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14. ABSTRACT

Our laboratory developed an animal model that replicates some of the symptoms of the chronic pain of GWI. Through this animal model we identified molecular targets in pain sensing neurons, which appear to have been chronically altered following exposure to insecticides and repellants that were present in the GW theater. K_v7 openers (KVOs) have known analgesic/palliative capacities that could serve as treatments for the chronic pain associated with GWI. We have previously shown that Retigabine (a KVO) can temporarily reverse signs of GWI pain. In an effort to improve upon these findings, we tested the capacity of a combination KVO treatment strategy to reverse pain-signs in our GWI pain model. Experiments completed in year 1 confirm that certain combinations of KVOs can significantly improve outcomes on molecular targets relative to the performance of individual KVOs. Experiments have further shown that these KVO combinations can greatly retard action potential discharges that occur following an acetylcholine challenge. In year 2 we will move toward behavioral testing in our rat model.

15. SUBJECT TERMS
pain, nociceptor, K_v7, Retigabine, pesticides, Gulf War Illness, treatment

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Introduction

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

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

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

It has been shown that certain FDA approved NSAID drugs also have the capacity to open K_v7 channels (Diclofenac; Meclofenamate; Celecoxib; Peretz et al., 2005; Peretz et al., 2007; Bruggermann et al., 2010; Frolov and Singh, 2011; Du et al., 2011). There is evidence that combinations of Retigabine with the NSAID KVOs can be more effective at opening K_v7 ion channels than either drug singly (Peretz et al., 2005; Khattab et al., 2017, 2018). If dose combinations can be shown to be more effective activators of K_v7 in deep tissue nociceptors, this could substantially improve the capacity of KVOs to be effective analgesics for the widespread deep tissue pain associated with GWI.

In the experiments funded for budget year 1, we examined the capacity of combinations of NSAID KVOs (Diclofenac, Meclofenamate and Celecoxib) and a classic K_v7 opener (Retigabine) to modulate the excitability of deep tissue nociceptors associated with the development of GWI pain.

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

3. Accomplishments

What were the major goals of the project?

Objectives: Year 1

1) **Determine the influence of Meclofenamate on voltage dependent opening of K_v7.**
2) **Determine the influence of Meclofenamate on dose dependent opening of K_v7.**
3) **Determine the influence of Diclofenac on voltage dependent opening of K_v7.**
4) **Determine the influence of Diclofenac on dose dependent opening of K_v7.**
5) **Determine the influence of Celecoxib on voltage dependent opening of K_v7.**
6) **Determine the influence of Celecoxib on dose dependent opening of K_v7.**
7) **Determine the influence of Retigabine on voltage dependent opening of K_v7.**
8) **Determine the influence of Retigabine on dose dependent opening of K_v7.**

What was accomplished under these goals?

A summary of accomplishments for year 1 (see below for details):

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

Although 4 months of work were lost due to the shutdown of our laboratory, we were able to finish Specific Aim 2 and begin studies of Specific Aim 1 and 3. The main hypothesis of the project was confirmed. It is possible to improve the performance of KVOs by use of a combination strategy (Specific Aim 2).

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 four KVOs we studied differentially modified voltage dependent and dose dependent opening of K_v7; they also differed with respect to maximal current potency. Two of the NSAID inhibitor KVOs (Diclofenac and Meclofenamate) appeared to be good candidates for combination therapies with Retigabine (RET). In those combination experiments performed in year 1, we demonstrated that sub-threshold doses of RET (5 uM) greatly enhanced the effect of subthreshold doses of DIC (50 uM). K_v7 activation, by this combination, evoked currents that exceeded those produced by 40 uM RET or 80 uM DIC alone. Similar results were obtained with combinations of RET (5 uM) with MEC (30 uM).

In preliminary studies of Specific Aim 3, we found that combinations of RET and DIC (5 and 50 uM) could substantially reduce or block deep tissue nociceptor action potential activity following a challenge with an acetylcholine agonist (Oxotremorine-M; OXO). We have recently begun behavioral studies to determine whether combinations of RET and DIC can reverse an established

pain-like behavior in our animal model of GWI chronic pain (Specific Aim 1). Detailed results are presented below.

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

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

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

This aim was designed to apply discoveries from Specific Aims 2 and 3 to identify KVO dose combinations as a treatment for rats that have developed pain-signs following exposure to GW pesticides. Based up our findings 60 below, rats have just undergone GW chemical exposures (permethrin, DEET, PB and chlorpyrifos or vehicle). Treatment with a RET and DIC combination will begin in January 2021, after the delayed onset pain behaviors have been established.

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

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

Background: There are three important ways in which a KVO can modify reactivity of a nociceptor that would result in reduction or elimination of pain symptoms: 1) depolarized shift in voltage dependent activation; 2) reduce dose dependent (direct) activation; and 3) increase in the

maximal conductance. K_v7 is a voltage activated channel whose activation opposes formation of action potentials. The amount of opposing current that flows is proportional to the applied voltage. That is, the more channels that are opened the more opposition current is realized and the less likely nociceptor action potentials are generated and pain is experienced. On the other hand, with direct (dose dependent) activation by a KVO, all the channels can be opened at once providing an optimal effect on nociceptor discharge by keeping nociceptor membrane strongly hyperpolarized. If maximal conductance is increased, a supramaximal effect on pain should result. We will determine whether combination KVO treatments can achieve one or more of these outcomes.

Subtask 1: Meclofenamate Dose Response

Timeline: Months 1-4

Using the protocol described in 'Methods' (Appendix, p. 42) we assessed the influence of the cyclooxygenase inhibitor, Meclofenamate (MEC), on deep tissue nociceptor K_v7. Briefly, voltage shifts from -55 to -40 mV were applied in the presence and absence of a KVO. The KVO influence on voltage activated current was assessed in deep tissue nociceptors implicated in GWI pain. Direct, dose dependent, activation of K_v7 was also assessed. Previous dose response studies, by Peretz and colleagues, were confined to special, non-neural, test systems (Chinese hamster ovary cells (CHO)). They were able to demonstrate both voltage sensitive and direct opening by meclofenamate (Peretz et al., 2005).

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 1A, B, C, D). Current measures were converted to conductance (pS; PicoSiemens) in order to control for 'driving force'. Conductance was normalized (divided) by a measure of cell volume (pF; picofarads) in order to account for differences in the size of individual nociceptors. Comparisons were made to vehicle exposed cases.

Shifts in Voltage Sensitivity. Doses of 20 to 100 uM were applied in separate studies. When the dose of MEC reached 70 uM, we observed a significant increase of K_v7 conductance at all test voltages (figure 1). As MEC doses increased through 100 uM, conductance increases were observed at nearly all levels that were tested (-40, -45, -50 and -55 mV).

Direct Activation. When MEC was applied in the absence of any voltage shifts (i.e., at the holding potential of -60 mV), it proved to be a very effective direct activator of K_v7. Currents developed rapidly in the presence of MEC. At doses as low as 40 uM, K_v7 current flowed and progressively increased with increasing doses. A maximum outward current was reached at 80 uM (~6.3 pA/pF; figure 2). Currents, evoked directly by MEC, thereafter declined (100 uM). The ED_{50} (effective dose for 50% activation) for MEC on deep currents nociceptors was determined to be 60.7 uM.

In summary, MEC strongly opened K_v7 channels in a manner consistent with a powerful effect on membrane excitability. Direct activation began at 30 uM, while voltage dependent shifts in conductance were amplified across much higher doses (70-100 uM). The appearance of voltage sensitivity shifts, and particularly the broad dose range over which they occurred, is important as

they occur in the voltage range at which action potentials are activated. We will examine the influence of MEC on membrane excitability in Specific Aim 3.

These studies were generally completed in the time frame of the SOW; minor additions may be needed to fill gaps for publication.

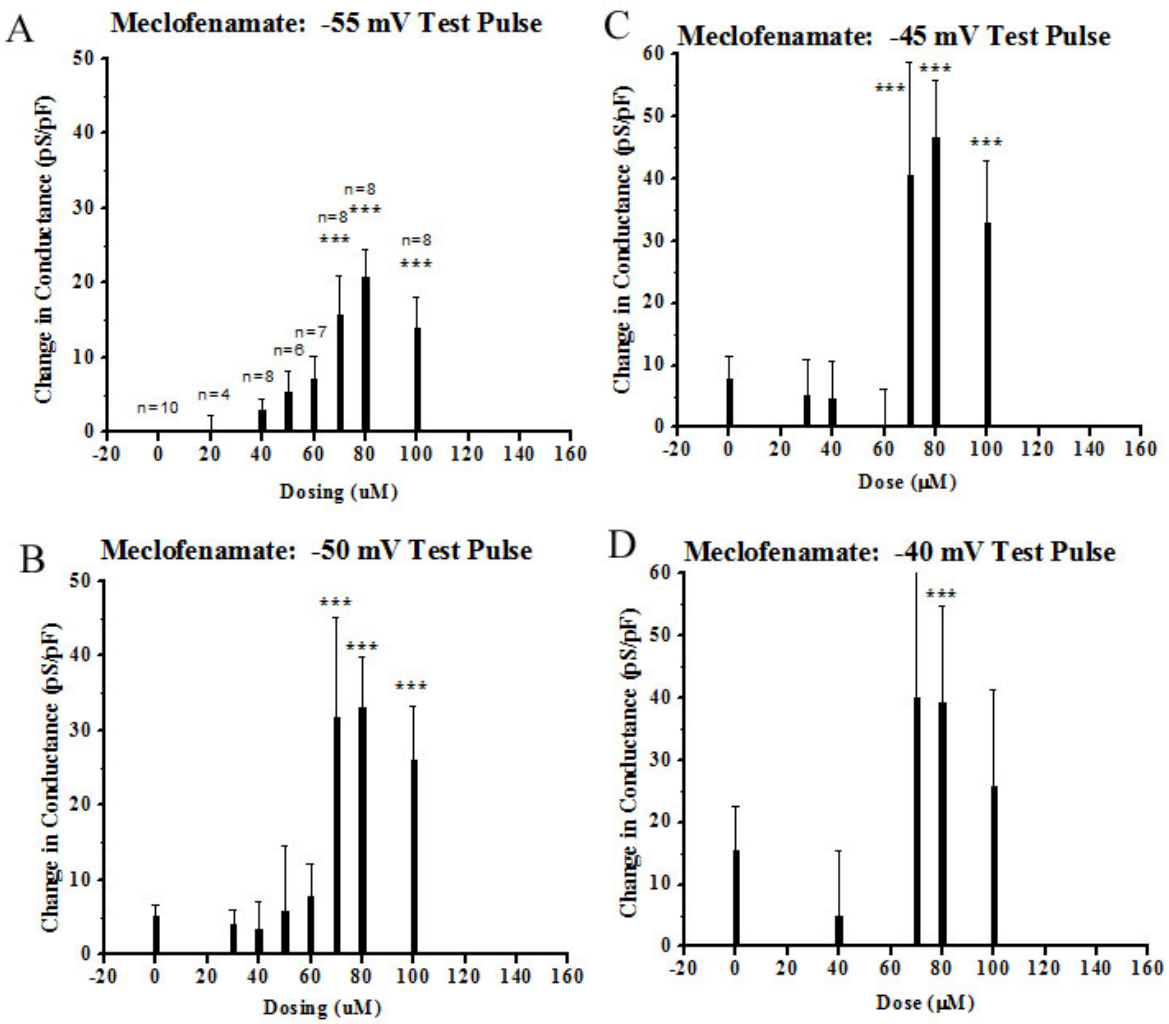

Figure 1. The Influence of Meclofenamate on K_v7 Voltage Sensitivity. MEC reliably increased the voltage sensitivity of K_v7 following a 2 minute application in a manner that generally paralleled direct activation (see figure 2). **A)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-MEC). Conductance was derived from currents evoked by a voltage step to -55 mV (V_h=-60 mV). **B)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-MEC). Conductance was derived from currents evoked by a voltage step to -50 mV (V_h=-60 mV). **C)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-MEC). Conductance was derived from currents evoked by a voltage step to -45 mV (V_h=-60 mV). **D)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-MEC). Conductance was derived from currents evoked by a voltage step to -40 mV (V_h=-60 mV). Data collected from voltage clamp experiments in a K^+ isolation solution. The number of cells contributing to each bar is indicated by 'n' appearing in panel 'A'. Statistical comparisons were made between vehicle-vehicle and vehicle-MEC tests. Vehicle: Water; *** p<.05 or greater

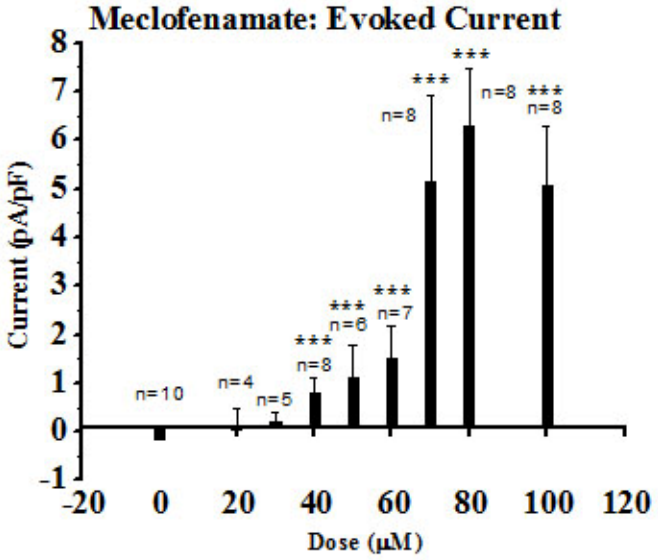

Figure 2. The Effect of Meclofenamate on Resting Currents Mediated by K_v7. MEC opened K_v7 channels without a shift in applied voltage. Relatively large outward (hyperpolarizing) currents were observed. **A)** Average normalized current produced at the holding potential (V_h=-60 mV) following a 2 minute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

Subtask 2: Diclofenac Dose Response

Timeline: Months 1-4

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

Shifts in Voltage Sensitivity. We tested whether DIC could alter voltage sensitivity. Doses of 50 to 140 uM were applied in distinct experiments. There was a strong influence of DIC on voltage sensitivity at a threshold dosage of 60 uM, thereafter little influence on voltage dependent opening was detected (figure 3A, 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 uM), little direct activation of the channel was apparent. Above this dose, DIC proved to be a reliable activator of K_v7. At 80 uM, significant K^+ currents developed rapidly and grew steadily through 120 uM (figure 4). At 120 uM K_v7 current appeared to be maximal (~1 pA/pF). Dose-current data fit to a Hill function

indicated an ED_{50} of 96.1 uM. This was considerably higher than the ED_{50} for MEC. It is also noteworthy that peak currents produced by DIC and MEC differed substantially (1.0 vs 6.3 pA/pF, respectively; $p<.001$). It is clear that MEC was much more potent as an opener of K_v7 than DIC.

In summary, DIC opened K_v7 channels over a range of 80-120 uM. Unlike MEC, DIC exerted narrow effects on voltage sensitivity. Direct activation of K_v7 by DIC was also far less potent than MEC as it produced less than 16% of the maximal current evoked by MEC; moreover, the threshold dose for opening channels was substantially higher for DIC. The ability of MEC treated cells to retain voltage sensitivity across a substantial range of doses suggests greater potential for MEC, relative to DIC, as an analgesic of deep tissue nociceptors. Nevertheless, as we demonstrate below, DIC is still a good candidate for combination studies of Major Task 2.

These studies were generally completed in the time frame of the SOW; minor additions may be needed to fill in gaps that may be needed for publication.

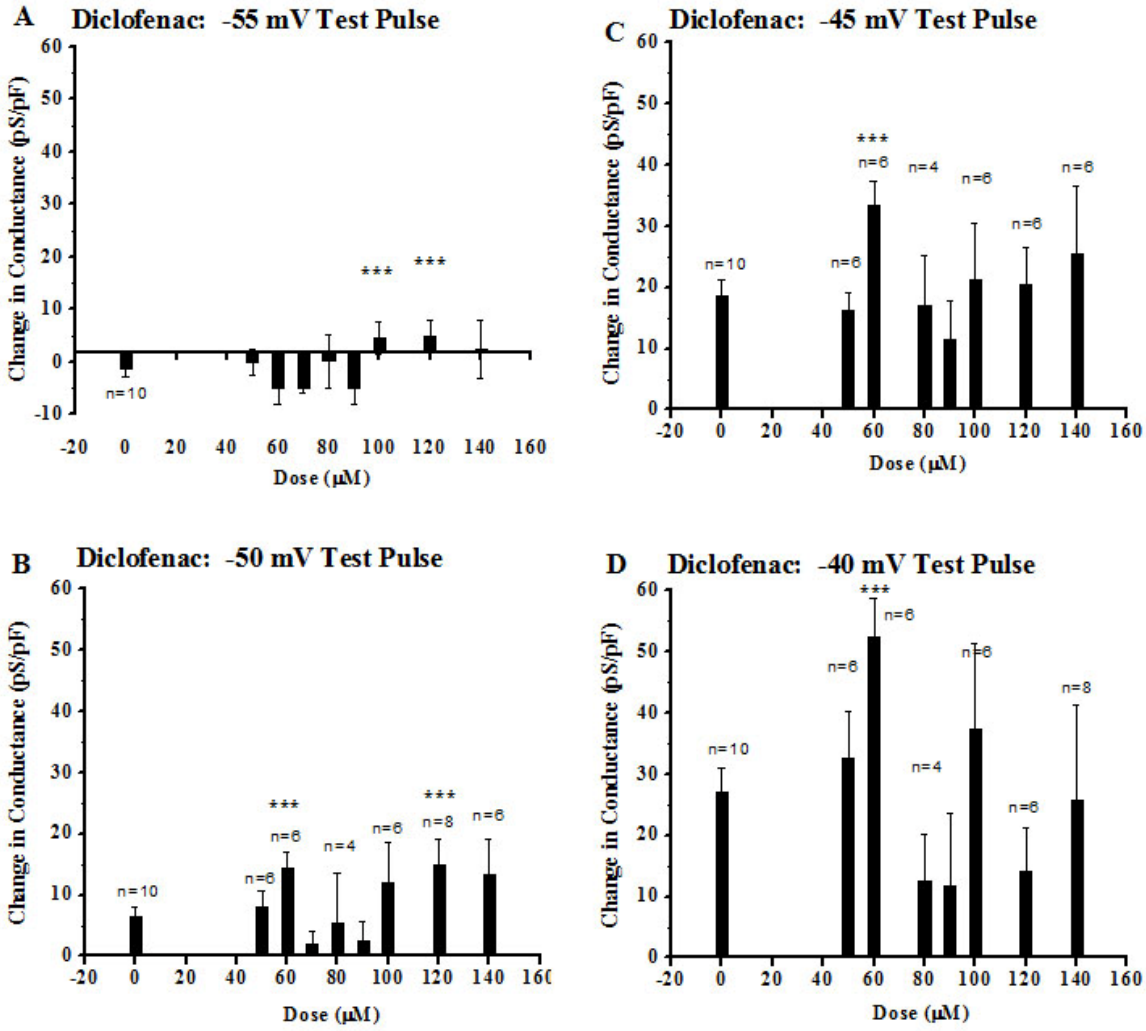

Figure 3. The Influence of Diclofenac on K_v7 Voltage Sensitivity. DIC had a narrow effect on the voltage sensitivity of K_v7. **A)** The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -55 mV (V_h=-60 mV). **B)**. The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -50 mV (V_h=-60 mV). **C)**. The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -45 mV (V_h=-60 mV). **D)**. The change in normalized conductance of deep tissue nociceptors following consecutive exposures to test agents (vehicle-vehicle or vehicle-DIC). Conductance was derived from currents evoked by a voltage step to -40 mV (V_h=-60 mV). Data collected from voltage clamp experiments in a K^+ isolation solution. Statistical comparisons were made between vehicle-vehicle and vehicle-MEC tests. Vehicle: DMSO; *** p<.05 or greater

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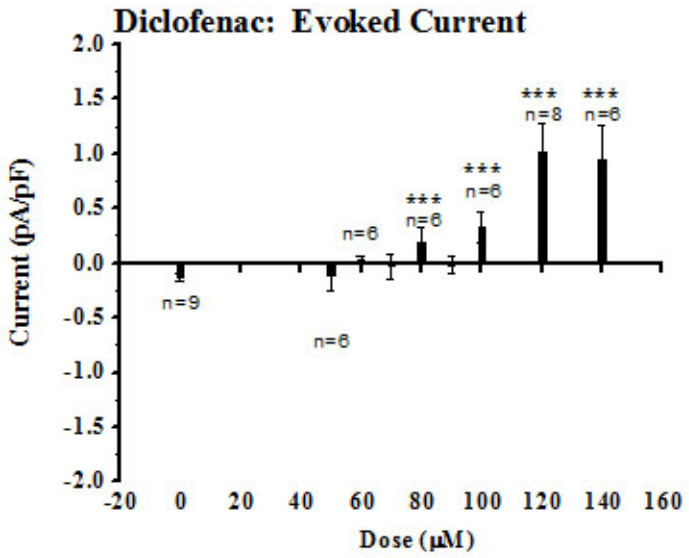

Figure 4. The Effect of Diclofenac on Resting Currents Mediated by K_v7. DIC opened K_v7 channels without a shift in applied voltage. Relatively small outward (hyperpolarizing) currents were observed. **A)** Average normalized current produced at -60 mV (V_h=-60 mV) following a 2 minute application of DIC. Evoked currents increased in proportion to the dose applied, but reached a maximum at 120 uM. Current was measured at the end of the 2 min application of DIC. Data collected from voltage clamp experiments conducted in a K^+ isolation solution. Statistical comparisons were made between vehicle and DIC tests. Vehicle: DMSO; *** p<.05 or greater.

Subtask 3: Celecoxib Dose Response

Timeline: Months 5-9

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

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

Subtask 4: Retigabine Dose Response

Timeline: Months 5-9

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

Shifts in Voltage Sensitivity. RET amplified the voltage sensitivity of K_v7 at doses as low at 5 uM (figure 5). No directly activated current could be demonstrated at this dose. Voltage effects were never observed above the threshold of 5 uM, as direct channel opening became the dominant effect of RET. This pattern was similar to that observed for DIC.

Direct Activation. As doses increased from 20 to 100 uM, evoked currents steadily increased reaching a peak at 80 pA (1.24 pA/pF; figure 6). An ED_{50} of 62 uM was determined. Dose dependent maximal maintained currents evoked by RET were similar in amplitude from those evoked by DIC. As with DIC, the maximal currents were significantly less that those evoked by MEC ($p<.001$).

In summary, the classic KVO, Retigabine was an effective opener of K_v7 channels in deep tissue nociceptors. Like DIC, voltage sensitivity to RET occurred over a narrow range, and was absent when channels were directly opened by the agent. Although RET exhibited a generally lower ED_{50} than the NASID/KVOs its evoked current maximum was considerably less that that observed with MEC. Accordingly, like DIC, the potential of RET to hyperpolarize nociceptors and thereby function as an analgesic was limited relative to MEC. On the other hand, the effective hyperpolarization could be sufficient, especially in combination with an NSAID KVO. This possibility will be examined is Specific Aim 3.

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

Subtask 1: Retigabine and Meclofenamate

Time Line: Months 10-11

Subtask 2: Retigabine and Diclofenac

Time Line: Months 10-11

Subtask 3: Retigabine and Celecoxib
Time Line: Months 10-11

Major Task 2

In sensory neurons, $K_v7.2$ combines with $K_v7.3$ to mediate the M-current (Jiang et al., 2013; Du et al., 2017). The M-current derives its name from the muscarinic acetylcholine receptor whose activation results in the closing of K_v7 channels, via a cytoplasmic pathway, and increased neuron excitability. KVOs are agents that open K_v7 channels and reduce excitability via direct binding to $K_v7.2$ or $K_v7.3$. KVOs with differential affinities for $K_v7.2$ or $K_v7.3$ could produce greater net hyperpolarizing M-current when acting in combination because they differ in binding sites and subunit affinity (Tatulian et al., 2001; Peretz et al., 2005; Huang et al., 2013). In fact, such demonstrations were made (RET and MEC) in non-neuronal host cells (CHO; Chinese hamster ovary; Peretz et al., 2005). In this project, we examined whether combination KVOs would be effective in mammalian nociceptor neurons that we have shown to be influenced by exposure to GW neurotoxicants (Cooper et al., 2018). While these studies are incomplete, we will show, below, that combinations of RET with DIC can amplify currents through K_v7 ion channels. This finding suggest that combination therapies could be effective analgesics in GW veterans. The lowered doses made possible with combination therapies should 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

Time Line: Months 10-11

Sub-threshold doses of RET and MEC were tested using procedures identical to those described in Major Task 1. When deep tissue nociceptors were exposed to combinations of sub-threshold doses of RET (5 uM) and MEC (30 uM), substantially larger currents were evoked than those evoked by RET or MEC alone (figure 5). However, with respect to MEC, these comparisons did not reach levels of significance due to substantial variation among the test neurons. In contrast, the RET (5 uM) and MEC (30 uM) combination did produce significantly more current than 40 uM RET alone. The combination of 5 uM RET and 30 uM MEC produce significantly more current than 40 uM RET alone and was roughly equivalent to 80 uM RET (figure 6). There was no evidence of an effect on voltage sensitivity. We will be pursuing other dose combinations in year 2.

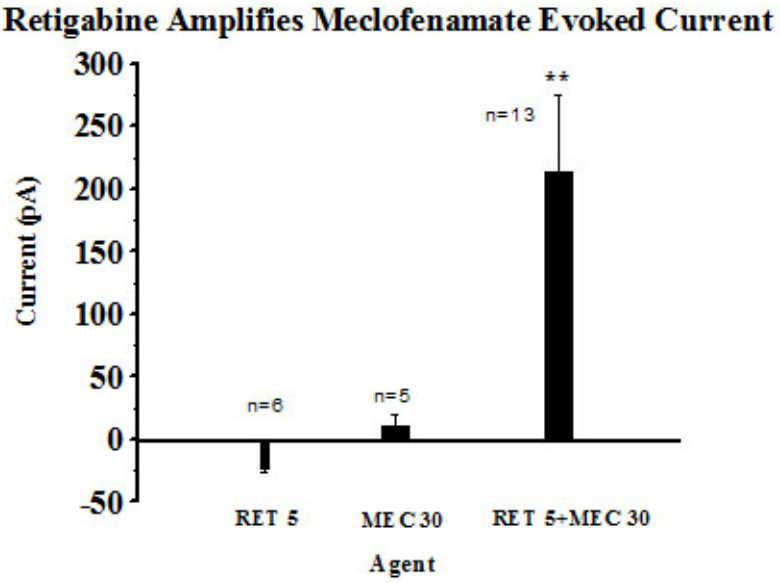

Figure 5. The combination of subthreshold doses of RET and MEC strongly activated K_V7. Currents measured at the end of the 2 min combined application exceeded those evoked by individual applications of REC or MEC. Data were collected from voltage clamp experiments conducted in a K^+ isolation solution. Statistical comparisons were made between RET/MEC, RET and MEC tests on different cells. For statistical tests, the cell currents were normalized for cell size (pA/pF). ** $p<.05$ vs RET.

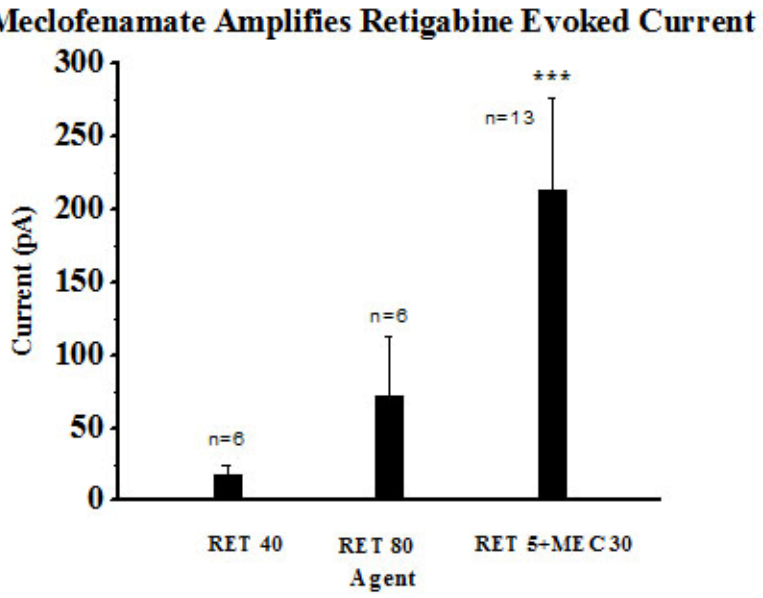

Figure 6. The combination of subthreshold doses of RET and MEC produced currents exceeding RET alone. Currents measured at the end of the 2 min combined application greatly exceeded those evoked by supra-threshold application of RET 40 uM. Roughly equivalent currents were evoked by RET 80 uM. Data collected 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). *** $p<.05$ vs RET 40 uM.

Major Task 2, Subtask 2: Retigabine and Diclofenac

Time Line: Months 12-14

Sub-threshold doses combinations of RET and DIC were tested using procedures identical to those described in Major Task 1. Combining 5 uM RET with 50 uM DIC significantly improved direct activation current amplitudes (figure 7). While the average evoked currents for 5 uM RET or 50 uM DIC applied singly were -22.7 + 3.7 pA and -26.7 + 20.2 respectively, the combination of 5 uM RET+50 uM DIC generated hyperpolarizing currents that averaged 59.5 + 18.1 pA. That is a net change of over 70 pA in the hyperpolarizing direction (figure 7). The combination of 5 uM RET and 50 uM DIC produce significantly more current than 80 uM DIC or 40 uM RET alone and was roughly equivalent to 100 uM DIC or 80 uM RET or (figure 8). However, co-application of 5 uM RET with 50 uM DIC did not significantly improve voltage sensitivity beyond that which could be obtained by single application of DIC or RET.

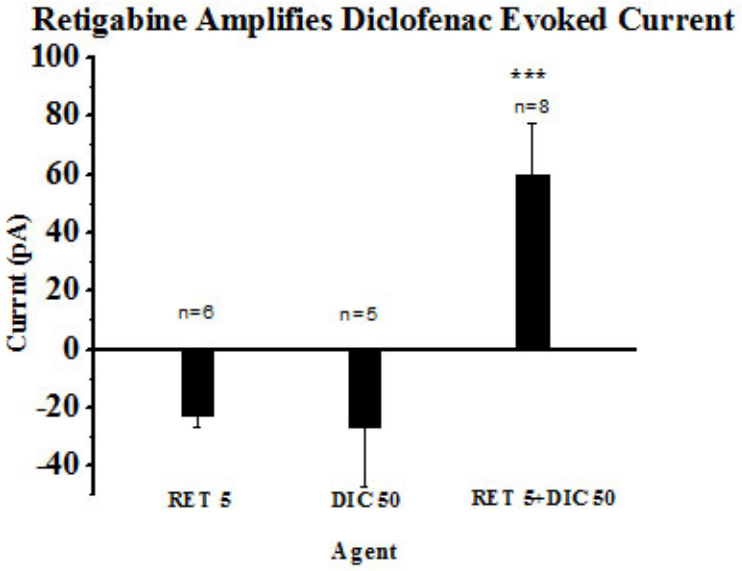

Figure 7. The combination of subthreshold doses of RET and DIC strongly activated K_V7. Currents measured at the end of the 2 min combined application greatly exceed those evoked by individual applications of REC 5 uM or DIC 30 uM. Data collected from voltage clamp experiments conducted in a K^+ isolation 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). *** $p<.05$.

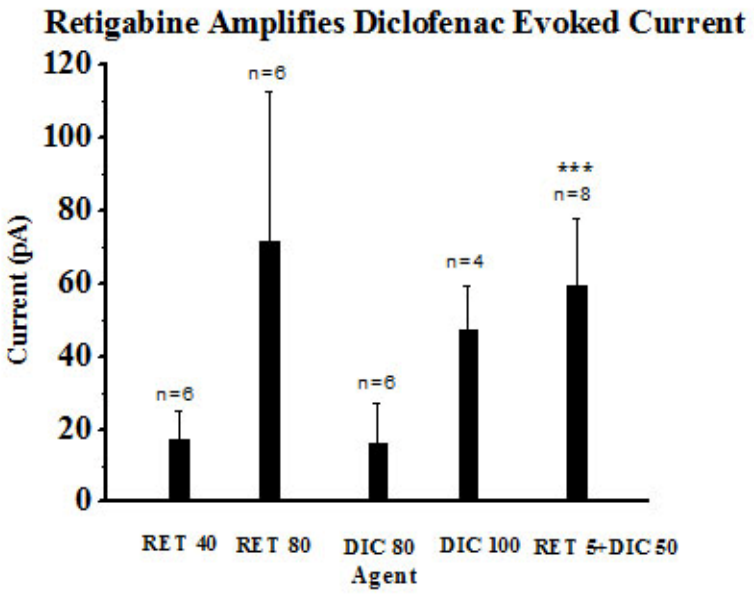

Figure 8. The combination of subthreshold doses of RET and DIC produced currents exceeding RET or DIC alone. Currents measured at the end of the 2 min combined application exceeded those evoked by supra-threshold applications of REC 40 uM or DIC 80 uM. RET 80 uM or DIC 100 uM evoked currents that were similar to those of RET 5 uM and DIC 50 uM combined. Data collected from voltage clamp experiments conducted in a K^+ isolation solution. Statistical comparisons were made between RET/DIC, RET and DIC tests on different cells. For statistical tests, the currents were normalized for cell size (pA/pF). *** $p<.05$ vs RET 40 uM or DIC 80 uM.

Subtask 3: Retigabine and Celecoxib

Time Line: Months 10-11

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

Summary for Major Task 2

Combination doses of RET and MEC or RET and DIC greatly amplified K_v7 currents of deep tissue nociceptors relative to individual applications of each KVO. Opening of these ion channels will produce strong hyperpolarization of nociceptor membranes and thereby prevent the action potential discharge experienced as pain. Importantly, the doses of the combination agents were, by themselves, below threshold for current activation. An adjunctive approach to pain control minimizes the likelihood of any side effects that might occur from long term use of high doses KVOs, but may not fully exploit the hyperpolarizing potential of KVO combinations. Given the far higher peak conductance possible when MEC interacts with K_v7 (6.3 vs 1 pA/pF), we should further explore combinations with MEC that might result in more effective pain control therapies.

Specific Aim 3. Optimizing KVO Functional Synergisms

The GW neurotoxicant Chlorpyrifos can produce long term changes in the function of acetylcholine esterase and the expression of muscarinic receptors (see discussion, Cooper et al., 2016). We have shown, in our rat model, that maladaptations in this muscarinic receptor pathway is related to the pathophysiology of GWI pain (Cooper et al., 2018). Muscarinic dependent burst discharges (MDBD) is a feature somewhat unique to deep tissue nociceptors (Cooper et al., 2016, 2018). Although initiated by acetylcholine binding, MDBD is a complex series of intracellular events that include the activation of TRPA1, the closing of K_v7, activation of $Na_v1.8$, $Na_v1.9$, and other muscarine dependent events. Muscarine dependent depolarization and burst discharges are amplified in rats that have been exposed to GWI chemicals. K_v7 modulates MDBD burst duration in GW agent exposed rats, but does not appear to be central to the pathophysiology (Nutter et al., 2015; Flunker et al., 2017).

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

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

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

Major Task 1: Cellular Synergisms of KVOs

Subtask 1: KVO combination Dose 1

Timeline: Months 12-14

Subtask 2: KVO combination Dose 2

Timeline: Months 12-14

Subtask 3: KVO combination Dose 3

Timeline: Months 12-14

Following cell characterization, cells were brought into current clamp mode. After a 2 minute presentation of vehicle, superfusion with the specific muscarinic agonist, Oxotremorine-M (OXO; 10 uM) depolarized muscle nociceptors resulting in substantial burst discharges in 3/3 cases (127 +/- 51.3; figure 9A). When RET+DIC preceded the OXO challenge test (5 and 50 uM, respectively; 2 min), action potential discharge was reduced to 30.5 +/- 30.4 (n=4), with 3 of 4 cases exhibiting complete block (figure 9B). Although these test results are preliminary, it is clear that combination therapy of RET and DIC has the potential to retard nociceptor discharge and pain.

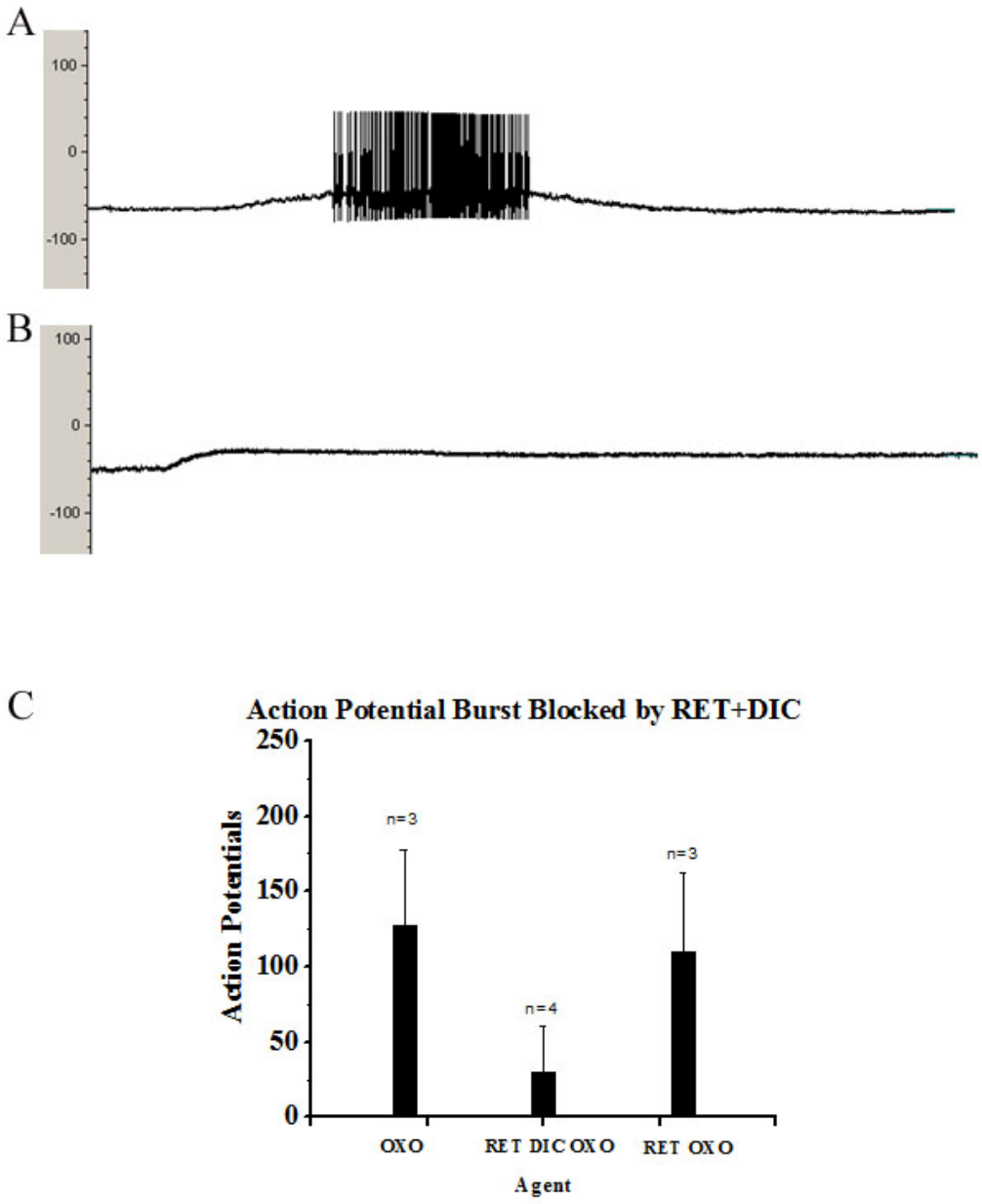

Figure 9. Action potentials evoked following exposure to Oxotremorine-M with and without KVOs. **A)** OXO produced powerful action potential bursts in deep tissue nociceptors. **B)** When RET (5 uM) and DIC (50 uM) was presented prior to the OXO application, action potentials were substantially reduced. **C)** Bar graph summary includes studies where RET alone (5 uM) preceded OXO application. Studies conducted in Tyrode's solution.

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?

With behavioral studies already started, we should have sufficient data by February to prepare a manuscript.

Assuming that Covid restrictions do not interfere, we will be submitting abstracts to the GWIRP and Neuroscience Society meetings in the spring and summer of 2021

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.

If we can demonstrate that this combination approach to treatment can substantially reduce the doses required to impact chronic pain, side effects associated with individual drugs, could be reduced or eliminated. Alternately, if we demonstrate that the potency of the agent on the target (K_v7) can be substantially amplified, the effectiveness of the treatment might be greatly enhanced.

What was the impact on other disciplines?

Control of acute and chronic pains that afflict active duty soldiers, veterans and the general public, is a high priority field of research. Activation of K_V7 is a recognized target for drug development. Hopefully, if KVO combination therapies prove useful in our animal model of GWI, other applications will be found for post-traumatic and disease related pain syndromes.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

Changes in approach and reasons for change

It is likely that CEL will be dropped from further studies due to substantial untoward inhibition of other K^+ channels.

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.

Changes that had a significant impact on expenditures

A substantial number of rats (37) were euthanized due to the enforced shutdown. This put some pressure on the expense portion of the budget. However, staff layoffs compensated for these budget losses in the short term. As we move forward, I anticipate the shutdown will result in a no-cost extension in the fall of 2021 to complete the proposed Tasks of the SOW.

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

Nothing to report

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. Products

Publications, conference papers, and presentations

Journal publications

Nothing to report

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.

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Personnel:
Name: Brian Y. Cooper, Ph.D., College of Dentistry
Project role: Principal Investigator,
Researcher Identifier (ORCID ID): 0000-0002-7592-588X

Nearest person month worked: 12

Contribution to Project:

Design, execution and analysis of physiology and behavior experiments
Preparation of Reports

Thomas J. Nutter, Ph.D., College of Dentistry
Project Role: Biological Scientist IV
Researcher Identifier: none
Person Months: 8
Contribution to Project:
Execution of physiology experiments

Linda Flunker, MS, College of Dentistry
Role on Project: Biological Scientist III
Research Identifier: none
Person Months: 8
Contribution to Project:
Preparation of solutions; Preparation of rats; ordering laboratory items; General laboratory duties

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

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. Special Reporting Requirements

Award Chart (see Appendix, p. 52)

9. Appendices

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

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

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

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

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

Potential Reduction Optimized			

Methods

Subjects. One hundred three (103) young adult male rats were used in physiology experiments. All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO (Animal Care and Use Review Office of the Army Medical Research and Materiel Command).

Preparation of Cells. Male Sprague-Dawley rats were anesthetized (Isoflurane) and euthanized by decapitation. The dorsal root ganglia were excised, digested in a solution containing, 2 mg/ml, type-A collagenase and 5 mg/ml Dispase II (Sigma-Aldrich). The procedure for digestion and plating of cells has been described, in detail, previously (Flunker et al., 2018). Isolated neurons were plated on 9, 35 mm Petri dishes. Cells were bathed continuously in Tyrode's solution, containing (in mM) 140 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. Electrodes were formed from boroscilate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm. Experiments began 2 hours after plating. Only one cell was used per Petri dish, and recording experiment 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., 2012; 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 Sigma-Aldrich (Meclofenamate; Diclofenac; Celecoxib) and Alomone Labs (Retigabine Cl). Agents were prepared in stock solutions (50-200 mM; in DMSO or H_2O as needed). Stock solutions were kept at -20 °C. On the day of the experiment, solutions of 5-140 uM were prepared in a K^+ isolation solution for application to isolated deep tissue nociceptors (see below). Each tested cell received only one dose of KVO or vehicle. Typically, a given KVO and its vehicle were both tested on individual DRG neurons during a single day experiment.

After achieving the whole-cell mode, cells were characterized and classified at type 5 or type 8 nociceptors. Cells not fitting these criteria were rejected. Series resistance was compensated and a K^+ isolation (K_{iso}) solution containing (in mM) 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 10 glucose, 10 HEPES, (adjusted to pH 7.4 with KOH) was applied, for 2 minutes, by close superfusion (1000 um).

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

KVO applications were made by close superfusion via a glass tube positioned approximately 1 mm from the cell. During superfusion, a series of brief 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 voltage sensitive K^+ currents that were present, we superfused the cell with the K_v7 specific inhibitor, linopirdine (20 uM; 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 were determined by subtraction.

The peak difference currents obtained were converted to conductances (G1 and G2) according to the formula: $I_L/(V_{step}-V_{rev})$. Where I_L is the measured peak of the linopirdine sensitive current; V_{step} is the applied test voltage and V_{rev} is the calculated reversal potential for K^+ (-86 mV). The influence of the KVO on voltage sensitivity was determined as the difference in conductance (G) measured in the pre-KVO (G_1) and post-KVO test (G_2) conductances: $G_{diff}=G_2- G_1$. For statistical analysis, the G_{diff} was normalized to cell size, where cell size was determined by capacitive charging (pF).

Identical procedures were carried out using only vehicle applications for both VS1 and VS2 protocols (water (meclofenamate, retigabine), DMSO (diclofenac, celecoxib)). Statistical tests

were made between the G_{diff} determined for cells tested in the presence of vehicle only contrasted with the G_{diff} determined in the presence of a KVO.

Current Clamp Studies

Membrane Excitability. Following cell characterization and classification, cells were brought into current clamp mode. Cells whose resting membrane potential did not fall between -55 and -65 mV were rejected. In current clamp mode, we assessed excitability using two distinct protocols. Type 8 cells were tested using current injection, while type 5 cells were tested by challenge with a muscarinic agonist (see Cooper et al., 2018). In our previous studies we had determined that a very high percentage of type 5 cells manifested burst discharges to muscarinic agonists (MDBD; muscarine dependent burst discharge). In contrast, only a subgroup of type 8 cells exhibited this characteristic.

Membrane excitability was assessed by a series of stepped current injections (0.1 to 1 nA; 250 ms; 10 consecutive steps; 1 sec interstep interval). Excitability was quantified as the total number of action potentials evoked in the 10 stepped current injections. All studies were carried out in standard Tyrode's solution supplemented with agent vehicle (DMSO or H_2O) or selected KVOs (2 minute applications). Current injection excitability was first tested in the presence of vehicle; and subsequently retested in the presence of single or multiple KVOs.

Measurements included action potential threshold (in nA), total number of action potentials (APs) evoked over the series of current injections, and shift of the RMP at the end of the two minute

administration of one or more KVOs. The influence of KVOs was determined as the pretest to posttest shift in threshold, total APs and RMP.

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 uM). KVOs (or vehicle) we co-applied with OXO. Membrane depolarization, action potential frequency, number and burst duration were quantified off-line for statistical analysis. Comparisons were made between vehicle treated and OXO-KVO cases.

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Award Chart

GW180038: Optimizing K_v7 Channel Openers for Treatment of GWI Pain

PI: Brian Cooper, PhD, University of Florida, Florida **Budget:** $1,028,938.00

Topic Area: Gulf War Illness Research Program 2018

Mechanism: Investigator Focused Research Award-Tier2-Applied Research Program W81XWH-18-GWIRP-IIFRA

Research Area(s): 1406-Pain

Award Status: Funded, W81XWH-19-1-0657 (9/01/2019-08/31/2021)

Study Goals:

Identify and optimize FDA approved Kv7 channel openers for treatment of Gulf War Illness pain symptoms

Specific Aims:

Specific Aim 1: Recovery from Pain Behaviors Using Multiple K_v7 Ion Channel Openers
Specific Aim 2. Optimizing Molecular Synergisms of KVOs on Deep Tissue Nociceptor K_v7
Specific Aim 3. Optimizing KVO Functional Synergisms

Key Accomplishments and Outcomes:

Specific Aim 2: Dose-Response and Maximum Conductance Data for Meclofenamate, Diclofenac and Retigabine on Deep Tissue Nociceptors Implicated in GWI Pain

Specific Aim 2: Combination Doses of Retigabine/Diclofenac or Retigabine/Meclofenamate Exceed Effectiveness of Either Drum Alone.

Specific Aim 3: Retigabine/Diclofenac Block Acetylcholine Muscarinic Discharge of Deep Tissue Nociceptors.

Publications: none to date
Patents: none to date
Funding Obtained: Start date of 09/01/2019