	2.60	21.85
OXOTREMORINE M		MECLOFENAMATE CONTROLS	
DOSING (uM)	VEHICLE: WATER RMP SHIFT (mV)	OXO RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
10	-0.35	36.5	54
10	1.68	32.5	39
10	-2.6	34.9	147
10	-0.78	27.9	124
10	-0.14	22.7	18
10	0.35	38.1	136
10	-0.2	22.6	194
10	0.25	15.2	0
10	1.83	20.2	51
MEAN	0.00	27.84	84.78
STD DEVIATION	1.32	8.09	66.89
N	9.00	9.00	9.00
STD ERROR	0.44	2.70	22.30

CURRENT CLAMP STUDIES: RETIGABINE AND DICLOFENAC

RETIGABINE +DICLOFENAC			
DOSING (uM)	RETIGABINE +DICLOFENAC RMP SHIFT (mV)	RETIGABINE +DICLOFENAC + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 5 DIC 1.25	-3.26	29.7	32
RET 5 DIC 1.25	-2.27	27.6	40
RET 5 DIC 1.25	-5.21	29.2	54
RET 7.5 DIC 1.25	-2.11	22.2	0
RET 7.5 DIC 1.25	-1.39	25.2	1
RET 7.5 DIC 1.25	-1.16	33.1	0
RET 7.5 DIC 1.25	-5.4	3.1	0
RET 7.5 DIC 1.25	-6.17	15.9	0
RET 7.5 DIC 1.25	-1.78	30.2	36
RET 7.5 DIC 1.25	-3.43	20.7	0
MEAN	-3.06	21.49	5.29
STD DEVIATION	2.01	9.96	13.55
N	7.00	7.00	7.00
STD ERROR	0.76	3.76	5.12
RETIGABINE +DICLOFENAC			
DOSING (uM)	RETIGABINE +DICLOFENAC RMP SHIFT (mV)	RETIGABINE +DICLOFENAC + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 5 DIC 2.5	-0.69	23	0
RET 5 DIC 2.5	-4.14	8.83	0
RET 5 DIC 2.5	-3.6	23.3	14
RET 5 DIC 2.5	-2.99	29.5	20
RET 5 DIC 2.5	-1.57	21.9	17
MEAN	-2.59	21.31	10.20
STD DEVIATION	1.45	7.58	9.37
N	5.00	5.00	5.00
STD ERROR	0.65	3.39	4.19

RETIGABINE +DICLOFENAC			
DOSING (uM)	RETIGABINE +DICLOFENAC RMP SHIFT (mV)	RETIGABINE +DICLOFENAC + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 5 DIC 20	-3.55	23.7	28
RET 5 DIC 20	-3.29	8.4	0
RET 5 DIC 20	-2.26	42.1	2
RET 5 DIC 20	-1.8	24.9	128
RET 5 DIC 20	-3.13	33.95	0
RET 5 DIC 20	-2.89	24.7	5
MEAN	-2.83	26.29	27.17
STD DEVIATION	0.65	11.31	50.53
N	6.00	6.00	6.00
STD ERROR	0.27	4.62	20.63
RETIGABINE +DICLOFENAC			
DOSING (uM)	RETIGABINE +DICLOFENAC RMP SHIFT (mV)	RETIGABINE +DICLOFENAC + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 5 DIC 50	3.16	42.4	0
RET 5 DIC 50	-4.07	39.2	0
RET 5 DIC 50	-6.87	35.9	64
RET 5 DIC 50	-8.597	36.1	1
RET 5 DIC 50	-6.79	40.8	0
RET 5 DIC 50	-0.01	25.1	9
RET 5 DIC 50	-7.26	23.6	0
RET 5 DIC 50	-4.58	24.8	2
MEAN	-4.38	29.77	9.50
STD DEVIATION	4.04	13.32	22.23
N	8.00	9.00	8.00
STD ERROR	1.43	4.44	7.86

RETIGABINE +DICLOFENAC			
DOSING (uM)	RETIGABINE +DICLOFENAC RMP SHIFT (mV)	RETIGABINE +DICLOFENAC + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 10 DIC 2.5	-4.077	27.5	32
RET 10 DIC 2.5	-2.83	13.1	0
RET 10 DIC 2.5	0.89	40.3	1
RET 10 DIC 2.5	-4.88	25.3	0
RET 10 DIC 2.5	0.07	28.7	17
RET 10 DIC 2.5	-3.72	28.5	10
RET 10 DIC 2.5	-4.69	23.5	14
MEAN	-2.54	26.70	10.57
STD DEVIATION	2.18	8.06	11.75
N	7.00	7.00	7.00
STD ERROR	0.82	3.05	4.44
RET 10 DIC 5	-2.03	24.3	12
RET 10 DIC 5	-3.56	16.47	0
RET 10 DIC 5	-3.4	25.9	59
MEAN	-2.34	22.22	23.67
STD DEVIATION	0.84	5.05	31.18
N	3.00	3.00	3.00
STD ERROR	0.49	2.91	18.00

RETIGABINE +DICLOFENAC			
DOSING (uM)	RETIGABINE +DICLOFENAC RMP SHIFT (mV)	RETIGABINE +DICLOFENAC + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 2.5 DIC 70	-8.57	27.2	0
RET 2.5 DIC 70	-4.77	40.6	3
RET 2.5 DIC 70	-10.7	34.1	0
RET 2.5 DIC 70	-8.5	30.1	8
RET 2.5 DIC 70	-11.7	36.4	6
RET 2.5 DIC 70	-7.7	36.6	0
RET 2.5 DIC 70	-8.5	23.3	0
RET 2.5 DIC 70	-7.1	26.5	0
MEAN	-8.44	31.85	2.13
STD DEVIATION	2.12	5.99	3.23
N	8.00	8.00	8.00
STD ERROR	0.75	2.12	1.14

CURRENT CLAMP STUDIES: RETIGABINE CONTROLS

RETIGABINE CONTROLS			
DOSING (uM)	RETIGABINE RMP SHIFT (mV)	RETIGABINE + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 10	4.1	19.7	20
RET 10	-3.59	25.3	0
RET 10	-1.4	36.8	132
RET 10	-2.4	26.5	6
RET 10	-1.63	23	32
RET 10	0.06	17.2	0
RET 10	-4.99	20.9	0
RET 10	-2.53	22.8	24
MEAN	-1.32	24.03	26.75
STD DEVIATION	2.64	5.96	44.30
N	8.00	8.00	8.00
STD ERROR	0.88	2.11	15.66
RETIGABINE CONTROLS			
DOSING (uM)	RETIGABINE RMP SHIFT (mV)	RETIGABINE + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 20	-1.69	32.3	177
RET 20	4.53	45.7	0
RET 20	-2.1	42.1	2
RET 20	-5.87	12.4	0
RET 20	1.17	20.3	0
MEAN	-0.79	30.56	35.80
STD DEVIATION	3.89	14.15	78.94
N	5.00	5.00	5.00
STD ERROR	1.74	6.33	35.30

RETIGABINE CONTROLS FOR DICLOFENAC AND MECLOFENAMATE

DOSING (uM)	RETIGABINE RMP SHIFT (mV)	RETIGABINE + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 5 for DIC	-0.46	26.7	148
RET 5 for DIC	-2.25	20.14	0
RET 5 for DIC	0.36	39.2	0
RET 5 for DIC	-2.17	23.3	81
RET 5 for MEC	-0.27	33.8	108
RET 5 for MEC	-4.21	32.8	32
RET 5 for MEC	-1.77	32.9	24
RET 5 for MEC	-2.63	28.6	25
RET 5 for MEC	-2.47	28.8	117
RET 5 for MEC	-2.2	38.9	10
RET 5 for MEC	-3.18	23.7	0
RET 5 for MEC	-0.98	19.8	0
MEAN	-1.85	29.05	45.42
STD DEVIATION	1.31	6.63	53.42
N	12.00	12.00	12.00
STD ERROR	0.38	1.91	15.42

RETIGABINE CONTROLS

DOSING (uM)	RETIGABINE RMP SHIFT (mV)	RETIGABINE + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 7.5	-2.68	9.5	0
RET 7.5	-4.41	20.4	0
RET 7.5	-1.14	22.6	9
RET 7.5	-4.06	25	41
RET 7.5	-1.77	18.5	0
RET 7.5	-3.43	18.6	0
MEAN	-2.92	19.10	8.33
STD DEVIATION	1.29	5.32	16.40
N	6.00	6.00	6.00
STD ERROR	0.53	2.17	6.70

RETIGABINE CONTROLS			
DOSING (uM)	RETIGABINE RMP SHIFT (mV)	RETIGABINE + OXOTREMORINE RMP SHIFT (mV) ACTION POTENTIALS (30 sec)	
RET 2.5	1.7	34.3	0
RET 2.5	3.4	36.3	33
RET 2.5	3.1	21.2	172
RET 2.5	0.81	23.6	65
RET 2.5	-2.97	21.2	101
MEAN	1.21	27.32	22.27
STD DEVIATION	2.56	7.38	66.28
N	5.00	5.00	5.00
STD ERROR	1.15	3.30	29.64

CURRENT CLAMP STUDIES: DICLOFENAC CONTROLS

DICLOFENAC CONTROLS			
DOSING (uM)	DICLOFENAC RMP SHIFT (mV)	DICLOFENAC+OXOTREMORINE	
		RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
DIC 70	-9.03	34	0
DIC 70	2.87	18.6	20
DIC 70	-7.1	12.2	0
DIC 70	-5.87	30.27	15
DIC 70	-9.87	21.9	0
DIC 70	-8.7	36.4	41
DIC 70	-13.1	37.4	9
MEAN	-7.26	24.66	12.14
STD DEVIATION	5.01	11.64	15.03
N	7.00	7.00	7.00
STD ERROR	1.89	4.11	5.68

DICLOFENAC CONTROLS			
DOSING (uM)	DICLOFENAC RMP SHIFT (mV)	DICLOFENAC+OXOTREMORINE	
		RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
DIC 50	-6.74	32.3	16
DIC 50	-6.94	23	8
DIC 50	-7.54	30.8	0
DIC 50	-4.88	25.5	8
DIC 50	-0.83	24.4	9
DIC 50	-8.69	38.4	8
DIC 50			
MEAN	-5.77	25.49	6.13
STD DEVIATION	2.59	10.88	5.72
N	6.00	6.00	6.00
STD ERROR	0.98	4.11	2.02

DICLOFENAC CONTROLS			
DOSING (uM)	DICLOFENAC RMP SHIFT (mV)	DICLOFENAC+OXOTREMORINE	
		RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
DIC 10	-3.7	33.9	18
DIC 10	-2.3	24.7	23
DIC 5	0.85	28.7	12
DIC 5	1.2	18.9	14
DIC 5	-5.23	32.9	10
DIC 5	0.24	13.1	0
DIC 5	-0.36	24	0
	0.39	27	51
	-1.06	30.5	18
MEAN	-0.57	25.01	15.00
STD DEVIATION	2.19	6.95	17.27
N	7.00	7.00	7.00
STD ERROR	0.83	2.63	6.53
DICLOFENAC CONTROLS			
DOSING (uM)	DICLOFENAC RMP SHIFT (mV)	DICLOFENAC+OXOTREMORINE	
		RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
DIC 2.5	2.73	20.3	39
DIC 2.5	-1.58	27.6	26
DIC 2.5	3.16	19.3	64
DIC 2.5	0.15	28.2	53
DIC 2.5	-1.64	35.9	47
DIC 2.5	2.18	24.5	46
DIC 2.5	-1.12	26.7	44
MEAN	0.56	26.07	45.57
STD DEVIATION	2.10	5.56	11.73
N	7.00	7.00	7.00
STD ERROR	0.79	2.10	4.43

CURRENT CLAMP STUDIES: MECLOFENAMATE CONTROLS

MECLOFENAMATE CONTROLS			
DOSING (uM)	MECLOFENAMATE RMP SHIFT (mV)	MECLOFENAMATE+OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
MEC 30	-8.4	10.75	0
MEC 30	-6.06	5.1	0
MEC 30	3.17	11.6	0
MEC 30	10.3	14.1	22
MEC 30	9.83	20.1	49
MEAN	12.78	12.33	14.20
STD DEVIATION	16.99	5.45	21.66
N	10.00	5.00	5.00
STD ERROR	5.37	2.44	9.69
MEC 15	-6.43	9.1	0
MEC 15	-5.37	31.9	1
MEC 15	0.19	23.7	9
MEC 15	-5.77	7.8	0
MEAN	-4.35	18.13	2.50
STD DEVIATION	3.05	11.67	4.36
N	4.00	4.00	4.00
STD ERROR	1.53	5.84	2.18

MECLOFENAMATE CONTROLS			
DOSING	MECLOFENAMATE	MECLOFENAMATE+OXOTREMORINE	
(uM)	RMP SHIFT (mV)	RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
MEC 10	4.9	19.5	0
MEC 10	6.1	12.3	32
MEC 10	-5.4	26.5	4
MEC 10	-3.2	24.2	3
MEAN	0.60	20.63	9.75
STD DEVIATION	5.75	6.27	14.93
N	4.00	4.00	4.00
STD ERROR	2.87	3.13	7.47
MEC 5	-1.8	27.2	9
MEC 5	-0.43	29.1	75
MEC 5	-3.4	17.7	0
MEC 5	-8.07	28.6	2
MEC 5	3.5	15.1	0
MEC 5	-8.17	24	0
MEC 5	-8.13	9.5	0
MEAN	-3.79	21.60	12.29
STD DEVIATION	4.56	7.60	27.85
N	7.00	7.00	7.00
STD ERROR	1.72	2.87	10.53

MECLOFENAMATE CONTROLS			
DOSING (uM)	MECLOFENAMATE RMP SHIFT (mV)	MECLOFENAMATE+OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
MEC 1	0.72	21.2	0
MEC 1	-1.08	9.5	0
MEC 1	-4.2	29.4	0
MEC 1	-7.1	32	0
MEC 1	1.44	9.6	0
MEAN	-1.66	20.34	0
STD DEVIATION	3.57	10.63	0
N	5.00	5.00	5
STD ERROR	1.60	4.75	0
MEC 0.5	4.07	29.8	26
MEC 0.5	-1.56	29.4	0
MEC 0.5	2.13	20.4	28
MEC 0.5	-2.37	32.3	71
MEC 0.5	-4.73	30.5	122
MEC 0.5	1.22	30.3	80
MEC 0.5	3.17	31.9	0
MEC 0.5	-0.54	20.2	0
MEC 0.5	6.13	23.6	66
MEC 0.5	5.77	22.1	92
MEC 0.5	4.083	14.5	0
MEAN	1.58	23.75	44.09
STD DEVIATION	3.51	9.40	43.84
N	11.00	12.00	11.00
STD ERROR	1.06	2.71	13.22

CURRENT CLAMP STUDIES: RETIGABINE AND MECLOFENAMATE

RETIGABINE + MECLOFENAMATE			
DOSING	RETIGABINE + MECLOFENAMATE	RETIGABINE + MECLOFENAMATE + OXOTREMORINE	
(uM)	RMP SHIFT (mV)	RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
RET 5 MEC 0.5	-2.23	30.8	15
RET 5 MEC 0.5	-3.43	32.1	1
RET 5 MEC 0.5	-5.2	11.3	0
RET 5 MEC 0.5	-4.03	9.5	0
RET 5 MEC 0.5	1.33	17.5	0
MEAN	-2.11	16.98	3.20
STD DEVIATION	3.30	9.20	6.61
N	5.00	5.00	5.00
STD ERROR	1.35	3.76	2.96
RET 5 MEC 1	-5	29.4	21
RET 5 MEC 1	-0.9	25	125
RET 5 MEC 1	-5.23	27.8	117
RET 5 MEC 1	-1.39	29.7	34
MEAN	-3.13	27.98	74.25
STD DEVIATION	2.30	2.15	54.34
N	4.00	4.00	4.00
STD ERROR	1.15	1.08	27.17

CURRENT CLAMP STUDIES: MECLOFENAMATE AND DICLOFENAC

MECLOFENAMATE + DICLOFENAC			
DOSING (uM)	MECLOFENAMATE + DICLOFENAC RMP SHIFT (mV)	MECLOFENAMATE + DICLOFENAC + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
MEC 0.5 DIC 4	-1.57	9.9	0
MEC 0.5 DIC 4	4.53	13.7	0
MEC 0.5 DIC 4	-5.2	31.9	19
MEC 0.5 DIC 4	-5.7	35.2	75
MEC 0.5 DIC 4	-5.97	4.1	0
MEC 0.5 DIC 4	-2.17	26.4	0
MEC 0.5 DIC 4	0.67	25.8	102
MEAN	-2.20	21.00	28.00
STD DEVIATION	3.86	11.80	42.63
N	7.00	7.00	7.00
STD ERROR	1.46	4.46	16.11

MECLOFENAMATE + DICLOFENAC			
DOSING (uM)	MECLOFENAMATE + DICLOFENAC RMP SHIFT (mV)	MECLOFENAMATE + DICLOFENAC + OXOTREMORINE RMP SHIFT (mV)	ACTION POTENTIALS (30 sec)
MEC 0.5 DIC 2.5	-3.3	32.2	50
MEC 0.5 DIC 2.5	-3	8.6	0
MEC 0.5 DIC 2.5	-3.1	11.6	0
MEC 0.5 DIC 2.5	-2.59	7	0
MEC 0.5 DIC 2.5	1.7	26.5	8
MEC 0.5 DIC 2.5	-4.03	1.8	0
MEC 0.5 DIC 2.5	-1.77	28.7	11
MEC 0.5 DIC 2.5	-1.48	16.7	0
MEC 0.5 DIC 2.5	-0.65	16.7	0
MEAN	-2.02	16.64	7.67
STD DEVIATION	1.74	10.54	16.42
N	9.00	9.00	9.00
STD ERROR	0.58	3.51	5.47

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 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.

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 μ M RET to 4 μ M 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 μ M, 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 μ M or 5 μ M RET; 2.5 μ M 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 $\pm$ 0.67 mV vs -2.54 $\pm$ 0.82, $p < .001$, n=7 and 7; -2.59 $\pm$ 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 $\pm$ 19.3 APs vs 10.5 $\pm$ 4.3 APs; $p < .009$, n=7 and 9; and 10.2 $\pm$ 4.27 APs; $p < .02$, n=5 and 9; vehicle, 4:1 and 2:1 RD molar ratios, respectively). Combination dosing with 5 μ M RET and 2.5 μ M DIC were significantly superior than either agent alone ($p < .002$ and $p < .02$ vs 5 μ M RET and 2.5 μ M 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.

Manuscripts

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 (ED₅₀: 62.7 ± 13.7 μM). Diclofenac produced weak voltage dependent effects but a similar maximum sustained current of 1.01 ± 0.26 pA/pF (ED₅₀: 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 μM) 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 (Nav, Kv7; 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-Elseyed et al., 2015; Du et al., 2017; Wu et al., 2017; Yu et al., 2018) and pain related behaviors in rodents (Abd-Elseyed 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 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). The pipette solution contained (in mM): 120 KCl, 5 Na₂-ATP, 0.4 Na₂-GTP, 5 EGTA, 2.25 CaCl₂, 5 MgCl₂, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm. 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 μm).

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 μ M; 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 (G_1 and G_2) 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:

$$G_{diff} = G_2 - G_1$$

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 μ M DIC was applied by close superfusion. At 60 μ M 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 μ M), standing currents were not detected (figure 2). Above this dose, DIC proved to be a reliable activator of K_v7 . At 80 μ M, significant K^+ currents developed rapidly and grew steadily through 120 μ M (figure 2). At 120 μ M 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 μ M. All standing K_v7 current was fully reversed by application of linopirdine (20 μ M).

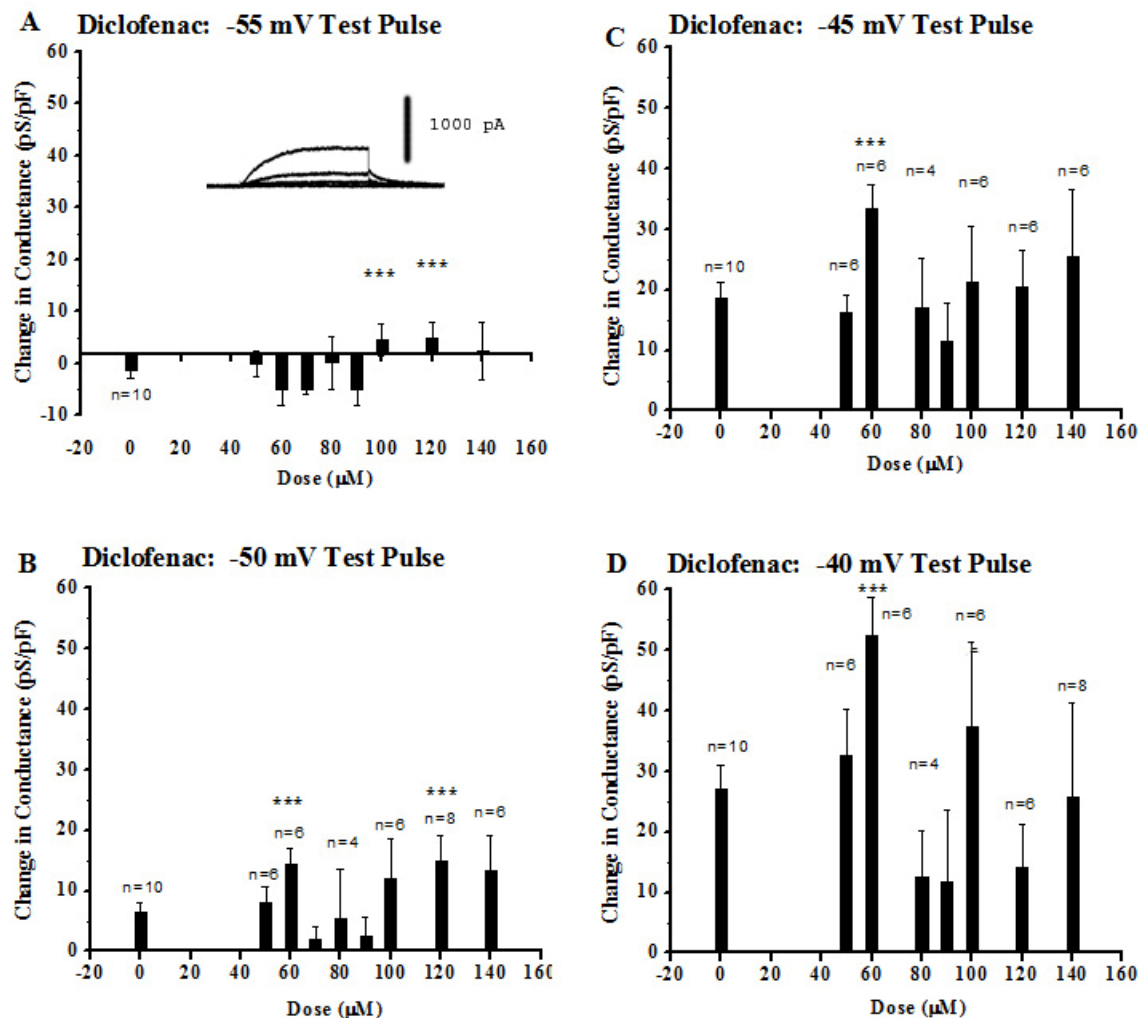


Figure 1. The Influence of Diclofenac on Kv7 Voltage Sensitivity. DIC had a narrow effect on the voltage sensitivity of Kv7. **A)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived 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

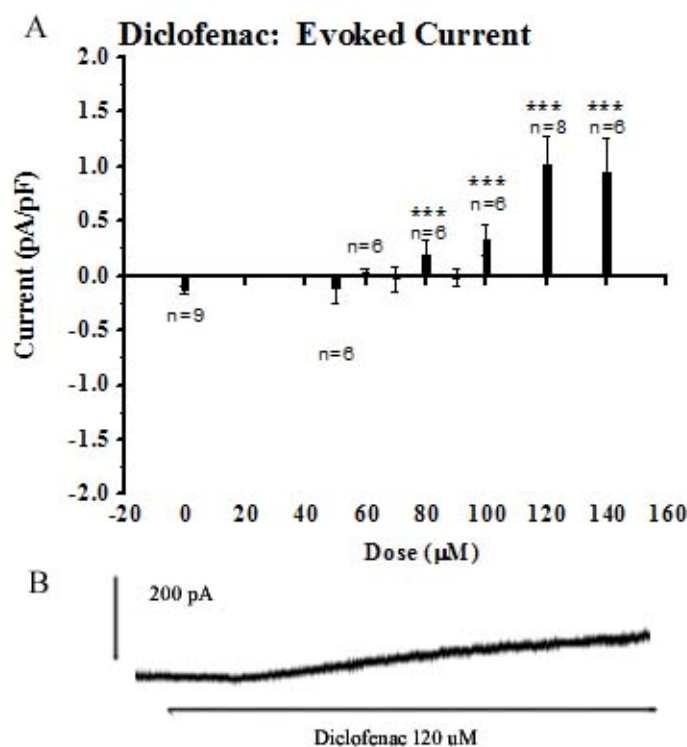


Figure 2. The Effect of Diclofenac on Resting Currents Mediated by Kv7. DIC opened Kv7 channels without a shift in applied voltage. Relatively small outward (hyperpolarizing) currents were observed. **A)** Average normalized current produced at -60 mV ($V_h = -60$ mV) following a 2 minute application of DIC. Evoked currents increased in proportion to the dose applied, but reached a maximum at 120 μ M (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 Kv7. In contrast to DIC, RET amplified the voltage sensitivity of Kv7 at doses as low as 5 μ M (figure 5). A persistent increase in resting current could not be demonstrated at this dose. As doses increased to 40 μ M, Voltage dependent conductance shifts were retained, but fell off as resting currents tended to dominate the effect. Persistent resting currents were observed at doses of 20 to 120 μ M; however some transient currents were present at 5 and 10 μ M. Transient currents reached a relatively rapid peak but subsided within the 2 minute test period. An ED_{50} of 62.7 ± 13.7 μ M was determined

based upon currents present at the 2 min timepoint. Evoked currents reached a maximum at 100 μ M (1.14 ± 0.15 pA/pF; figure 4). Resting currents produced by application of RET were completely reversed by linopirdine (20 μ M). Although the influence on voltage sensitivity and ED₅₀ differed markedly from DIC, maximal maintained currents evoked by RET were similar in amplitude from those evoked by DIC.

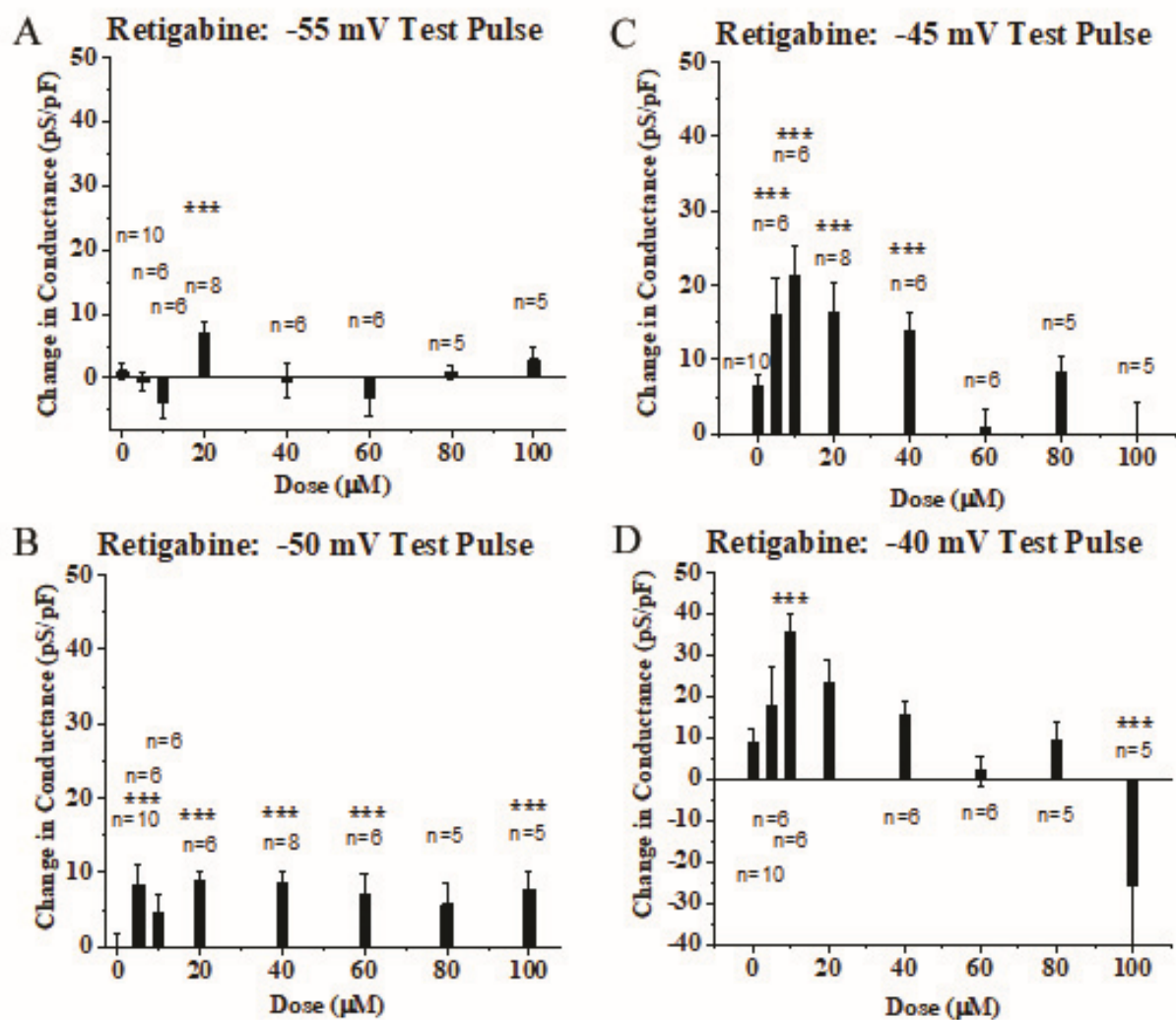


Figure 3. Retigabine Shifts Kv7 Voltage Sensitivity. A-D) The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-RET). Conductance was derived from currents evoked by a voltage step to -55, -50, -45, and -40, respectively. 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.

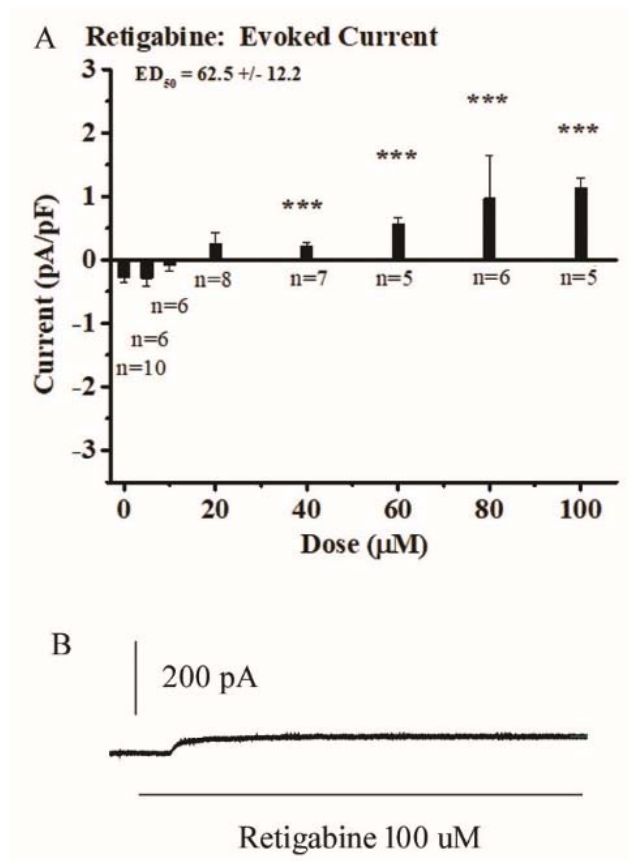


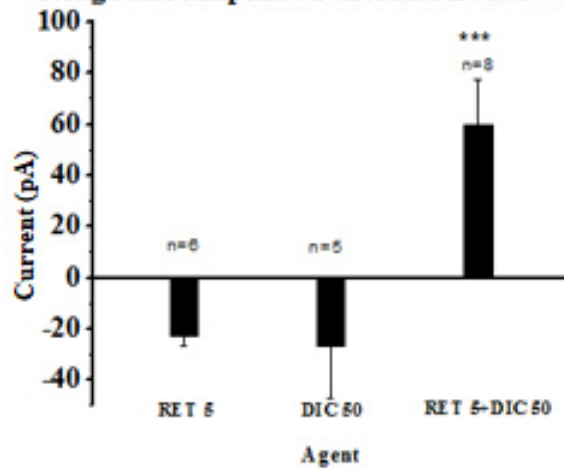
Figure 4. Resting K^+ Currents Evoked by Retigabine. **A)** Average normalized current produced at -60 mV ($V_h = -60$ mV) following a 2 minute application of RET. Evoked currents increased in proportion to the dose applied, but reached a maximum at 100 μ M. **B)** A typical current trace produced by RET. Currents grew rapidly and were stable. $ED_{50} = 62.5 \pm 12.2$ μ M; *** $p < .05$ 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 μ M RET with 50 μ M DIC significantly improved resting current amplitudes (figure 5). The average evoked currents by 5 μ M RET or 50 μ M DIC (applied singly) were -22.7 ± 3.7 pA and -26.7 ± 20.2 respectively, the combination of 5 μ M RET and 50 μ M DIC generated outward currents that averaged 59.5 ± 18.1 pA; an increase of over 70 pA. The combination of 5 μ M RET and 50 μ M DIC produce significantly more current than 70 μ M DIC or 40 μ M RET alone and was roughly equivalent to a dose of 80 μ M DIC or 60 μ M RET (see figures 2 and 4).

At this combination, we observed only sporadic improvements of voltage sensitivity relative to DIC alone (vs 70 μ M DIC; not shown) or RET alone (vs 40 μ M or 100 μ M 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 capacity to improve voltage dependent current, combinations treatments failed to improve on these.

A Retigabine Amplifies Diclofenac Evoked Current



B

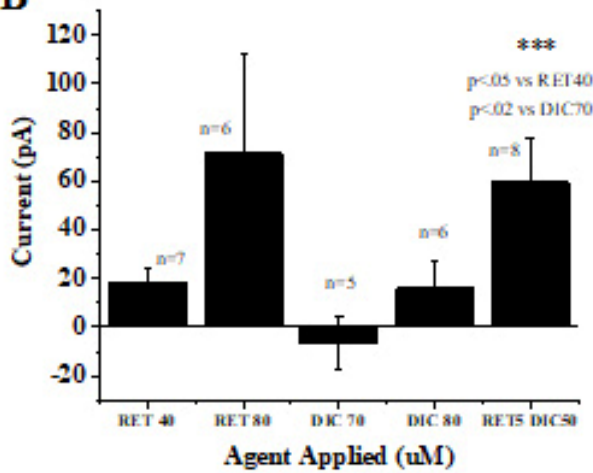


Figure 5. The combination of subthreshold doses of RET 5 μ M and DIC 50 μ M produced currents exceeding those of RET or DIC alone. **A)** Currents measured at the end of the 2 min combined application greatly exceed those evoked by individual applications of sub-threshold doses (RET 5 μ M or DIC 50 μ M). **B)** Currents measured at the end of the 2 min combined application also exceeded those evoked by supra-threshold applications of RET 40 μ M or DIC 70 μ M. RET 80 μ M or DIC 80 μ M evoked currents that were similar to those of RET 5 μ M and DIC 50 μ M 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 μ M RET with 20 μ M DIC produced a similar pattern of outcomes at a lower dose (figure 6). The combination of 5 μ M RET with 20 μ M 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 μ M and 50 μ M DIC. Despite the high variability, the combination of 5 μ M RET and 20 μ M DIC produced significantly more current than 80 μ M DIC or 40 μ M RET alone and was roughly equivalent to a dose of 100 μ M DIC or 60 μ M RET (see figures 2 and 4). In contrast to the 50 μ M DIC combination, co-application of 5 μ M RET with 20 μ M DIC did not significantly improve voltage sensitivity beyond that which could be obtained by a single application of DIC or RET.

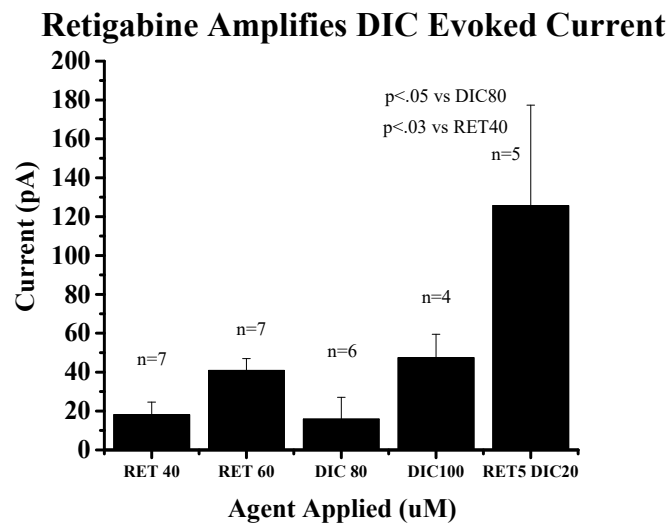


Figure 6. The combination of doses of RET 5 μ M and DIC 20 μ M produced currents exceeding those of RET or DIC alone. Currents measured at the end of the 2 min combined application exceeded those evoked by supra-threshold applications of RET 40 μ M or DIC 80 μ M. RET 60 μ M or DIC 100 μ M evoked currents were similar to those of RET 5 μ M and DIC 20 μ M 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 μ M), 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 μ M 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 μ M (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 μM 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 μM (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 μ M RET with 20 μ M 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 μ M RET (89.8 ± 26.8 APs; median 94.5 APs; $n=6$) nor 20 μ M 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 μ M; however, the combination of RET 5/DIC 20 μ M was not significantly better at reducing AP discharges than DIC 20 μ M alone. The better performance achieved with a lower level of DIC may have resulted from the significantly larger OXO dependent depolarizations observed when 50 μ M 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).

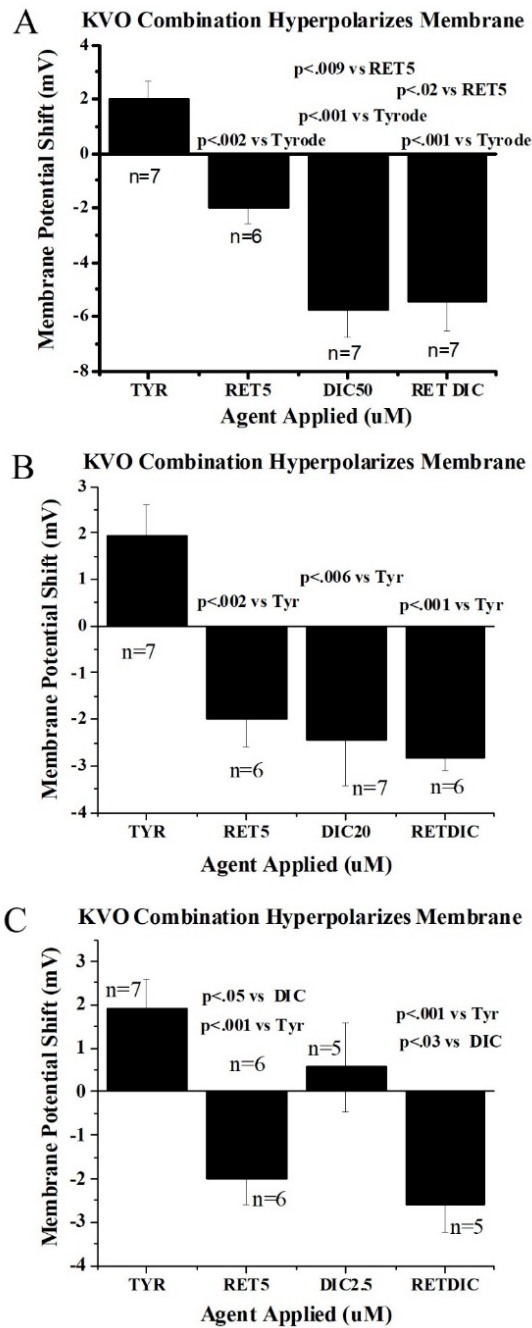


Figure 7. Combinations of RET and DIC Hyperpolarized Nociceptor Membranes. **A)** RET 5 μ M and DIC 50 μ M produce powerful shifts in the membrane potential that exceeded that of RET alone. **B)** Hyperpolarization by RET 5 μ M and DIC 20 μ M were significantly less than that of RET 5 and DIC 50 μ M.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

Ultimately, the best overall results were obtained with a combination dose of 5 μ M RET and 2.5 μ M 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 μ M treatment combination was significantly superior to either RET 5 or DIC 2.5 μ M single applications and did not differ from those of the above combinations (RET 5/DIC 50 μ M; RET 5/DIC 20 μ M).

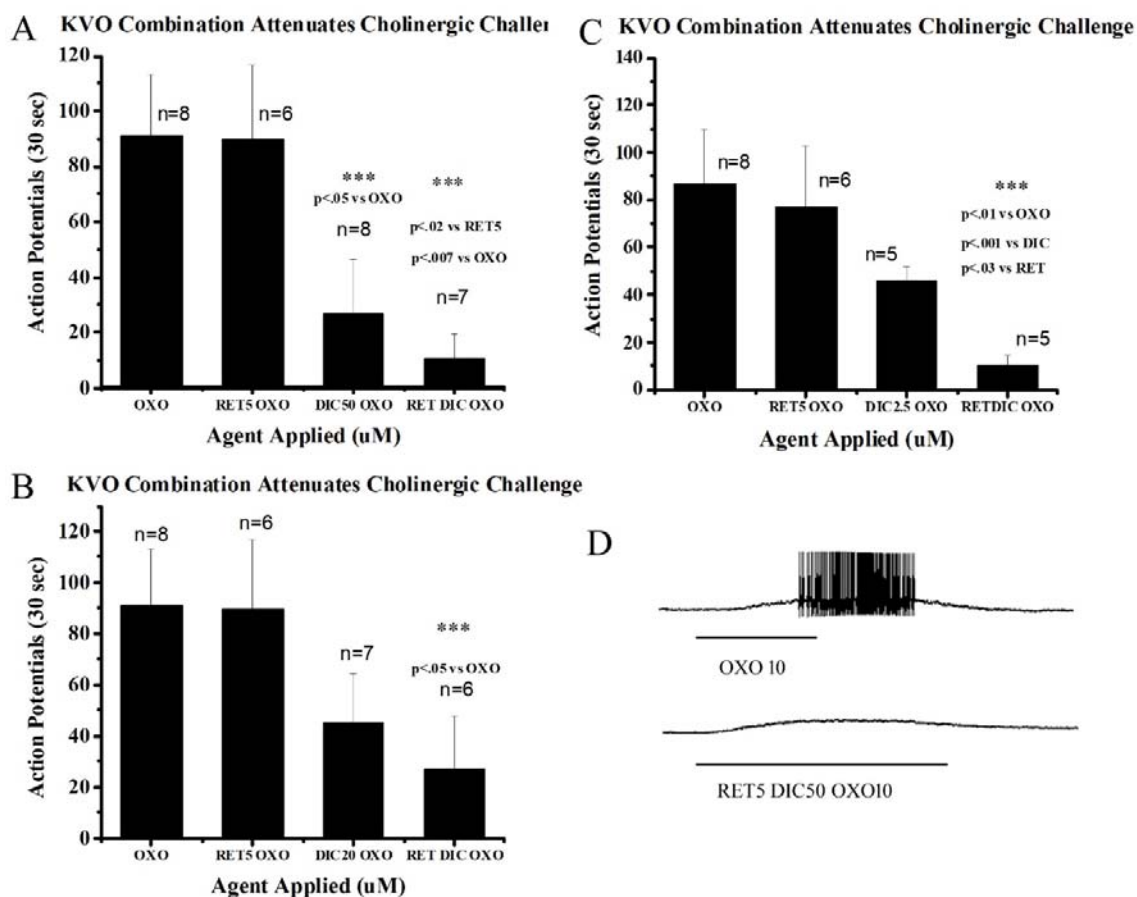


Figure 8. Combination KVOs Reduce Deep Tissue Nociceptor Discharge Consequent to a Cholinergic Challenge. **A)** The number of AP's evoked by 10 μ M OXO were significantly reduced by RET 5 μ M and DIC 50 μ M. RET 5 μ M had no influence. **B)** RET 5 μ M and DIC 20 μ M significantly reduced APs evoked by 10 μ M OXO. Neither 5 μ M or DIC 20 μ M had any effect on AP discharge. **C)** RET 5 μ M and DIC 2.5 μ M significantly reduced APs evoked by 10 μ M OXO. Neither 5 μ M or DIC 2.5 μ M had any effect on AP discharge. **D)** Representative traces illustrate powerful burst evoked by 10 μ M OXO (upper trace). AP bursting was completely abrogated by the application of RET 5 μ M and DIC 50 μ M (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 μ M and DIC 1.25 μ M 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) with regard to membrane hyperpolarization or action potential suppression.

Behavioral Studies

GW 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 μ M RET and 20 μ M 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.

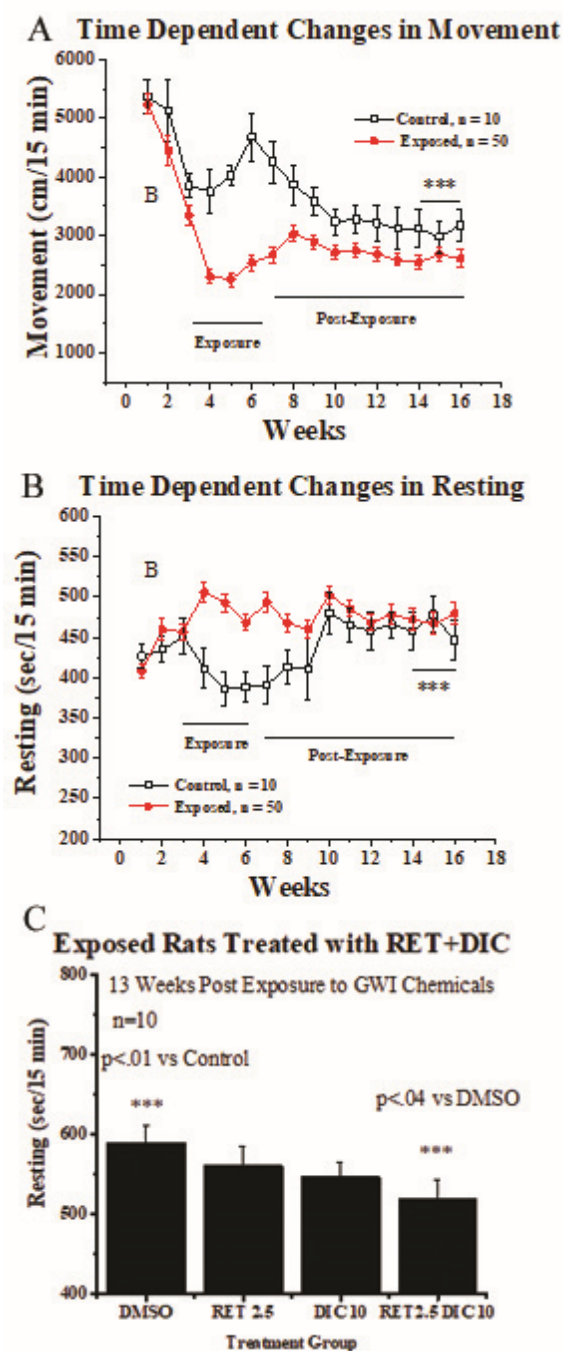


Figure 9. The Combination of RET and DIC (1:4) Reduced Pain-Like Behavior. **A)** Weekly progression of movement distance data before, during and following exposure to GW chemicals. **B)** Weekly progression of rest time scores before, during and following exposure to GW chemicals. **C)** The influence 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.

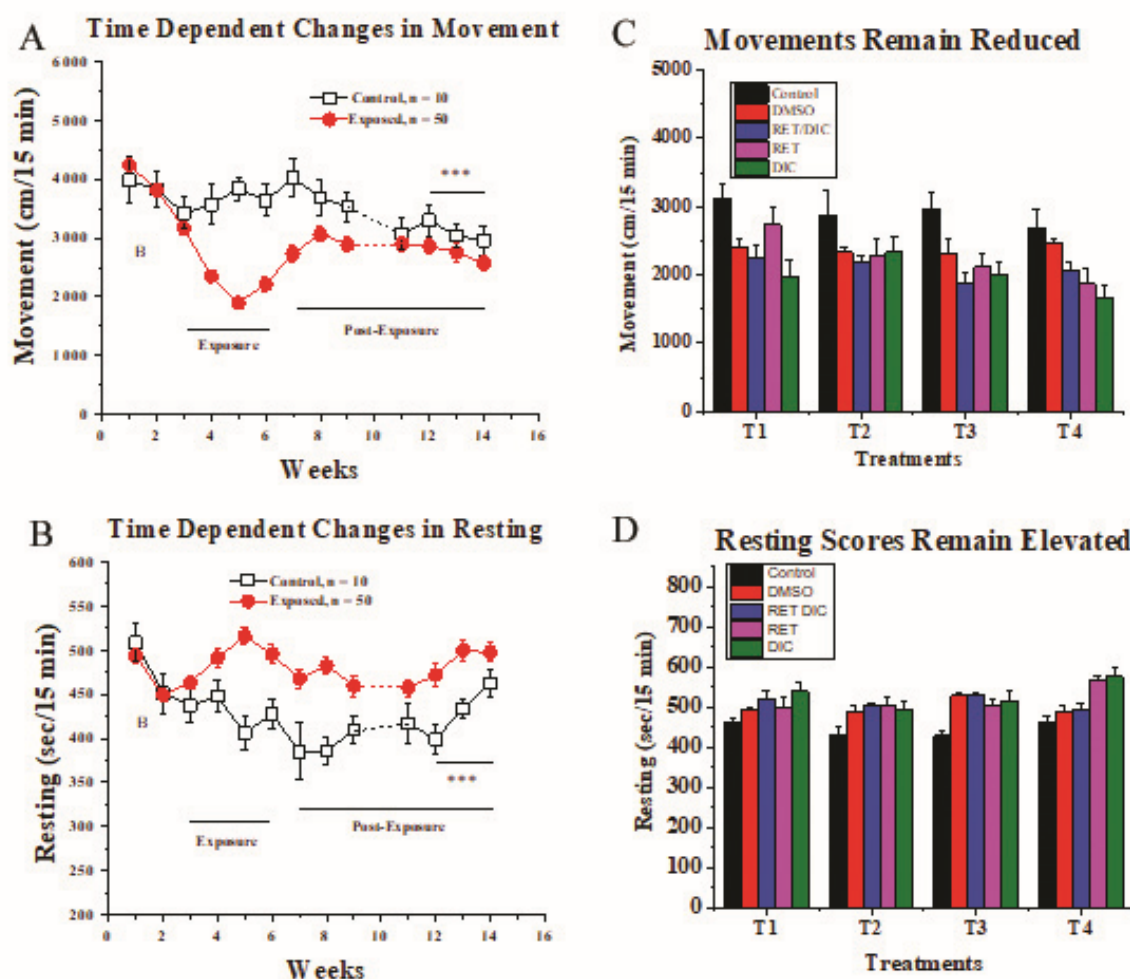


Figure 10. Multiple Dose Combinations of RET and DIC Fail to Modify Pain-Like Behaviors. A) Weekly progression of movement distance scores before, during and following exposure to GW chemicals. B) Weekly progression of rest time scores before, during and following exposure to GW chemicals. C) The influence of single and combination treatments on movement distance scores; D) The influence of single and combination treatments 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.

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) ED₅₀s 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. ED₅₀s and maximal currents were determined for individual KVOs expressed in two classes of deep tissue nociceptors (RET: ED₅₀ of 62.7 μ M, I_{max}: 1.14 pA/pF; DIC: ED₅₀ 93.2 μ M; I_{max}: 1.05 pA/pF; Cooper et al.,

2014). The nociceptor based ED₅₀ for RET varied considerably from those reported for K_v7.2/K_v7.3 heteromultimers expressed in CHO cells or *Xenopus* Oocytes (1-4 μ M; 3.6 μ M Main et al., 2000; 1.7 μ M, Rundfelt and Netzer 2000; 1.6 μ M, Wickendon et al., 2000; 2 μ M, Peretz 2005). These values, with the exception of Main et al., reflect calculations based on V₅₀ 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 μ M). However, one report that did base ED₅₀ calculations on evoked current, obtained results similar to those reported in CHO cells (~1 μ M; 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 ED₅₀s 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 particularly 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 uniformly 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’ ED₅₀ for DRG nociceptors.

Regulatory factors could also play a role in determining the sensitivity of nociceptor K_v7 channels to RET (e.g., phosphorylation status and interaction with membrane PIP₂; Erdem et al., 2017; Salzer et al., 2017). ED₅₀s 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 neurotoxicant 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 μ M and DIC 2.5 μ M. This combination significantly suppressed AP discharge, and was significantly better than either RET 5 μ M or DIC 2.5 μ M alone. Other combinations were arguably more effective than 5 RET+2.5 DIC at membrane hyperpolarization (i.e., RET 5 μ M and DIC 50 μ M) 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 especially a concern when DIC alone was shown to suppress AP discharge (50 μ M 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 μ M (Nakamura and Jang, 2016; but see Lee et al., 2003, 96 μ M). DIC exerts a much greater influence on TTXs channels (I_{50} = 14 μ M; 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 μ M 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 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 nociceptor 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; possibly via inhibition of cyclooxygenase and TTXs Nav. 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; Yekkiralala et al., 2017; Eid and Gurney, 2018).

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Further Investigations into KVO Treatment Strategies for Chronic Pain in a Rat Model of Gulf War Illness

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Abstract

In a companion paper we examined whether combinations of K_v7 channel openers (Retigabine and Diclofenac; RET, DIC) 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. In the present report, we examined the combinations of Retigabine/Meclofenamate (RET/MEC) and Meclofenamate/Diclofenac (MEC/DIC). Voltage clamp experiments were performed on deep tissue nociceptors isolated from rat DRG (dorsal root ganglion). In voltage clamp studies, 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. MEC greatly enhanced voltage dependent conductance and produced exceptional maximum sustained currents of 6.01 ± 0.26 pA/pF (ED₅₀: 62.2 ± 8.99 μ M). Combinations of RET/MEC, and MEC/DIC substantially amplified resting currents at low concentrations. MEC/DIC also greatly improved voltage dependent conductance. In current clamp experiments, a cholinergic challenge test (Oxotremorine-M, 10 μ M; OXO), associated with our GWI rat model, produced powerful action potential (AP) bursts (85 APs). Optimized combinations of RET/MEC (5 and 0.5 μ M) and MEC/DIC (0.5 and 2.5 μ M) significantly reduced AP discharges to 3 and 7 Aps, respectively. Treatment of pain-like ambulatory behavior in our rat model with a RET/MEC combination (5 and 0.5 mg/kg) successfully rescued ambulation deficits, but could not be fully separated from the effect of RET alone. Further development of this approach is recommended.

Key Words: Gulf War Illness; Chronic Pain; K_v7; Meclofenamate; Retigabine; Diclofenac

1. Introduction

Approximately 30% of veterans returning from the 1991 Persian Gulf War (ODS; Operation Desert Storm) developed a multisymptom illness that was subsequently labelled Gulf War Illness (GWI). Although it is widely believed that symptom onset occurred only after warfighters returned from deployment, about 25% of GWI sufferers developed symptoms during their deployment in the Persian Gulf (Kroenke et al., 1998). From the point at which GWI was first recognized, categorized and tracked, these symptoms have tended to worsen over time (Hotopf et al., 2003; Dursa et al., 2021; Zundel et al., 2020; Kregel et al., 2022). After more than two decades, an effective treatment for GWI has not been identified (Nugent et al., 2020; Dickey et al., 2021).

GWI is a multisymptom disorder that can be clustered into 3 major, but overlapping, syndromes with primary features of chronic pain, confusion-ataxia and cognitive deficits (Haley and Kurt, 1997). The symptoms of chronic pain are a diverse mixture of deep tissue pains, that can include muscle, back, joint, visceral and headache pains (Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013; White et al., 2016; Maule et al., 2018; Porter et al., 2018; Dursa et al., 2019). Over the last decade, our laboratory has investigated the cellular and molecular pathophysiology of GWI pain (Nutter, et al., 2015; Flunker et al., 2017; Cooper et al., 2018).

While the pathophysiology of GWI remains speculative, a large body of evidence points to exposure to multiple insecticides and other neurotoxicants encountered in the region (Binns et al., 2008; RAC2014; O'Callaghan et al., 2017; Janulewicz et al., 2018; Naughton and Terry, 2018; Nizamutdinov et al., 2018; Michalovicz et al., 2019; Dickey et al., 2021). During ODS, the military used a number of organophosphate (anti-cholinesterase) based insecticides as areal sprays, while other insecticides were embedded in uniforms (i.e., Permethrin, a pyrethroid). Additional agents were obtained by individual soldiers for their personal use (e.g., DEET, Chlorpyrifos flea collars). In all, over 64 insecticides and

repellants containing 37 distinct ingredients of various chemical composition were used in both officially sanctioned and unsanctioned manners to combat insects encountered in the region (U.S. Department of Defense Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008; RAC2014; Brady et al., 2021). This highly varied exposure pattern was layered upon a backdrop of prophylactic, anticholinesterase, treatment.

Given the real threat of chemical attack (e.g., Sarin, Soman), soldiers were prescribed 3 daily doses of pyridostigmine bromide tablets (PB), taken over a period of months, in order to counter the fatal sequelae of nerve agent attack (Weinbroum, 2004; Newmark, 2005; Weissman and Raveh, 2011). In a series of investigations prior to the war, it had been determined that PB, a reversible anticholinesterase, in combination with 2-PAM, Atropine, or similar agent, could lessen the effects of anticholinesterase based nerve agents (Gordon et al., 1978; Gall, 1981; Maxwell, et al., 1988; von Bredow et al., 1991; Ray et al., 1991; see also Koplovitz et al., 1992; Koplovitz and Stewart, 1994; Kassa and Fusek, 1998; Kassa and Vachek, 2002; Kassa and Krejeova, 2003). As a result, soldiers were exposed to multiple sources of anticholinesterases in a short period of time.

Both pyrethroid and organophosphate insecticides interact with portions of the human nervous system that are critical to pain perception. Specific interactions include multiple ionic and metabotropic channels that are expressed on neurons of the peripheral pain detection network (nociceptors), including: 1) voltage gated Na^+ and K^+ channels (Nav , K_v7 ; Narahashi et al., 1998; Soderlund et al., 2002; Jiang et al., 2013; Nutter et al., 2013; Nutter et al., 2015; Cooper et al., 2016; Cooper et al., 2018; Boethe and Lampert, 2021); 2) transient receptor potential channels (TRPA1; Cooper et al., 2016; Ding et al., 2017; Cooper et al., 2018); 3) ionic and metabotropic cholinergic channels and receptors (Nostrandt et al., 1997; Liu et al., 1999; Huff et al., 2001; Zhang et al., 2002; Abou-Donia et al., 2003; Padilla et al., 2005; Pung et al., 2006; Proskocil et al., 2010); and 4) generation of pro-inflammatory mediators ($\text{IL1}\alpha$, IL2 , IL6 ; Michalovicz et al., 2019).

Over the last 10 years, our laboratory developed and refined an animal model of GWI pain (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al., 2015; see also Lacagnina et al., 2021; Nguyen et al., 2021). This model made it possible to produce rats with GWI pain signs from which neurons could be extracted for molecular and cellular studies. In 2015, we reported that a 4 week exposure to Permethrin, Chlorpyrifos and PB (pyridostigmine bromide) produced a delayed manifestation of pain-like ambulation deficits that developed many weeks after exposures had ceased (Nutter et al., 2015). When the 3 agent exposure set was augmented with DEET (N,N-diethyl-meta-toluamide), pain behaviors were more robust, lasted longer and appeared much earlier following exposures (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 we demonstrated that co-application of DEET was a magnifying factor, only the anticholinesterases (PB and Chlorpyrifos) were shown to be necessary for the development of pain-like behaviors (Flunker et al., 2017; see also Kregel et al., 2022).

A series of studies conducted on nociceptors harvested from the dorsal root ganglia (DRG) of rats manifesting GWI pain-like behaviors, indicated that maladaptations were present in Nav1.8, Nav1.9, TRPA1, and Kv7 expressing neurons (Nutter et al., 2015; Cooper et al., 2016; Cooper et al., 2018). Given the determinant role of anticholinergics in the development of GWI, and the demonstrated contribution of muscarinic sensitive Kv7, we began a series of studies to determine whether a family of Kv7 opening drugs (KVOs) could be utilized as analgesic treatments for the chronic pain of GWI.

Nociceptor Kv7 is assembled from a combination of subunits designated as Kv7.2, Kv7.3 and Kv7.5 (Hernandez et al., 2008; Brown and Passmore, 2009; Du et al., 2017; Yu et al., 2018). Closing of Kv7, via activation of the muscarinic class of cholinergic receptors, depolarizes membranes and increases nociceptor excitability. Inversely, activation of Kv7, via its inherent voltage dependence, acts as a break on nociceptor discharge by driving membrane potential in a hyperpolarizing direction (Brown, 1988; Brown and Passmore, 2009; Jepps et al., 2021). The potential for analgesic applications is clear.

Classical K_v7 openers, such as Retigabine (RET) and Flupirtine can shift K_v7 voltage dependence, open K_v7 channels, and thereby retard action potential (AP) discharge (Peretz et al., 2005; Abd-Elseyed et al., 2015; Du et al., 2017; Wu et al., 2017; Yu et al., 2018). These KVOs reduce pain behaviors in animals (Abd-Elseyed et al., 2015; Teng et al., 2016; Wu et al., 2017; Yu et al., 2018), and have been shown to be effective treatments for some forms of chronic pain (Luben et al., 1994; Worz et al., 1996; Uberall and Muller-Schwefe, 2011; Mishra et al., 2013; Uberall et al., 2013; Worz, 2014; see also Blackburn-Monro et al., 2005). In a test of the analgesic potential of RET in our model of GWI pain, we reported that a daily dose of RET (7.0 mg/kg; 4 weeks), reversed established ambulation deficits that emerged 9 weeks following exposure to GW neurotoxins. While pain-like behaviors significantly and rapidly diminished, the improvement was transitory. Pain- signs returned over the 4 week treatment period (Cooper et al., 2018).

Diclofenac (DIC) and Meclofenamate (MEC) are FDA approved agents with powerful cyclooxygenase inhibitory activity (Conroy et al., 1991; see also Hill and Zawia, 2021). Recent investigations have shown that both of these agents act secondarily as KVOs (Peretz et al., 2005; Brueggemann et al., 2010; Huang et al., 2013), but exert their KVO activity via preferential binding to distinct subunits of the K_v7 complex. The anti-inflammatory KVOs target K_v7.2 preferentially over K_v7.3 (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). In contrast, Retigabine preferentially targets K_v7.3 over K_v7.2 (Tatulian et al., 2001; Peretz et al., 2005; Varghese et al., 2021). Given these distinct modes of action, it was possible that combinations of RET and MEC or RET and DIC could act cooperatively to amplify the voltage dependent K⁺ currents beyond which either agent could produce in isolation. In studies of assemblies of K_v7.2/K_v7.3 in CHO cells (Chinese hamster ovary), Peretz and colleagues demonstrated that MEC amplified the voltage dependent and resting current effects of RET (Peretz et al., 2005; see also Peretz et al., 2007; Peretz et al., 2010). Following this lead, our laboratory demonstrated that cooperative

actions of the classic and secondary KVOs, RET and DIC, could be demonstrated in mammalian nociceptive neurons expressing native K_v7, and that this *in vitro* demonstration could be translated into analgesic activity in behaving rats in our model of GWI pain. Although evidence of recovery for GWI pain was obtained with combined RET and DIC treatment, these behavioral experiments were less than totally successful due to toxic side effects of these combinations that emerged with repeated treatments (Flunker et al., 2022). More importantly, this proof of concept, *in vitro*, encouraged further exploration of the underlying hypothesis that these distinct binding sites could be independently utilized to improve the analgesic potential of K_v7.

In the experiments described below, we examined whether combinations of primary and secondary KVOs (RET/MEC and MEC/DIC): 1) optimized activity of K_v7 channels expressed in deep tissue nociceptors; 2) suppressed muscarinic dependent AP burst discharges; and 3) relieved pain-like behaviors in rats exposed to GW neurotoxicants.

2. Methods

2.1 Subjects

A total of 172 young adult male rats were used in all studies (electrophysiology: n=132; behavior: n=40). An additional 11 rats were used for pilot studies and/or were excluded for erratic behavior. All rats were obtained from Envigo (Sprague-Dawley) and weighed 90-110 grams on arrival. Terminal weights of rats, used in the behavioral studies, did not differ due to their exposure to GWI neurotoxicants (404.6 ± 7.6 vs 405.3 ± 1.8 grams; control and exposed, respectively). We did not observe any signs of acute pesticide toxicity typically associated with permethrin or chlorpyrifos during the execution of these studies. 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). Methods were consistent with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2 Electrophysiology

2.2.1 Preparation of Cells

Male Sprague-Dawley rats were anesthetized (Isoflurane) and rapidly euthanized by decapitation (Harvard Apparatus). The dorsal root ganglia were excised, and subsequently 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., 2017). Isolated neurons were plated on nine, 35 mm, Petri dishes. Cells were bathed continuously in Tyrode's solution, containing (in mM) 140 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 M Ω) by a Sutter P1000 (Sutter Instruments, Novato, CA). The pipette solution contained (in mM): 120 KCl, 5 Na₂-ATP, 0.4 Na₂-GTP, 5 EGTA, 2.25 CaCl₂, 5 MgCl₂, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm. Experiments began 2 hours after plating. Only one cell was used per Petri dish, and recording experiments were completed within 10 hours after plating. Studies were conducted at room temperature (20° C).

2.2.2 Recording and Characterization of Neurons

Whole cell recordings were conducted using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and a Digidata 1322A (Molecular Devices). Whole cell resistance and capacitance were assessed by the Clampex

software utility. Series resistance (R_s) was compensated 65-80% with Axopatch compensation circuitry. During voltage clamp studies, voltage dependent currents were leak-corrected on line using the P/4 procedure of pClamp 9.0. Series resistance compensation was checked every 2-4 minutes during the procedure. 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 prior to series resistance 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 deep tissue nociceptors (Rau et al., 2007; Rau et al., 2012; 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), and a Na^+ current that was broad at its base (> 3.0 msec at 0 mV) and was fully activated 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 (CP2), and a Na^+ current that exhibited a broad base (>2.4 msec at 0 mV) and was fully activated 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 (Petruska et al., 2000; 2002). Cells not fitting the classification criteria were discarded.

2.2.3 Voltage Clamp Studies

KVOs were obtained from Alomone Labs, Adooq Biosciences (Retigabine) and Sigma-Aldrich (MEC, DIC). Agents were prepared in stock solutions of 10-50 μ M (DMSO or H_2O as needed). Stock solutions were kept at -20 °C. RET stocks were discarded after 3 months. 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 mm).

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 at the holding potential. For voltage sensitivity tests, a 1000 msec conditioning pulse to -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, the series resistance was corrected if 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: $G = I_{\text{peak}} / (V_m - V_{\text{rev}})$, where I_{peak} was the test current, V_m was 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:

$$G_{\text{diff}} = G_2 - G_1$$

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 and MEC; 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. Type 5 and Type 8 data were pooled for the conductance analysis.

Analyses of KVO sustained currents at $V_H = -60$ mV, were conducted on the net change in the current produced by application of KVO for 2 minutes relative to those shifts present during application of the appropriate vehicle. Currents were normalized for cell capacitance for statistical analysis. Type 5 and Type 8 data were pooled for the resting current analysis.

An ED_{50} was determined for MEC by fitting the normalized currents (I/I_{MAX}) to a sigmoidal dose-response function (Origin 9.0) of the form:

$$Y=A1 + (A2 - A1)/(1 + 10^{(\text{Log}((X0-X)*p)})$$

Where A1 and A2 are the minimum and maximum asymptotic values, P is the Hill Slope and Log X0 is the ED₅₀.

2.2.4 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) were co-applied with OXO. The resting membrane potential was recorded continuously. Application of OXO to Type 5 or Type 8 cells typically produces a rapid depolarization and a powerful burst discharge (Cooper et al., 2016). Statistical comparisons were made between vehicle treated and OXO-KVO cases.

2.3 Behavioral Studies

2.3.1 Exposure Protocol

Over a period of 4 weeks, 32 rats were exposed to GW chemicals (or vehicles) that were previously demonstrated to produce a complex of pain-like signs (Nutter et al., 2015; Flunker et al., 2017). GRP 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 (n=8) 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.

2.3.2 Assessment of Ambulation

Open field testing was conducted before, during and after GWI chemical exposures. Upon arrival, rats were acclimated to their cage for 1 week and then to the behavioral procedures for 1 week prior to exposures. Activity levels (movement distance in cm; movement rate in cm/sec; rest time duration in 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, Pyridostigmine) and vehicle exposed (ETOH, corn oil, water) animals over an identical time course. Each rat was tested once per week on the behavioral tasks.

2.3.3 Treatment Protocol

Twelve weeks after exposure to GW chemicals, the 32 toxicant exposed rats were divided into 4 groups of 8 each. Two hours prior to ambulation testing: 1) GRP R rats were treated with RET; 2) GRP M rats were treated with MEC; 3) GRP RM rats were treated with the combination of the KVO dosages;

4) GRP EXP were given water vehicle. GRP CON rats (not exposed to GWI chemicals) were also with treated water. All treatments were by oral gavage. Behavioral testing began 2 hours after treatments.

2.4 Statistics

2.4.1 Electrophysiology

Student's T-test was used to assess the influence of KVOs on: 1) the amplitude of K_v7 resting currents; 2) the change in voltage dependent conductance; 3) membrane hyperpolarization; and 4) action potential discharge. The alpha level was set at .05.

2.4.2 Behavioral Testing

During the final 4 weeks of post-exposure testing, and prior to the beginning of KVO treatments, a repeated measures ANOVA (QIMacros) was used to assess the influence of chemical exposure on dependent measures of Movement Distance (cm/15 min), Movement Rate (cm/sec) and Rest Duration (sec). During the treatment phase (post-exposure weeks 12, 13 and 14), a one way ANOVA was used to assess Main effects of the Treatment factor (KVOs). Fisher LSD follow-up tests compared individual groups ($p < .05$).

3. Results

Given indications of multiple binding sites on K_v7 subunits, cooperative activation could be manipulated for analgesic applications. Enhanced K_v7 activation features could include potentiation of voltage dependent activation, resting current production or both. In this report, we examined whether we could identify a specific combination of KVOs that both enhanced therapeutic potential and lowered effective dosing. In our last report, we identified combinations of DIC and RET that reached this goal,

in vitro, but we could not safely transfer this potential to a behaving animal (Flunker et al., 2022). The present report extends these studies to include the cyclooxygenase/KVO, Meclofenamate.

3.1 Voltage Clamp Studies on Meclofenamate

3.1.1 Shifts in Voltage Sensitivity

Following isolation and characterization of two deep tissue nociceptive cell types (Type 5 and Type 8; Petruska et al., 2000; Petruska et al., 2002), we examined the dose dependent influence of a known KVO, Meclofenamate (MEC) on resting current and voltage conductance. Concentrations of 10 to 100 μ M MEC were applied by close superfusion (1 mm). Each nociceptive neuron was exposed to only one concentration, and the dish was discarded thereafter. The influence of MEC was assessed in a K_{iso} solution that was applied for 2 minutes after cell characterization. Voltage probe steps were limited to those near AP activation threshold (-40, -45, -50 and -55 mV).

As MEC doses increased from 10 to 100 μ M, significant increases in voltage dependent conductance were observed at nearly all levels tested (figure 1). Surprisingly, voltage dependent effects remained pervasive despite the fact that a considerable portion of K_v7 channels were directly opened by MEC.

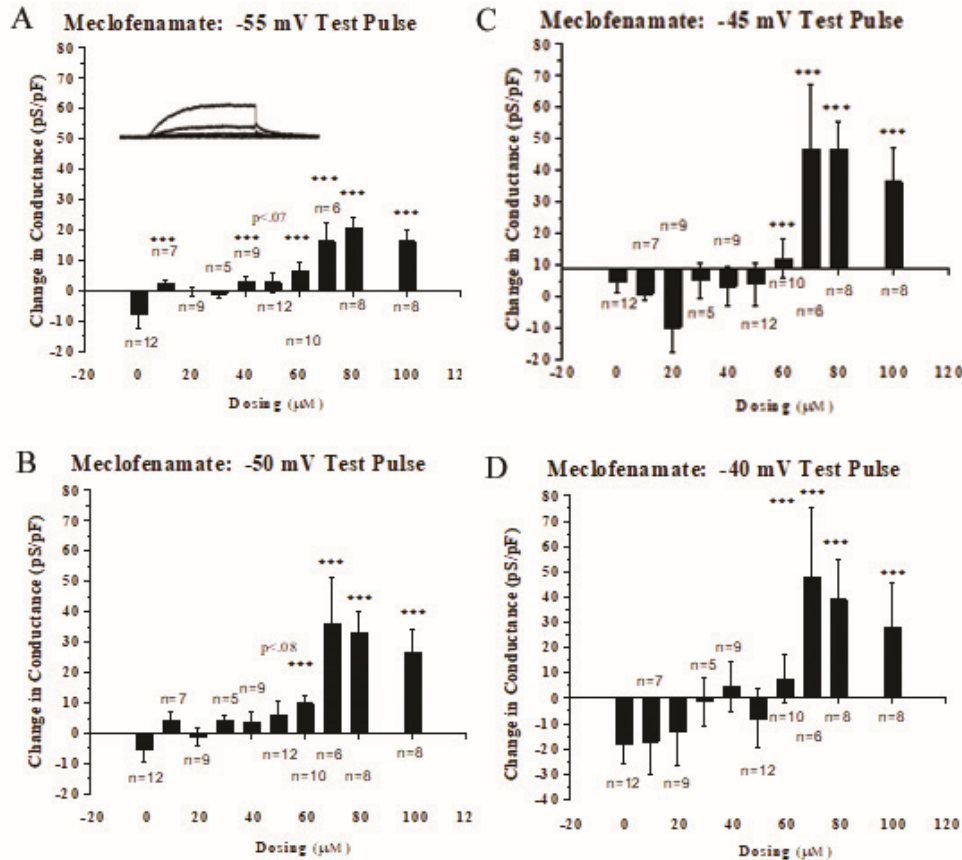


Fig. 1. The influence of Meclofenamate on Kv7 voltage dependent conductance. The dose dependent change in normalized conductance of deep tissue nociceptors following exposure to MEC or vehicle (vehicle-vehicle or vehicle-MEC). **A)** Conductance derived from currents evoked by a voltage step to -55 mV ($V_h = -60$ mV). **B)** Conductance derived from currents evoked by a voltage step to -50 mV ($V_h = -60$ mV). **C)** Conductance derived from currents evoked by a voltage step to -45 mV ($V_h = -60$ mV). **D)** Conductance 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.

3.2 Activation

When MEC was applied at the holding potential of -60 mV, it proved to be a very effective activator of Kv7. At doses as low as 30 μ M, a weak Kv7 current could be detected (~ 11 pA; 0.22 ± 0.18 pA/pF). Evoked current grew substantially with increasing concentration, and reached a maximum at 80 μ M (6.3 ± 1.2 pA/pF, $n=8$; figure 2). The ED₅₀ (effective dose for 50% activation) for MEC was determined to be 62.2 ± 3.2 μ M. Evoked currents were completely reversed by the specific Kv7 inhibitor, Linopirdine (20 μ M; 3 minutes). The maximal evoked current was substantially higher than those we

reported during tests of a structurally similar secondary KVO (Diclofenac; 1.01 ± 0.26 pA/pF) or the classic KVO, Retigabine (figure 2B and C; data presented from Flunker et al., 2022 for comparison). The relatively more powerful influence of MEC on K_v7 activity suggested it would be superior to DIC or RET in the suppression of nociceptor activity.

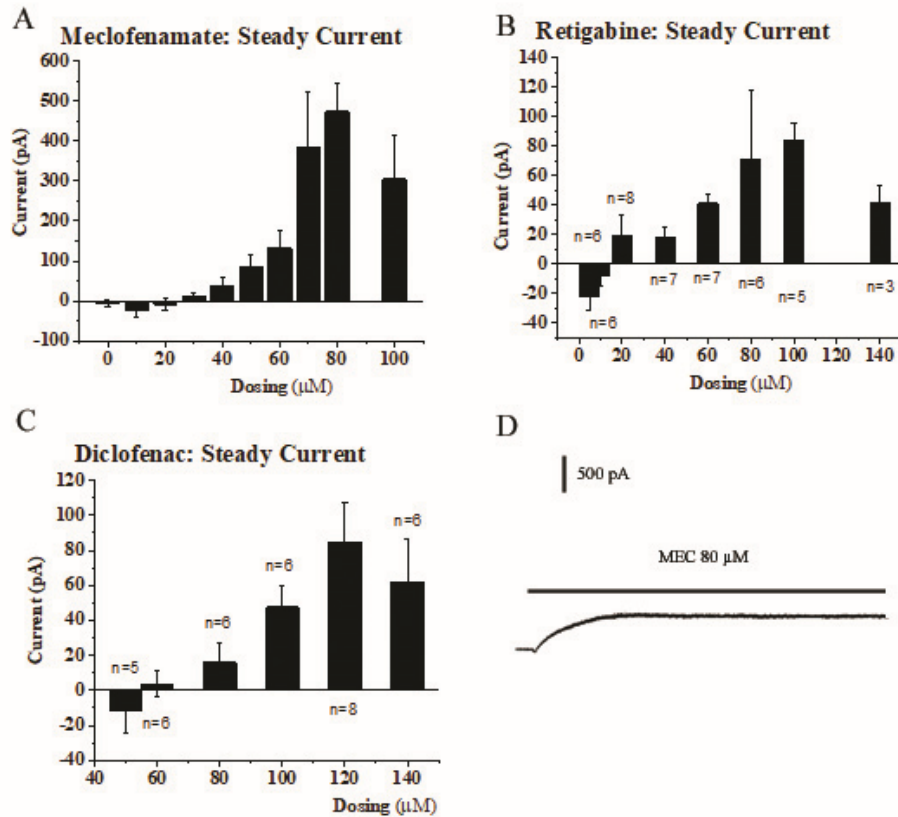


Fig. 2. The influence of Meclofenamate on resting currents. MEC opened $\text{K}_{\text{v}}7$ channels independent of any shift in applied voltage. Relatively large outward (hyperpolarizing) currents were observed. **A)** Steady current produced at the holding potential ($V_{\text{h}} = -60$ mV) following a 2 minute application of MEC. Evoked currents increased in proportion to the dose applied, but reached a maximum at 80 μM . **B and C)** Steady currents produced by Retigabine and Diclofenac, respectively (data reprinted from Flunker et al., 2022 for comparison). In ‘C’ note the test at 10 μM that is partially obscured. Note differences in scales. **D)** A representative trace shows the rapid increase of K^+ current with MEC (2 min application). Data collected from voltage clamp experiments in a K^+ isolation solution. Current was measured at the end of the 2 min application. The number of cells contributing to each bar is indicated as ‘n=’. Each cell was exposed to only one concentration.

3.3 Combinations of Meclofenamate with Retigabine or Diclofenac

Using methods identical to those above, we examined whether a combination of KVOs could synergize to produce current and voltage effects that substantially exceeded those that could be produced by individual applications of either agent. In the first series of studies, we examined the influence of MEC on RET activated currents when these agents were combined at sub- or near threshold levels. Although the threshold for a RET activated current was previously shown to be 20 μM (Flunker et al., 2022; see

figure 2B), the combination of 5 μM RET with near threshold 30 μM MEC could strongly activate nociceptor K_v7 (figure 3A). Sustained currents at this combination significantly exceeded those that could be obtained at 80 μM RET or 60 μM of MEC alone (figure 3B). There was also a weak and inconsistent enhancement of voltage activated currents at the 5 RET/30 MEC combination (not shown).

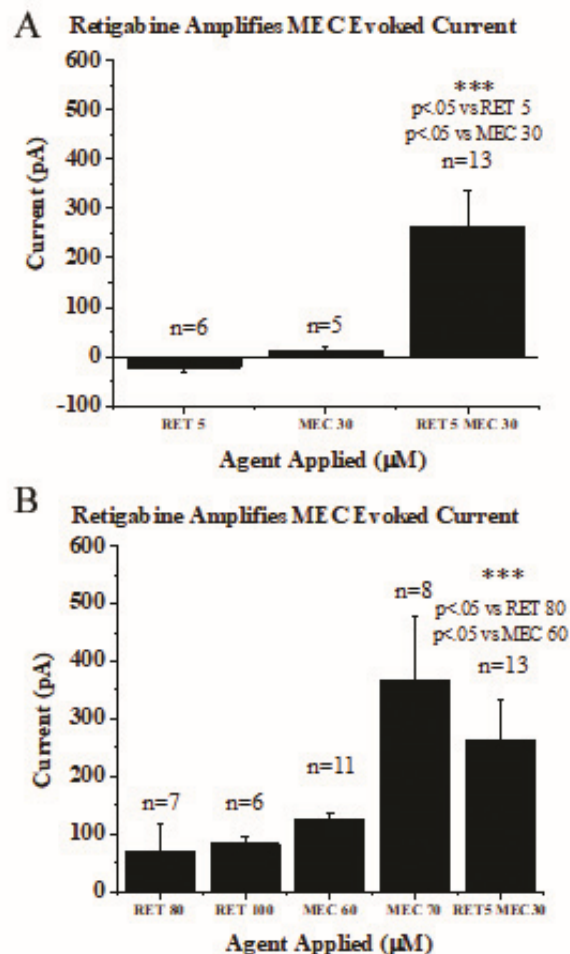


Fig. 3. The combination of doses of RET 5 μM and MEC 30 μM produced currents exceeding those of RET or MEC alone. **A)** Currents measured at the end of the 2 min combined application greatly exceeded those evoked by individual applications (RET 5 μM or MEC 30 μM). **B)** Currents measured at the end of the 2 min combined application significantly exceeded those evoked by supra-threshold applications of RET 80 μM or MEC 60 μM . RET 100 μM or MEC 70 μM evoked currents that were similar to those of RET 5 μM and MEC 30 μM 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). RET data from Flunker et al., 2022, but experiments were conducted in the same time period.

Although DIC and MEC have similar structures and K_v7 binding patterns, we explored the possibility that DIC and MEC combinations could potentiate K_v7 activation. A large number of combinations were tested, with the best results obtained with subthreshold levels of DIC (40 μ M; see Flunker et al., 2022 and figure 2C) and minimally supra-threshold levels of MEC (see figure 2A). As shown in figure 4A, the combination of MEC 40 μ M with DIC 40 μ M evoked large currents in deep tissue nociceptors (363 ± 109 pA). This combination exceeded the maximum current that could be evoked by DIC alone (120 μ M; Flunker et al 2022) or 60 μ M MEC (figure 4B). Good results were also obtained by the combination of 5 μ M MEC with 40 μ M DIC. Although both of these concentrations were considerably below threshold, when they were presented in combination, sustained outward currents could be produced (figure 4C), and exceeded 50 μ M DIC alone.

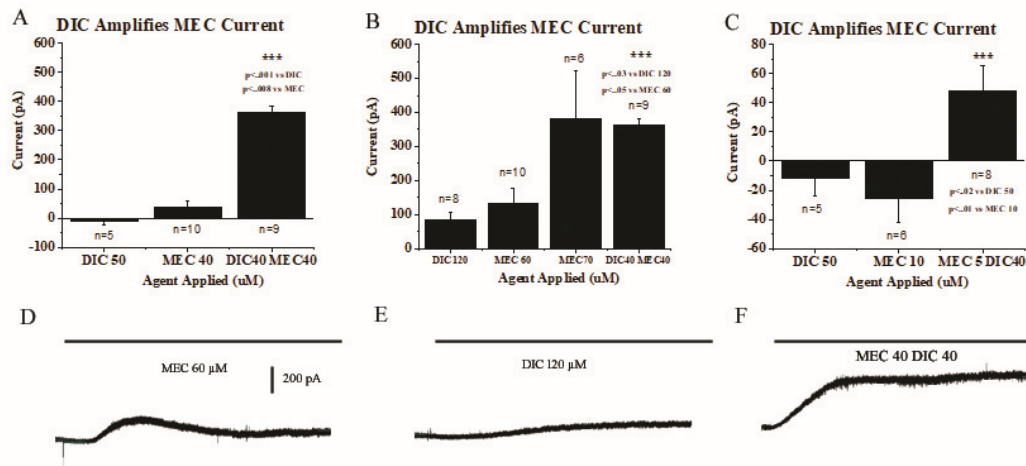


Fig. 4. Combinations of DIC and MEC produced currents exceeding those of DIC or MEC alone. **A)** Currents measured at the end of the 2 min combined application greatly exceed those evoked by individual applications of sub- and near- threshold doses (DIC 40 μ M, MEC 40 μ M, respectively). **B)** Currents measured at the end of the 2 min combined application exceeded those evoked by applications of DIC 120 μ M or MEC 60 μ M. **C)** Application of MEC 5 μ M and DIC 40 μ M exceeded those evoked by subthreshold applications of DIC 50 μ M or MEC 10 μ M. We did not conduct tests of DIC at 40 μ M or MEC 5 μ M specifically, but these were well below thresholds for current activation. **D, E and F)** Representative traces of MEC 60 μ M, DIC 120 μ M or DIC40 μ M /MEC40 μ M. Note that for MEC60 μ M, the peak current was substantially higher. This was not uncommon for low dose applications. Data were collected from voltage clamp experiments conducted in a K⁺ isolation solution. Statistical comparisons were made between DIC/MEC, DIC and MEC tests on different nociceptive cells. For statistical tests, the currents were normalized for cell size (pA/pF).

We recently reported that DIC had a negligible influence on voltage dependent responding at doses ranging from 50 to 120 μ M (Flunker et al., 2022). However, when DIC was combined with MEC (DIC 40/MEC 40) there was a substantial potentiation of conductance at voltage steps relative to MEC 40 μ M alone (figure 5A). No potentiation was found at DIC 40/MEC 5, DIC 40/MEC 20, or DIC 30/MEC 40. Voltage dependent increases in conductance, when combined with potentiated resting current should substantially increase analgesic potential of these DIC/MEC combinations.

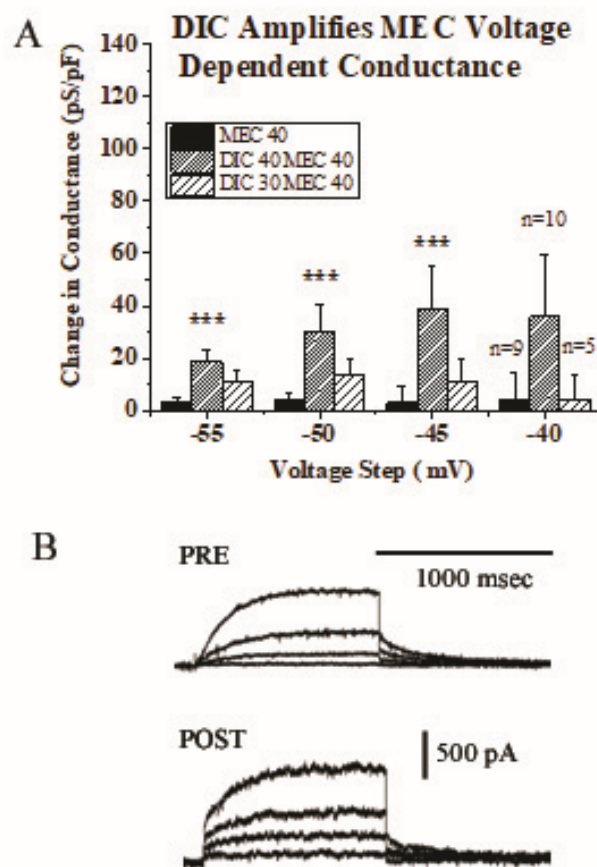


Fig. 5. K_v7 conductance enhanced by a DIC/MEC combination. A) DIC 40/MEC 40 showed significant voltage dependent effects while DIC30/MEC 40 did not (voltage steps as in figure 1). Other combinations with MEC40 also failed. **B)** Representative voltage traces prior to and following DIC40/MEC40 (same cell; leak corrected traces). Data were collected from voltage clamp experiments conducted in a K⁺ isolation solution. Statistical comparisons were made between DIC/MEC and MEC tests on different nociceptive cells.

3.4 Current Clamp Studies with Meclofenamate and Retigabine

In a recent publication, we reported that RET/DIC combinations amplified K⁺ currents and hyperpolarized nociceptor membranes. It was further demonstrated that a persistent hyperpolarization could suppress nociceptor discharge to a muscarinic stimulus (MDBD; Cooper et al., 2016; Cooper et al., 2018). Using methods identical to those described above, we isolated deep tissue nociceptors from other neurons plated in acute culture conditions. Following the current signature characterization procedures (see Methods), the cell was brought into current clamp mode. After a 1 minute observation/settling period, we adjusted the RMP to ~-65 mV (maximum injected current of <2 pA). Once established, current injections were never readjusted.

One minute following current injection, the cell was exposed to either RET or MEC alone or a RET/MEC combination. The influence of the KVO(s) on membrane potential was evaluated at the end of the 2 minute exposure. Immediately afterward, OXO (10 µM), containing the test KVO(s), was superfused for a maximum of 1 minute. If AP discharge occurred, the APs evoked over a 30 second window were counted offline. AP discharges rarely exceeded 30 sec. Some studies were conducted using KVO vehicle alone (water).

In these tests, we began with MEC concentrations that were most effective in voltage clamp combination experiments. However, it became clear that concentrations of MEC, that were ineffective, or minimally effective in voltage clamp, but very effective in combination with RET, could be fatal in physiological solutions. At 40 µM, all cells tested became irreversibly *depolarized* in excess of 35 mV and therefore not further testable. At 30 µM, 4 cells also suffered irreversible depolarization while 3 others remained viable with lesser depolarizations of 3-10 mV. The remaining 3 cells hyperpolarized (-4 to -8.4 mV). The net shift in RMP was an average depolarization of 12.8 ± 5.3 mV. Nociceptors that survived the 30 µM MEC exposure underwent additional testing with OXO. APs were significantly

depressed in these OXO tested cells relative to vehicle exposed controls (84.7 ± 22.3 vs 14.2 ± 9.7 APs; $p < .03$; $n=9$ and 5 , respectively; data not shown).

As MEC doses were further decreased (15 , 5 and $1 \mu\text{M}$), progressively fewer neurons manifested irreversible depolarization; and a net hyperpolarization was observed (figure 6). Over the range of 1 to $15 \mu\text{M}$, 15 cells hyperpolarized or depolarized less than 2 mV , 4 others depolarized more than 2 mV , but only one died from exposure to MEC (figure 6A). During OXO testing, APs were strongly and significantly suppressed, in surviving cells, at all three concentrations (0 , 2.5 and 12.8 APs evoked; 1 , 5 and $15 \mu\text{M}$, respectively; not shown). At a dose of $0.5 \mu\text{M}$, MEC application resulted in a weak depolarization ($n=4$) or hyperpolarization ($n=7$) resulting in a net depolarization of ($1.58 \pm 1.06 \text{ mV}$; figure 7A). There was no influence MEC $0.5 \mu\text{M}$ on OXO evoked APs (figure 7B). Accordingly, we chose $0.5 \mu\text{M}$ MEC to test potential synergisms with RET.

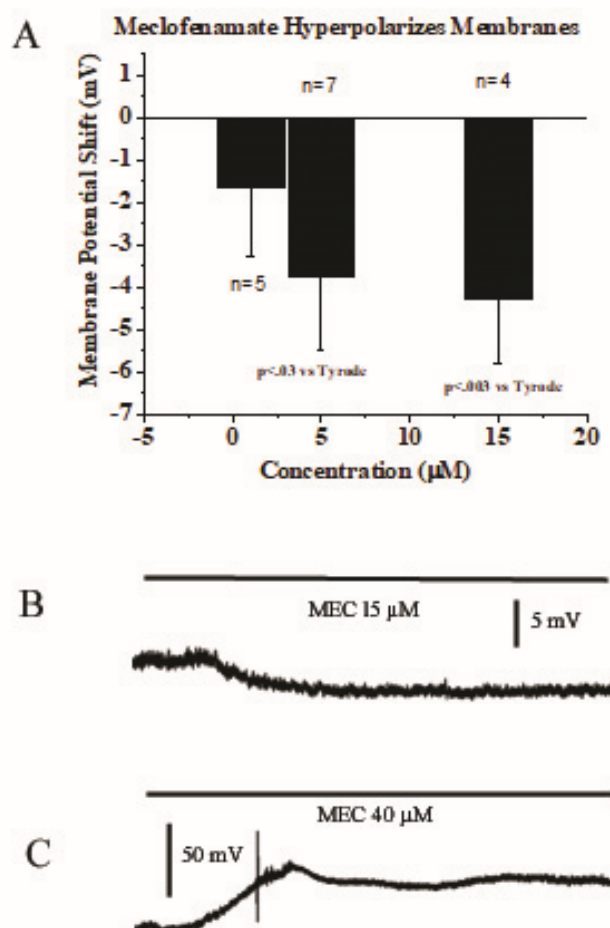


Fig. 6. Meclofenamate hyperpolarizes nociceptor membranes. **A)** Nociceptors exhibited hyperpolarization to MEC 1 to 15 μ M. Cells manifesting strong depolarization were excluded in this figure ($n=3$; 1 cell excluded from each dose). **B)** A representative trace of strong hyperpolarization to MEC 15 μ M. **C)** A representative case of catastrophic, irreversible, depolarization to MEC 40 μ M. Note the AP evoked during depolarization.

The combination of RET (5 μ M) with MEC (0.5 μ M) did not improve hyperpolarization over that which could be produced from RET alone (figure 7A), but significantly surpassed that of vehicle and MEC 0.5 μ M. Neither RET 5 μ M, nor .05 μ M MEC alone were able to significantly reduce AP discharges during OXO testing, but strong trends were observed (figure 7B). The RET MEC combination of 5 μ M and 0.5 μ M significantly reduced OXO dependent nociceptor APs relative to vehicle or individual KVOs (figure 7B).

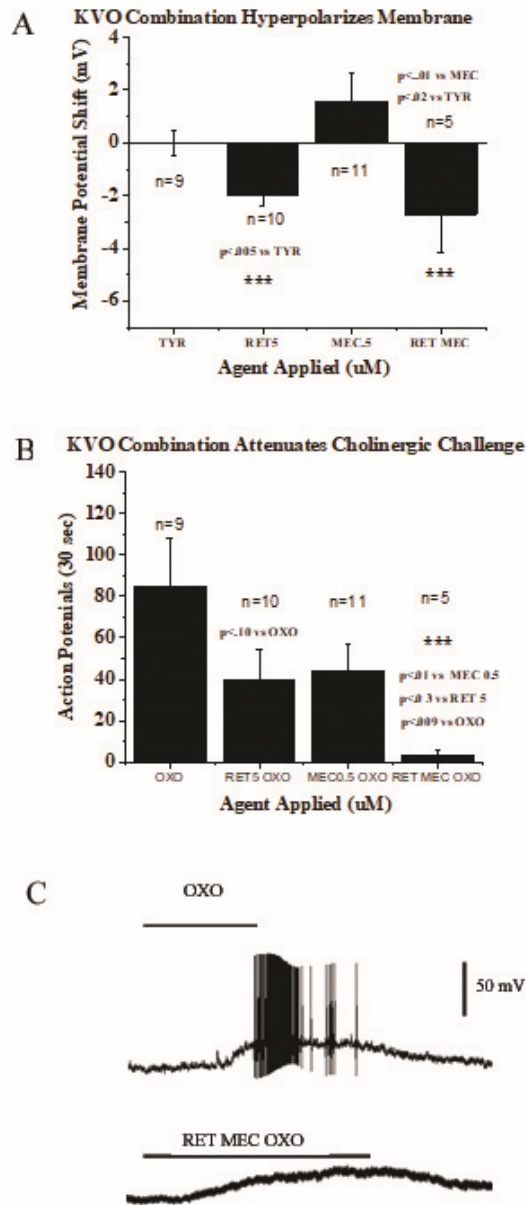


Fig. 7. Combinations of RET and MEC hyperpolarized membranes and suppressed AP discharges. **A)** RET 5 μ M and MEC 0.50 μ M shifted the membrane potential to more negative levels. Shifts were larger for the KVO combination relative to MEC 0.5 alone. **B)** Action potential generation was significantly suppressed by the same RET/MEC combination. APs produced by OXO in the presence of RET or MEC trended lower but failed to reach significance ($p < .10$ and $p < .14$ for RET 5 μ M and MEC 0.5 μ M, respectively). **C)** Representative traces from a test of OXO 10 μ M (upper) and RET5/MEC0.5/OXO 10 μ M (lower). Both traces were shortened to preserve detail.

3.4.1 Current Clamp Studies with Meclofenamate and Diclofenac

Voltage clamp experiments revealed distinct outcome patterns when MEC was combined with RET relative to combinations with DIC. In particular, larger resting currents could be produced with MEC/DIC. Both of these outcomes indicated that MEC/DIC would perform well in current clamp studies designed to evaluate the capacity to suppress muscarinic dependent AP discharges of deep tissue nociceptors.

Nociceptive neurons were isolated as previously described and all testing procedures were identical to those above. Combinations of MEC/DIC were adjusted to respect the powerful depolarizing actions of MEC at concentrations exceeding 5 μ M, as well as to strive for the lowest effective concentrations. In the above studies, 0.5 μ M produced negligible depolarizations, and failed to suppress nociceptor discharges. Of the two combinations with DIC that were tested, the best results were obtained with MEC 0.5 and DIC 2.5 μ M.

Within 2 min after application, nociceptor membranes were significantly hyperpolarized by MEC/DIC ($-2.02 \pm .58$ mV; figure 8A). While modest, these were similar to hyperpolarizations achieved by RET/MEC combinations described above; and consistent with hyperpolarizations previously associated with AP suppression by RET 5 μ M /DIC 2.5 μ M (-2.6 mV; Flunker et al., 2022). When challenged with the OXO burst discharge test, MEC0.5/DIC2.5 was highly effective. Evoked APs were strongly suppressed (7.66 ± 5.4), and significantly exceeded the effectiveness of either MEC 0.5 or DIC 2.5 μ M alone (figure 14B). Statistical contrasts between APs evoked during MEC 0.5/DIC 2.5/OXO with RET 5/MEC 0.5/OXO were not significant. Given the lower clinical side-effect potential for RET over DIC, behavioral testing focused first on RET/MEC combinations. Dosing was anchored by previous behavioral tests with RET (Flunker et al., 2017) and molar ratios that were proven effective in current clamp studies.

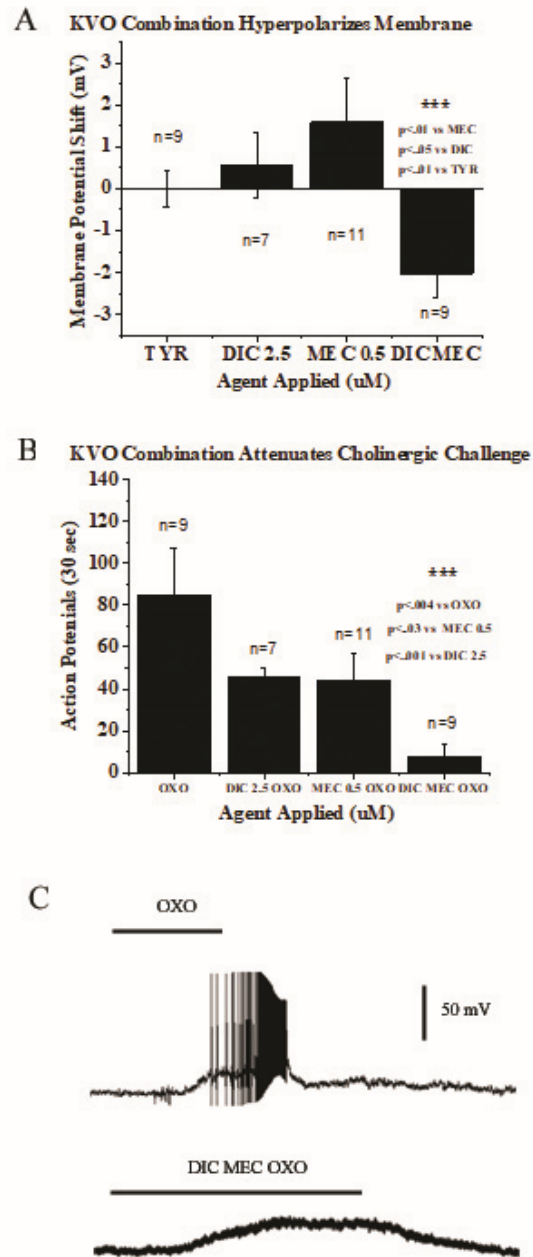


Fig. 8. Combinations of DIC and MEC hyperpolarized membranes and suppressed AP discharges. **A)** MEC 0.5 and DIC 2.5 μ M shifted the membrane potential to more negative levels. Shifts were larger for the KVO combination relative to MEC 0.5 or DIC 2.5 μ M alone. **B)** Action potential generation was significantly suppressed by the same DIC/MEC combination. APs produced by OXO in the presence of DIC or MEC trended lower but failed to reach significance ($p<.12$ and $p<.14$ for DIC 2.5 μ M and MEC 0.5 μ M, respectively). **C)** Representative traces from a test of OXO 10 μ M (upper) and DIC2.5/MEC0.5/OXO 10 μ M (lower). Both traces were shortened to preserve detail.

3.5 Behavior Testing with Combinations of Retigabine and Meclofenamate

Based upon the above findings, we initiated studies to examine whether a combination treatment regimen could be used to reverse behavioral signs of pain in an animal model of GWI pain. We hypothesized that, if KVOs were used in combination, doses targeting nociceptor Kv7 could be substantially reduced and/or the analgesic performance of the KVOs could be boosted beyond that achievable by a single KVO.

Following a 4 week exposure to GW chemicals (Permethrin, DEET, PB and Chlorpyrifos or vehicle; see Methods), rats (n=32) developed pain-like signs manifested as decreased Movement Distance, average Movement Rate and increased Resting Duration (figure 9). Eight additional rats served as unexposed controls (GRP CON). Twelve weeks following neurotoxicant exposure, the 32 exposed rats were randomly divided in 4 groups (GRP EXP, GRP RETMEC, GRP RET, GRP MEC). Treatments began in Week 17. Each group of 8 rats received a KVO treatment or water vehicle (oral gavage). Two different dose combinations were administered in weeks 17 and 18 and one was repeated in week 19 (post-exposure weeks 13, 14 and 15). Both treatments were based upon the 10:1 ratio of RET to MEC that was shown to be highly effective in current clamp studies (figure 7; the actual mg/kg doses varied slightly due to small differences in the molecular weight of RET and MEC). Since we had previously shown successful, though transient, reversal of pain-like signs at a RET dose of 7 mg/kg (Cooper et al., 2018), we used this dose as an anchor point around which to design the KVO combination treatments (Week 17, Treatment 1: RET 7 mg/kg, MEC 0.7 mg/kg; Week 18, Treatment 2 and Week 19, Treatment 3: RET 5 mg/kg, MEC 0.5 mg/kg. With these combinations, we hoped to improve the analgesic performance of RET, as demonstrated in Cooper et al., (2018), and/or sustain its effectiveness through repeated treatments. Groups of 8 rats received single and combination treatments of KVOs as indicated by GRP names, above.

In order to confirm the presence of pain-like signs prior to Treatment testing, we subjected free range activity scores to a repeated measures ANOVA on GRP CON and GRP EXP (n=8) across the time

period of weeks 13-16 (post-exposure weeks 9-12). Significant main effects of neurotoxicant exposure were confirmed for activity measures of Movement Distance and Rate ($F=4.3$, $p<.04$ and $F= 13.64$, $p<.001$, respectively); indicating, that pain-signs were present and persistent prior to treatment testing (figure 9A and B). Analysis of the Rest Duration times failed to reveal a significant effect for weeks 13-16 (data not shown). Accordingly, Rest Durations were not analyzed for subsequent Treatment effects.

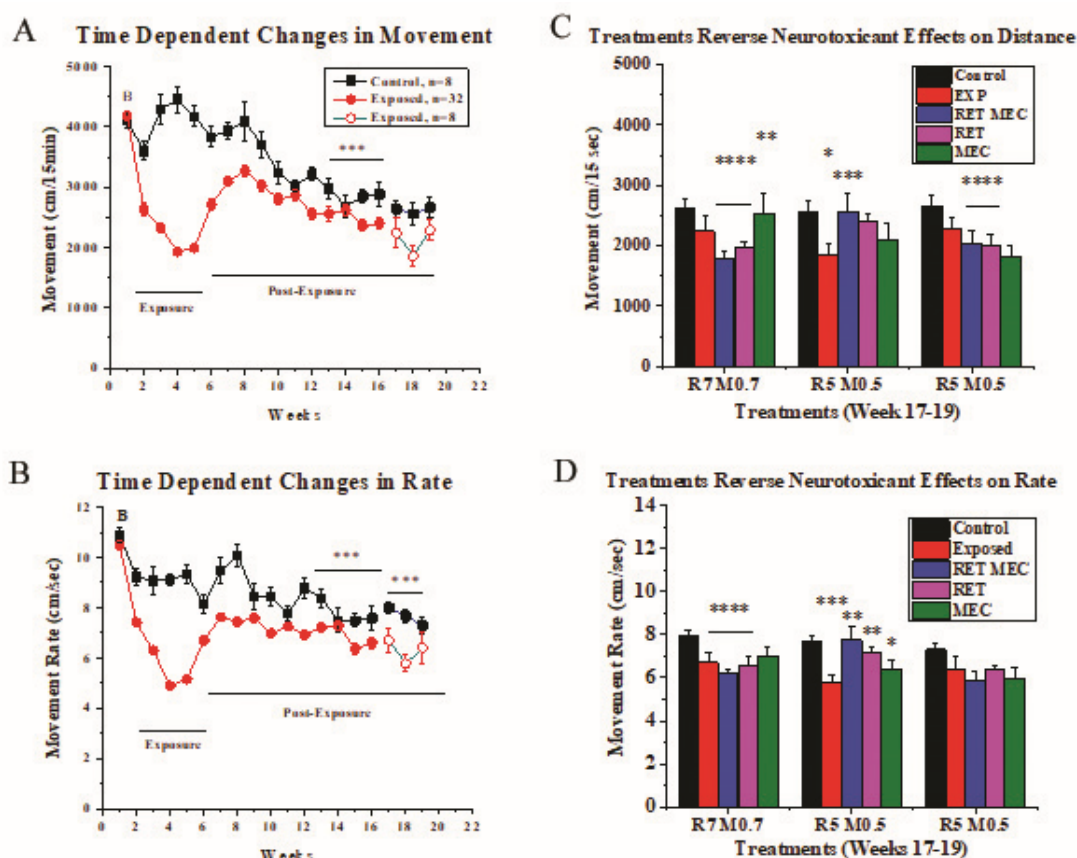


Fig. 9. The combination of RET and MEC reduced pain-like behaviors. A) and B) Weekly progression of Movement Distance and Rate data before, during and following exposure to GW chemicals. Repeated measures ANOVA verified significant pain-like changes for rats in the last 4 weeks prior to treatment testing (***). C) The influence of Treatment 1, 2 and 3 on Movement Distance scores. In Treatment 2 (Week 18), RETMEC treated rats significantly improved over water treated GRP EXP. D) The influence of Treatment 1, 2 and 3 on Movement Rate scores. In Treatment 2 (week 18) RETMEC treated rats significantly improved over water treated GRP EXP and MEC treated rats but did not differ from GRP RET. GRP RET also improved relative to GRP EXP but not relative to GRP MEC. B: baseline testing. **** significantly different from GRP C. *** significantly different from GRP EXP. ** significantly different from GRP RETMEC; * significantly different from GRP CON. $P<.05$, Fisher LSD.

KVO treatment studies (Treatment 1: 7 mg/kg RET, 0.7 mg/kg MEC) were initiated in Week 17. ANOVA conducted on the 5 GRPS of Treatment 1 (CON, EXP, RETMEC, RET, MEC) revealed a significant main effect for Movement Rate, with Movement Distance barely failing the Main effect test ($F=3.02$, $p<.03$ and $F=2.5$, $p<.06$, respectively). Follow-up tests (Fisher LSD) revealed a significant effect of exposure (GRP CON vs GRP EXP), for Movement Rate, but not Movement Distance (figure 9C and D; Week 17). In any case, Treatment GRP RECMEC did not improve on Movement Rate or Distance relative to GRP EXP. Given that Treatment 1 tended to decrease Movement Distance and Rate relative to GRP EXP, we lowered the base dose of RET to 5 mg/kg (0.5 mg/kg MEC) for Treatment 2 studies.

In Treatment 2, groups of 8 rats were administered, 5 mg/kg RET and/or 0.5 mg/kg MEC singly and in combination. GRPs EXP and CON received only water. All treatments were administered by oral gavage, and behavior testing was conducted 2 hrs after gavage. As before, we conducted a one-way ANOVA on GRPs CON, EXP, RETMEC, RET, and MEC. Significant main effects of Treatments were achieved for Movement Rate ($F=4.47$, $p<.005$) and approached significance for Movement Distance ($F=2.02$, $p<.11$). We chose to examine GRP differences in both analyses, given our *a priori* plan to examine individual group pairings.

Post-hoc testing (Fisher LSD) indicated partial support for the hypothesis that combination treatments would produce improved analgesic outcomes in our GWI model. In the Movement Rate analysis, we confirmed a significant pain effect manifested as decreased Rate for GRP EXP relative to GRP CON (figure 9D, Week 18). Importantly, those rats treated with RET/MEC demonstrated improved Movement Rate performance relative to GRP EXP. That is, the average Movement Rate increased to levels that were significantly better than GRP EXP, and improved to the point that they were not significantly lower than the non-exposed GRP CON (figure 9D, Week 18). GRP RETMEC was also significantly improved relative to GRP MEC (7.7 ± 0.67 vs 6.39 ± 0.39 cm/sec; figure 9D). However, GRP RET also exhibited some analgesic response. Specifically, GRP RET manifested improved

performance relative to GRP EXP. Although improvements trended better for GRP RETMEC relative to GRP RET (7.7 ± 0.67 vs 7.13 ± 0.27 cm/sec) a difference could not be supported statistically.

A priori follow-up testing of the Movement Distance data lent further support to a potential RET/MEC synergism. Tests on paired GRPs identified a significant effect of pain (GRP CON vs GRP EXP), and a significant improvement of GRP RETMEC relative to GRP EXP (figure 9C, Week 18). That is, Movement Distance scores significantly increased for RET/MEC treated rats compared to GRP EXP, and did not differ from those rats of GRP CON, that had not been exposed to toxicants. None of the other Treatment contrasts (i.e., GRP MEC or GRP RET) were significantly different from, water treated, toxicant exposed, GRP EXP.

In Treatment 3, we attempted to replicate/clarify the RET 5/MEC 0.5 mg/kg outcome. Treatments were carried out in an identical manner. Unfortunately, we were unable to confirm a significant pain effect in week 19 (GRP EXP vs GRP CON; figure 9C and D); as a result, the treatment data was difficult to interpret. Additional testing in Week 20 also failed to confirm that a significant pain effect was still present. Behavioral tests ceased at this point, and we were unable to conduct further Treatment tests that would be meaningful.

4. Discussion

In 2017 our laboratory turned from investigations of the pathophysiology of GWI pain to the development of GWI treatment strategies. Our approach was based upon molecular findings that identified enhanced muscarinic AP bursting through a mechanism that included both TRPA1 activation and suppression of K_v7 resting activity (MDBD; muscarine receptor dependent burst discharge; Nutter et al., 2015; Cooper et al., 2016; Cooper et al., 2018; see also Pant et al., 2021). We chose to focus on the K_v7 component due to the availability of tools to manipulate K_v7 activation. As several of these tools, at that time, were FDA approved, they offered the attractive possibility of rapid repurposing of these

compounds as analgesics for GWI pain in veterans (Retigabine, Flupertine, Diclofenac, Meclofenamate, Celecoxib). Given evidence that these different families of K_v7 activators exhibited differential binding patterns on K_v7 forming subunits (K_v7.2, K_v7.3; Peretz et al., 2005; see Introduction), the potential existed for cooperative activation of K_v7. Such activation could produce enhance voltage and/or ligand activated resting currents that could suppress nociceptor action potentials with an analgesic effect. Moreover, due to reduction of dosing requirements for K_v7 activity, deleterious side effects, that were otherwise associated with human use of these compounds, might be avoided (Singh et al., 1994; Fries, 1996). The studies of Peretz et al., laid groundwork for examination of KVO synergisms (Retigabine, Meclofenamate; Peretz et al., 2005). As these studies were carried out in CHO cells, we began a series of studies to characterize KVO influences on, native, nociceptor K_v7 in well characterized populations of deep tissue nociceptors. We had shown that maladaptations in these nociceptor subpopulations were associated with molecular maladaptations consequent to GWI chemical exposure (Nutter et al., 2015; Cooper et al., 2016; Cooper et al., 2018).

The present investigations are a continuation of studies published earlier this year (Flunker et al., 2022). In those experiments, we characterized, for the first time, the dose-response activation of nociceptor K_v7 by Retigabine and Diclofenac. This characterization permitted a detailed evaluation of the potential of combined, cooperative activation and its impact on nociceptor discharge. The underlying hypotheses and methods of these experiments are identical to those presented here. In short, we reported that: 1) DIC could amplify RET evoked current, but had no identifiable impact on voltage dependent conductance; 2) combinations of RET and DIC could hyperpolarize membranes and suppress AP bursts to a cholinergic challenge at relatively low doses; and 3) RET/DIC combinations could reduce some manifestations of pain-like behaviors in our GWI model, but repeated use of these doses were accompanied by serious side effects, including death.

In the present report, we confirmed and extended our previous findings. Nociceptor characterization of single and combined KVO activation of Kv7 revealed: 1) MEC could amplify RET evoked current, but did not improve voltage dependent conductance; 2) Low dose combinations of RET and MEC hyperpolarized membranes and suppressed AP bursts to the cholinergic challenge test; 3) RET/MEC combinations reduced manifestations of pain-like behaviors in a rat model of GWI, however, the pattern of changes only partially supported a critical role of combination treatment; 4) DIC could greatly amplify MEC evoked current, and also improve voltage dependent conductance at voltages near AP activation threshold; and 5) Low dose combinations of MEC and DIC failed to hyperpolarize membranes but strongly suppressed AP bursts on the cholinergic challenge test.

4.1 Kv7 Activation Synergisms and Suppression of Nociceptor Discharge

In a companion report, we characterized the reactivity of nociceptor Kv7 to an alternative KVO combination (RET and DIC; Flunker et al., 2022). Contrasting that data set with the current one reveals some surprising findings. Based upon the binding preferences of DIC and MEC (Peretz et al., 2005; Corbin-Leftwich et al., 2016; Zhang et al 2019; Borgini et al., 2021), we expected to observe similar potencies and efficacies from these two, structurally similar, KVOs. In contrast, we observed starkly different outcome patterns. Namely, MEC interacted with nociceptor Kv7 with far greater potency ($ED_{50}=62.2$ vs $93.2 \mu M$), efficacy (6.3 pA/pF vs 1.0 pA/pF); and voltage dependent influence than DIC (Flunker et al., 2022). We expected combinations of MEC and DIC to reflect competition for the same binding site, possibly reducing the influence of the much more active compound (MEC), but the effect was more consistent with synergism due to binding at multiple independent sites. Possibly the binding of DIC promoted a conformational change that improved MEC binding, altered pore characteristics or shifted open state probability when bound to complementary subunits of the Kv7 complex. In any case, before this interaction can be understood, more detail is needed about the specific binding site(s) for these two

KVOs (Peretz et al., 2010; Qi et al., 2011; Corbin-Leftwich et al., 2016; Zhang et al 2019; Borgini et al., 2021).

Optimized combinations of RET/MEC or MEC/DIC were highly effective against nociceptor burst discharge on the cholinergic challenge test. The search for optimization was designed to minimize KVO concentrations while maximizing suppression of AP responding. This was reasonably successful, but despite the greater potency of MEC relative to DIC, the extent of RET/MEC or MEC/DIC AP suppression did not exceed that of RET/DIC in our companion study (Flunker et al., 2022). At the optimized combination, RET/MEC pretreatment (RET 5 μ M; MEC 0.5 μ M) reduced AP generation on the OXO test to 3 APs relative to control OXO tests that produced 85 APs. This mirrored our observations with RET/DIC, where mean AP discharges were reduced from 76 to 10 APs (RET 5 μ M; DIC 2.5 μ M), and did not differ from that of MEC/DIC (MEC 0.5 μ M; DIC 2.5 μ M; 7APs). In all three combinations, membranes were hyperpolarized only about 2 mV. Regardless of evidence from voltage clamp studies that clearly separated combination influences on resting currents, we could not distinguish any RET, MEC or DIC combination statistically on the OXO test nor the net hyperpolarization that preceded it. This may have resulted from limitations on MEC concentrations used due to the strong depolarizing currents that we encountered at concentrations above 5 μ M.

MEC and DIC belong to a large family of fenamate NSAIDS. The fenamates interact with several ion channels, expressed in DRG nociceptors, which might impact on excitability independently of any actions on KVOs (TRPA1, Na_v , K_{Ca}). All fenamates, that have been tested, are activators of TRPA1 (diclofenac, flufenamic, niflumic, and mefenamic acid). TRPA1 is an important pro-inflammatory ion channel, which is sensitive to both endogenous and exogenous stimuli, resulting in strong depolarization and pain sensations (Julius, 2013; Meents et al., 2019; Talavera et al., 2020; Manolachi et al., 2021).

The reported fenamate TRPA1 $\text{EC}_{50\text{s}}$ vary widely depending on host cells but range from 100-500 μ M for rat TRPA1 expressed in *Xenopus* Oocytes (activation thresholds as low as 10 μ M) to as little

as 25-60 μM for human TRPA1 expressed in HEK293 cells. For DIC, an $\text{EC}_{50} > 200 \mu\text{M}$ was reported (Hu et al., 2010). We never observed any catastrophic depolarization during extensive testing with DIC at concentrations as high as 50 μM and 70 μM (Flunker et al., 2022). MEC has not been independently tested against TRPA1, but shares strong structural similarity to the fenamate activators of TRPA1 (Hill and Zawia, 2021). It is possible that the catastrophic depolarizations we observed during current clamp tests with MEC were due to activation of TRPA1. Both Type 5 and Type 8 nociceptors express TRPA1 (Cooper et al., 2016; Cooper et al., 2018).

DIC and related fenamates (mefenamic acid, flufenamic acid, and tolfenamic acid) also inhibit voltage gated Na^+ channels (Na_v), TTXs and TTXr (tetrodotoxin sensitive and resistant), that are expressed in rat DRG nociceptors (Lee et al., 2003, Acosta et al. 2007; Gwanyanya et al., 2012; Nakamura and Jang, 2016). TTXr $\text{Na}_v1.8$ is the primary AP forming channel in most nociceptors, including Type 5 and Type 8 nociceptors (Rush et al., 2007; Nutter and Cooper, 2014; Cooper et al., 2018). Although inhibition of TTXr Na_v could suppress AP discharge, the influence of DIC on TTXr requires concentrations approaching 1000 μM (Nakamura and Jang, 2016; see also Sun et al., 2019). This far exceeds the 2.5 μM DIC or 0.5 μM MEC used in our current clamp, AP suppression, experiments.

DIC and other fenamates exert a much greater influence on TTXs Na_v ($\text{IC}_{50\text{s}}$ ranging from 16-80 μM ; Sun et al., 2019; see also Lee et al., 2003). A major antagonism of TTXs Na_v would certainly be fatal systemically, but a weak antagonism could suppress nociceptor discharge in whole cell recordings. MEC and DIC have not been directly (MEC) or fully (DIC) tested against TTXs Na_v , (DIC 100 μM ; Lee et al., 2003). Due to the effects on closely related compounds, it is possible that the combination of DIC and MEC exerted some AP suppression through a synergistic action on TTXs Na_v . With concentrations of 2.5 μM (DIC) and 0.5 μM (MEC), and no independent evidence for fenamate synergisms on TTXs Na_v , there is little support for this interpretation of our data (see also Gribkoff et al., 1996).

Finally, although DIC activates most members of the K_v7 family, it *inhibits* currents mediated through K_v7.5 (Brueggemann and Byron, 2009; Brueggemann et al., 2011). As this channel is associated with the K_v7 complex in DRG (Yu et al., 2018), it could explain, in part, the low efficacy and potency of DIC relative to MEC.

4.2 KVO Combinations and Behavior

The capacity of RET/MEC and MEC/DIC combinations to suppress nociceptor AP discharge predicted that administration of these KVOs, as treatments, could reduce pain responding on our model of GWI chronic pain. Accordingly, rats were tested 13 weeks after exposure to the GWI toxicants. We used molar ratios, which were established in current clamp studies, to form dosing 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 translate dose ratio combination treatments from *in vitro* to *in vivo*. Slight corrections were made due to small differences in molecular weights.

Although the initial combination of RET/MEC (Treatment 1: 7 mg/kg and 0.7 mg/kg) failed to alter pain-like behaviors, the subsequent dosing plan (Treatment 2: 5 mg/kg RET and 0.5 mg/kg MEC) produced encouraging results. The pattern of these results were less than ideal, leaving some doubt as to whether the combination treatment actually represented a synergistic effect. Two hours following Treatment 2 gavage (Week 18), exposed rats recovered Movement Distance deficits to normal levels. Control groups for RET or MEC showed no improvement, but GRP RETMEC treatments did not significantly exceed the influence of RET or MEC alone either. Perhaps this was due to excessive variability and/or insufficient power. Similarly, Movement Rate deficits were also rescued by this RET/MEC combination in week 18. In this instance, improved Rate scores significantly exceeded those of GRP MEC alone. This was a strong indication of synergism. However, significant effects also emerged for GRP RET alone, making it unclear whether we were actually revealing a transitory RET influence on

activity measures, as we had previously reported in 2018 (Cooper et al., 2018). Although GRP RETMEC scores nominally exceeded those of GRP RET (7.75 vs 7.12 cm/sec), these two groups could not be separated statistically. The failure to replicate, or otherwise clarify, this finding in the third treatment week (Week 19) was inconclusive as we were no longer able to demonstrate any exposure-pain effect on Movement Distance or Movement Rate. We were unable to pursue Treatment testing further as statistical evidence of pain-like signs continued to be absent in Week 20. It should be recognized that Movement Distance and Rate are related measures coincident in time and subject. Looking at these two measures as a complementary reflection of activity deficits, the only significant treatment outcome that appeared in both the Rate and Distance analyses, in Week 18, was the normalization of activity in GRP RETMEC.

The pattern of outcomes in Treatment 2 implicated a real influence of the 10:1 ratio treatment plan on established pain-like behaviors of GWI. To some extent the main study hypothesis was confirmed. Namely, KVO combination treatments have applications for treatment of chronic pain of GWI. Doses can be reduced to low levels by using drugs with convergent actions on the same receptor to amplify receptor response beyond which a single drug can be used safely. Other chronic pain applications could also benefit from this KVO strategy. Continued pursuit of this approach is recommended, but perhaps with alternative synthetic KVOs, lacking unwanted side effects on TRPA1 and Nav, but designed to bind at the MEC or DIC sites that seemed critical to activation synergisms. A behavioral test system that is less demanding would also help to clarify outcomes.

Clearly, preparing dozens of rats for 16 weeks in order to reach a testing window is not ideal circumstances for testing dosing regimens. In addition to time/efficiency considerations, behavioral measures of pain always come with a high level of variability. Ideal outcome patterns will be challenging when adjunctive therapies are investigated as multiple groups are required for adequate control measures. The problems are more complicated when deep tissue pain is the issue. Deep tissue pain is especially difficult to detect in animal subjects, and even more so when it is a relatively moderate pain. GWI pain

presents as a multisymptom complex with patients differentially reporting pain in multiple tissues and combinations (i.e. joint pain, muscle pain, headache pain, visceral pain; see Introduction). These pains are sourced from distinct nociceptor families expressing a wide variety of mechanical, thermal and chemical detection mechanisms that can be specific to the tissue being innervated (Rau et al, 2007; Rau et al., 2012; Rau et al., 2014; Cooper et al., 2014). In our various studies on nociceptors and tissue innervation, only the Type 8 nociceptor group was shown to innervate multiple tissues (skin, muscle, viscera). This nociceptor/tissue diversity can be a critical factor for the development of analgesics, but the research community often chooses to overlook this diversity in the pursuit of ‘the silver bullet’ analgesic. The relationship between tissue type, nociceptor innervation and nociceptor capacities is a vastly understudied area which our laboratory pursued, but most other laboratories have not; favoring instead the silver bullet approach. Hopefully that will change moving forward.

In our physiology studies, we used nociceptive types we have shown to innervate muscle and other deep tissues (Type 5 and Type 8; Rau et al., 2007; Cooper et al., 2014; Rau et al., 2012; Rau et al., 2014). We further selected those nociceptors with cholinergic sensing mechanisms, in order to discern how their cholinergic sensing may have been altered by exposure to GWI toxicants that impact cholinergics (PB, Chlorpyrifos). The present studies sought to identify means to correct or counteract these maladaptations. Although we reported patterns of maladaptation in these Type 5 and Type 8 nociceptors (Nutter et al., 2015; Cooper et al., 2016; Cooper et al., 2018), they might not be the most important nociceptors for GWI pain and almost certainly not the full nociceptor population contributing to GWI pain symptoms.

The adjunctive treatment method we have explored in this and the related report faces other unique challenges (Flunker et al., 2022). Obviously, experimenters can administer agents in a 10:1 ratio that was derived from demonstrations, *in vitro*, that were highly effective in that setting. Achieving these dose ratios, *in vivo*, to nociceptor endings is a different matter. At this point, we have no idea how absorption, and catabolism of co-administered RET and MEC affected blood and tissue distribution patterns consistent

with our intent. An additional set of experiments would be required to move accurately from a cell in a dish to a behaving animal. A distinct approach, to this issue, would require multiple dose approximations to reach a behavioral outcome. Our historical experience with this animal model of GWI indicated we had several viable weeks of testing remaining in which to adjust doses. In this particular rendition, we found that our testing window was more narrow than we had anticipated, and we could not pursue multiple dosing or test other combinations that appeared promising for RET/MEC or MEC/DIC. Despite these limitations, we can assert that therapy strategies designed to promote synergistic activation of Kv7 at multiple binding sites is a promising approach.

5. Summary and conclusions

Combination therapies with KVOs can greatly improve activation of Kv7 ion channels in nociceptors, greatly reduce concentrations required for maintained activation, and improve voltage dependent conductances. All of these are consistent with improved analgesic potency. Whether MEC and DIC are the optimal adjunctive agonists for RET is questionable, as unwanted side-effects on TRPA1 and TTXs Nav, are problematic. This was brought home starkly by the observation of rat fatalities that occurred with RET/DIC combinations in our previous report (Flunker et al., 2022). While we never observed anything close to that with RET/MEC, we did not pursue daily dosing regimens, in this study, as we had in the Flunker et al., where 7-10 consecutive days of dosing with RET/DIC proved to be fatal (Flunker et al., 2022). Clearly, daily testing is needed to validate the efficacy and safety of a given combination. The development of KVO synthetics that are designed to bind to diverse Kv7 binding sites could fulfill the promise of improved analgesics for GWI or other chronic pain conditions where Kv7 expressing nociceptors may play a role in the symptoms of chronic pain (Bock and Link, 2017; Miceli et al., 2018; Jones et al., 2021; Liu et al., 2021).

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