

RESEARCH ARTICLE

Novel characterization of endogenous transient receptor potential melastatin 3 ion channels from Gulf War Illness participants

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Abstract

Gulf War Illness (GWI) is a chronic condition characterized by multisystem symptoms that still affect up to one-third of veterans who engaged in combat in the Gulf War three decades ago. The aetiology of GWI is mainly explained by exposure to multiple toxic agents, vaccines, and medications. As there is a significant overlap in symptoms between GWI and Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS), the objective of this study was to investigate a biomarker widely reported in Natural Killer (NK) cells from ME/CFS patients, the Transient Receptor Potential Melastatin 3 (TRPM3) ion channel. NK cells from 6 healthy controls (HC) and 6 GWI participants were isolated, and TRPM3 function was assessed through whole-cell patch-clamp. As demonstrated by prior studies, NK cells from HC expressed typical TRPM3 function after pharmacomodulation. In contrast, this pilot investigation demonstrates a dysfunctional TRPM3 in NK cells from GWI participants through application of a TRPM3 agonist and confirmed by a TRPM3 antagonist. There was a significant reduction in TRPM3 function from GWI than results measured in HC. This study provides an unprecedented research field to investigate the involvement of TRP ion channels in the pathomechanism and potential medical interventions to improve GWI quality of life.

Introduction

Gulf War Illness (GWI) is a chronic, debilitating, and multisystem disorder that affects people who served in the Gulf War (GW). In 1990 and 1991, almost one million defence personnel from 41 countries engaged in combat in the GW, where they were exposed to diverse known and unknown/undefined hazardous environmental stressors, biological and chemical agents (insecticide, pesticide, insect repellent, organophosphate nerve agents, smoke from oil-well fires, depleted uranium) [1–4]. Prophylactic strategies, several mandatory vaccines and drugs

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were also administered concomitantly to reduce conflict risk, such as botulinum toxoid and anthrax vaccines and pyridostigmine bromide to protect from nerve agent exposures [5–8] in addition to the hypervigilance and mental stress that life-threatening military conflict brings.

Despite the fact that defence personnel returned from the GW more than three decades ago, it is estimated that from 25% to 32% of GW veterans continue to suffer from health issues and disabling symptoms likely caused by exposures during the GW [4,9,10]. In general, GWI symptomatology includes chronic fatigue, pain, inflammation, sleep disturbances, neurological and cognitive impairment, gastrointestinal and respiratory disorders, and post-exertional malaise, but not all affected individuals develop the entire array of symptoms, which makes diagnosis difficult [9,11–13]. Currently, the most recommended consensus definitions for GWI are: Chronic multisymptom illness from the Centers for Disease Control and Prevention (CDC) [14] and Kansas definition [15].

To date, GWI aetiology remains not completely understood, however, scientific evidence suggests the most reasonable cause of GWI is the exposure to the abovementioned combination of toxicant agents (insecticide, pesticide, insect repellent, organophosphate nerve agents, smoke from oil-well fires, depleted uranium) and prophylactic strategy [9,16,17]. Interestingly, GWI symptoms spectrum significantly overlaps Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS), fibromyalgia and long COVID-19, all conditions in which complex pathomechanisms are only partially defined, including some GWI patients who meet the clinical criteria for ME/CFS and Fibromyalgia [6,13,18,19]. A remarkable feature of ME/CFS is the post-exertional neuroimmune exhaustion caused by an inappropriate response to stressors and involves symptoms exacerbated following mild physical or mental activity [20–22], a characteristic also reported in GWI patients [12,23].

Our previous studies have demonstrated the role of Transient Receptor Potential (TRP) ion channels in the pathophysiology of ME/CFS which also promoted research into novel therapies [24–29]. The Mammalian TRP ion channels family is compounded by six subfamilies totaling 28 members: ankyrin (TRPA), canonical (TRPC), melastatin (TRPM), mucolipin (TRPML), polycystin (TRPP), and vanilloid (TRPV) [30–32]. Furthermore, a large number of the TRP superfamily members are highly sensitive to diverse chemical, physical and biological stimuli, therefore these ion channels act as molecular sensors to perceive modifications in the internal and external environment for the purpose of maintaining homeostasis [33–38]. In addition, many TRP channels participate in the regulation of calcium (Ca^{2+}) signalling to preserve cellular homeostasis. Overall, TRP channels also contribute to crucial processes, including neurogenesis, plasticity, immune functions, inflammation control, cell proliferation and survival, and pain perception [32,37,39–45].

Although GWI symptoms are the result of the long-term effects of exposure during military service, and the potential for TRP channels to be modulated by various environmental and toxic stimuli, to our knowledge this is the first study to analyse a TRP channel function in veterans with GWI. We specifically selected TRPM3 as the first TRP channel to be investigated in the GWI pathomechanism due to TRPM3's association with ME/CFS, even though other TRP channels may also be associated with the symptomatology presented by patients with GWI. Hence, in this pilot study, the aim was to evaluate TRPM3 ion channel activity in NK cells from GWI participants in comparison with the same cells from healthy controls (HC).

Materials and methods

Participant characteristics

Participants diagnosed with GWI and HC participants were recruited between 06th June and 30th November 2023 to be included in this study. The GWI group was composed of six

Australian males who engaged in combat at the GW, were diagnosed with GWI previously, and met CDC Case Definition [14] and Kansas criteria [15] for GWI. Six HC male participants were selected for this study, all those reported to be in good health, absence of illness and no fatigue episodes. All volunteers were between 18 and 65 years and non-smokers. Participants were excluded from this investigation if they were previously diagnosed with a chronic illness or reported alcohol abuse, use of opioids, medication, or supplements that interfere with TRPM3 ion channels or Ca^{2+} signalling. However, participants had the option to cease taking conflicting medications in accordance with the half-life of pharmacological compounds and if authorised by their physician. This investigation was approved by the Griffith University Human Research Ethics Committee (GU HREC 2022/666) and all participants provided their written consent.

Participant symptoms and disability

All participants completed a questionnaire created by the National Centre for Neuroimmunology and Emerging Diseases (NCNED) to provide their medical history, sociodemographic background, illness symptoms, and disability information. To assess disability data the questionnaire includes the World Health Organization Disability Assessment Schedule (WHODAS).

In this study, symptoms from people with GWI were classified in ten subtypes: (1) cognitive difficulties (e.g. cognitive overload, confusion, disorientation, impaired concentration, forgetfulness and memory problems); (2) pain (e.g. headaches, muscle aches and multi-joint pain); (3) sleep disturbances (e.g. unrefreshing sleep, frequent awakenings, prolonged sleep, reversed sleep cycle); (4) cardiovascular symptoms (e.g. orthostatic intolerance, cardiac arrhythmias, heart palpitations, light headedness and dizziness); (5) respiratory symptoms (e.g. air hunger, difficulty breathing); (6) thermostatic intolerances (e.g. subnormal body temperature, abnormal sweating episodes, hot flushes and cold extremities); (7) neurosensory or perceptual symptoms (e.g. inability to focus vision, impaired depth perception, sensitivity to touch, light, odour, taste, sound, vibration and poor balance or coordination); (8) urinary changes (e.g. changes to urination frequency and urgency to urinate); (9) immune disturbances (e.g. sore throat, tender lymph nodes, new allergies/sensitivities); and (10) gastrointestinal disturbances (e.g. nausea, abdominal pain, bloating, diarrhoea and irritable bowel syndrome).

The WHODAS indicates the level of disability from each participant and data is combined by groups. WHODAS is subdivided into seven domains of life: (1) Communication and understanding; (2) Mobility; (3) Self-care; (4) Interpersonal connections; (5) Life activities; (6) Work or School participation; and (7) Participation in society. WHODAS items were scored on a five-point scale (none, mild, moderate, severe, and extreme or cannot do). The subscale scores were determined in accordance with the WHODAS 2.0 manual, first converting each item score into the corresponding, predefined weighted values [46]. Scores converted from 0% to 100%, disability are inversely proportional to the scale, whereby lower scores indicate less disability and correspond 100% to full disability [47].

Peripheral blood mononuclear cell and natural killer cell isolation

Each participant donated between 40 ml and 84 ml of whole blood. All blood collections were conducted by a qualified phlebotomist, via venepuncture, using ethylenediaminetetraacetic acid (EDTA) tubes. A sample of 4 ml of whole blood from each participant was sent to a pathology laboratory for full blood count (FBC).

The remaining whole blood samples were used to isolate peripheral blood mononuclear cells (PBMCs) by centrifugation over a density gradient medium (Ficoll-Paque Premium, GE

Healthcare, Uppsala, Sweden). PBMCs total cell count, live cell count and viability were assessed using trypan blue dye (Invitrogen, Carlsband, CA, USA) and automatic cell counter (TC20 Automated cell counter, Bio-Rad, Laboratories, Hercules, CA).

For NK cell isolations, PBMCs were adjusted for a concentration of 5×10^7 cells/ml. NK cell isolations were conducted by immunomagnetic selection using an EasySep Negative Human NK Cell Isolation Kit (Stem Cell Technologies, Vancouver, BC, Canada).

Flow cytometry was performed to identify the NK cell purification from each NK cell isolation. Immediately after NK cell isolation, cells were incubated with CD56 APC (0.25g/20l) and CD3 PE Cy7 (0.25g/5l) monoclonal antibodies (Becton Dickinson (BD) Bioscience, San Jose, CA, USA) for 20 minutes in the dark at room temperature. NK cells were washed and resuspended in 350 ml of stain buffer (BD Bioscience, New Jersey, USA) and acquired at 10,000 events using the BD LSR- FortessaTM X-20 flow cytometer (BD Biosciences, San Diego, CA, USA). The NK cell population was then identified using phenotypic surface expression as CD3⁺ CD56⁺. For this study, acceptable NK cells purity was $\geq 90\%$. [S1 Fig](#) shows and compares purity results from HC and GWI groups. Importantly, there was no statistical difference between groups.

Electrophysiological experiments

The gold standard patch-clamp technique was conducted to determine TRPM3 ion channel activity in NK cells freshly isolated from HC and people with GWI. In this study, borosilicate glass capillaries (Harvard Apparatus, Holliston, MA, USA, GC150F-15, outside diameter = 1.5 mm, inside diameter = 0.86 mm) were pulled to obtain glass pipette (Sutter Instrumental, model P-97) and polished posteriorly (Narishige, Micro Forge MF-900). When filled with pipette solution, membrane resistance was 8 to 12 M Ω . A CV203BU head-stage (Molecular Devices, Sunnyvale, CA, USA) connected to a 3-way coarse manipulator and a micromanipulator (Narishige, Tokyo, Japan) were used in these experiments. To amplify and record electrical signals, an Axopatch 200B amplifier and pClamp 10.7 software (Molecular Devices, Sunnyvale, CA, USA) were used, with data filtered at 5 kHz and sampled digitally at 10 kHz via a Digidata 1440A analogue to digital converter (Molecular Devices, Sunnyvale, CA, USA). The voltage-ramp protocol was a step from a holding potential of +10 mV to -90 mV, followed by a 0.1 s ramp to +110 mV, before returning to +10 mV (repeated every 10 seconds). The liquid junction potential between the pipette and bath solutions (10 mV) was corrected and no leak current component was subtracted.

The intracellular pipette solution contained: 30 mM CsCl, 2 mM MgCl₂, 110 mM L-Aspartic acid, 1 mM EGTA, 10 mM HEPES, 4 mM ATP disodium hydrate, 0.1 mM GTP sodium salt hydrate (pH = 7.2, adjusted with CsOH; Osmolality = 290 mOsm/L, adjusted with D-mannitol), filtered with 0.22 μ m membrane filter (Sigma-Aldrich, St. Louise, MO, USA), aliquoted and stored at -20°C. The possibility of chloride current involvement in TRPM3 assessment was minimized by using L-Aspartic acid in the intracellular pipette solution. The extracellular solution contained: 130 mM NaCl, 10 mM CsCl, 1 mM MgCl₂, 1.5 mM CaCl₂ 2H₂O, 10 mM HEPES, (pH = 7.4, adjusted with NaOH; Osmolarity = 300 mOsm/L, adjusted with D-glucose) freshly prepared.

As previously validated by NCNED, pharmacological agents were included in the extracellular solution to assess TRPM3 ionic currents [26]. Briefly, a gravity perfusion system was used to apply extracellular solution for 50 seconds to establish a baseline current. Subsequently, extracellular solution containing 100 μ M of PregS was added to stimulate TRPM3 ion channels for 2.5 minutes. Following on from this addition extracellular solution with 10 μ M Ononetin and 100 μ M of PregS was applied for another 2.5 minutes to block TRPM3 ion channels. After

the conclusion of the drugs application, another cycle of 100 seconds of only extracellular solution was applied to remove the drugs. ATP and GTP were purchased from Sapphire Bioscience Reagents, PregS and Ononetin were purchased from Tocris Bioscience, while all other reagents and chemicals were ordered from Sigma-Aldrich. PregS and Ononetin were resuspended and stored in accordance with the manufacturer's instructions. Electrophysiological experiments were conducted at room temperature (22–24°C).

All recordings were analysed individually by a blinded researcher and posteriorly data was reviewed one by one by another blinded researcher, as detailed in [S2 Fig](#). Additionally, any unstable currents or chloride contamination was excluded from the analysis.

Statistical analysis

Questionnaire data were analysed through the Statistical Package for the Social Sciences (SPSS) software, version 27 (IBM Corp, Armonk, NY, USA) and purity results with GraphPad Prism v9 (GraphPad Software Inc., La Jolla, CA, USA). For electrophysiological data analysis and data presentation, pCLAMP 10.7 software (Molecular Devices, Sunnyvale, CA, USA), Origin 2021 (OriginLab Corporation, Northampton, MA, USA), and GraphPad Prism version 9 were used. Shapiro-Wilk normality test was performed to identify the distribution of data. ROUT method was conducted to determine outliers and they were removed from analysis. The independent nonparametric Mann-Whitney U test was performed to identify the statistical significance between GWI and HC groups in PregS and Ononetin amplitude. The Fisher's exact test (applying Bonferroni method) was conducted to determine statistical significance regarding sensitivity to Ononetin in NK cells. Significance was set at $p < 0.05$ and the data are presented as mean \pm standard error of the mean (SEM) unless otherwise stated.

Results

Participant characteristics and full blood count

In general, there were no significant differences between GWI and HC participants regarding age (HC = 47.33 ± 9.24 and GWI = 52.33 ± 2.07), body mass index (BMI) (HC = 29.22 ± 4.20 and GWI = 25.93 ± 1.90), employment status and education level. An overview of participants' features is detailed in [Table 1](#).

[Table 2](#) provides the WHODAS and FBC results compared between groups. There were no differences in FBC results between HC and GWI participants. Meanwhile, there were significant differences between HC and GWI results in communication and understanding ($p = 0.029$), mobility ($p = 0.007$), self-care ($p = 0.022$), interpersonal connections ($p = 0.024$), life activities ($p = 0.022$), work participation ($p = 0.007$) and participation in society ($p = 0.004$). GWI participants had higher scores in all WHODAS domains, which indicates a significant increase in disability levels.

[Table 3](#) describes the symptoms experienced by GWI veterans in the month prior to blood donation, these symptoms might fluctuate over time as previously described [48]. All veterans reported fatigue, cognitive difficulties, pain and sleep disturbances, while respiratory disturbances were the less identified symptoms in this cohort, with only one patient referring respiratory problems. Furthermore, sensory, gastrointestinal and urinary disturbances were reported by 5 GWI participants (83.33%), while cardiovascular and immune symptoms by 4 GWI (66.66%). Thermostatic instabilities were reported by half of GWI participants. Interestingly, GWI participants from this study met ME/CFS clinical criteria, specifically all GWI met Canadian Consensus Criteria (CCC) for ME/CFS [49] and one GWI met the International Consensus Criteria (ICC) for ME/CFS [20].

Table 1. Participant characteristics.

		HC	GWI	P-value
Age (years)		47.33 ± 9.24	52.33 ± 2.07	0.180
BMI (kg/m ²)		29.22 ± 4.20	25.93 ± 1.90	0.200
Employment Status	Full Time	5 (83.33%)	4 (66.66%)	0.400
	Part Time	1 (16.67%)	-	
	Casual	-	1 (16.67%)	
	Retired	-	1 (16.67%)	
	Illness/Disability	-	-	
Education	Primary Education	-	-	0.277
	High School	-	1 (16.67%)	
	Professional Training	2 (33.33%)	2 (33.33%)	
	Undergraduate	1 (16.67%)	3 (50.0%)	
	Postgraduate/Doctoral	3 (50.0%)	-	

Data presented as mean ± SD or N (%). Values of $p < 0.05$ are bolded. Abbreviations: BMI, body mass index; GWI, Gulf War Illness; HC, healthy controls.

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Electrophysiological experiments

The gold standard patch-clamp technique for studying ion channels was performed to characterize TRPM3 using whole-cell configuration in this study. In line with extensive literature [26,50–55], the TRPM3 agonist pregnenolone sulfate (PregS) activates these ion channels inducing an increase in intracellular Ca^{2+} concentration in HC cells, as represented in Fig 1A and 1B. Under voltage-clamp conditions, 100 μ M PregS induced small outward rectifying

Table 2. Disability and full blood count results.

	HC	GWI	P-value
WHODAS			
Communication and Understanding	5.55 ± 8.19	28.47 ± 21.96	0.029
Mobility	0.0 ± 0.0	28.33 ± 21.13	0.007
Self-Care	0.0 ± 0.0	12.50 ± 16.77	0.022
Interpersonal Connections	3.12 ± 5.23	31.25 ± 20.16	0.024
Life Activities	0.0 ± 0.0	26.04 ± 22.51	0.022
Work Participation	0.0 ± 0.0	23.96 ± 18.72	0.007
Participation in Society	0.52 ± 1.28	37.50 ± 26.66	0.004
Full blood count			
White Cell Count (4.0–11.0 $\times 10^9/L$)	5.90 ± 0.64	7.02 ± 1.69	0.109
Lymphocytes (1.0–4.0 $\times 10^9/L$)	1.80 ± 0.58	1.78 ± 0.39	0.873
Neutrophils (2.0–8.0 $\times 10^9/L$)	3.45 ± 0.88	4.46 ± 1.19	0.150
Monocytes (0.1–1.0 $\times 10^9/L$)	0.42 ± 0.15	0.57 ± 0.18	0.054
Eosinophils (< 0.6 $\times 10^9/L$)	0.17 ± 0.12	0.15 ± 0.11	0.376
Basophils (< 0.2 $\times 10^9/L$)	0.04 ± 0.02	0.05 ± 0.03	0.373
Platelets (140–400 $\times 10^9/L$)	227.5 ± 17.44	271.0 ± 81.39	0.261
Red Cell Count (3.8–5.2 $\times 10^{12}/L$)	5.22 ± 0.29	5.06 ± 0.67	0.749
Haematocrit (0.33–0.47)	0.45 ± 0.03	0.45 ± 0.04	0.747
Haemoglobin (115–160 g/L)	154.7 ± 10.97	154.0 ± 16.89	0.873

Data presented as mean ± SD. Reference ranges for full blood count parameters have been included in the table. Values of $p < 0.05$ are bolded. Abbreviations: DAS, disability assessment schedule; GWI, Gulf War Illness; HC, healthy controls; WHO, World Health Organization.

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Table 3. GWI participants symptom.

		GWI
Fatigue	Yes	6 (100.0%)
	No	0 (0.0%)
Cognitive Difficulties	Yes	6 (100.0%)
	No	0 (0.0%)
Pain	Yes	6 (100.0%)
	No	0 (0.0%)
Sleep Disturbances	Yes	6 (100.0%)
	No	0 (0.0%)
Sensory Disturbances	Yes	5 (83.33%)
	No	1 (16.67%)
Immune Disturbances	Yes	4 (66.66%)
	No	2 (33.34%)
Gastrointestinal Disturbances	Yes	5 (83.33%)
	No	1 (16.67%)
Cardiovascular Disturbances	Yes	4 (66.66%)
	No	2 (33.34%)
Respiratory Disturbances	Yes	1 (16.67%)
	No	5 (83.33%)
Thermostatic Instability	Yes	3 (50.0%)
	No	3 (50.0%)
Urinary Disturbances	Yes	5 (83.33%)
	No	1 (16.67%)

Data presented as mean \pm SD and N (%). Abbreviations: GWI, Gulf War Illness.

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currents in most NK cells isolated from HC which showed characteristic TRPM3 current–voltage relationship (I–V) (Fig 1B). However, in NK cells from the GWI group, the application of 100 μ M of PregS stimulated only a few NK cells. To statistically compare TRPM3 ion channel function between both groups, amplitudes were determined for each recording as a change in amplitude from baseline to PregS induced peak, as represented in time-series graphs (Fig 1A and 1D). In this investigation a significantly smaller amplitude of PregS-evoked currents was found in NK cells isolated from GWI participants in comparison to cells from HC (Fig 1G, $p < 0.0001$), a result that indicates people diagnosed GWI have TRPM3 impaired function. Fig 1 provides examples of recordings in a NK cell from a HC (Fig 1A–1C) and GWI participant (Fig 1D–1F).

To confirm the presence of TRPM3, 10 μ M of the antagonist Ononetin was applied in the presence of PregS whereby a reduction in ionic currents indicated sensitivity to Ononetin and consequently the presence of TRPM3 [56]. As expected, PregS-evoked ionic currents were successfully suppressed during Ononetin application in NK cells from HCs (Fig 1A) and an outward rectification I–V curve was observed (Fig 1C). However, there was a significant reduction in Ononetin amplitude in currents obtained in NK cells from GWI patients compared to HC individuals (Fig 1H, $p = 0.0008$). In addition, there was a significant reduction in the number of NK cells from GWI participants sensitive to Ononetin compared with HC (30.8%, Fig 2A $p < 0.0001$). In Fig 2, scatter plots demonstrate each current amplitude with PregS and Ononetin modulation, to show changes during patch-clamp protocol in NK cells from HC (Fig 2B) and GWI (Fig 2C). These results confirmed the involvement of TRPM3 ion channels in PregS-evoked currents in NK cells isolated from HC and the TRPM3 dysfunction in cells from GWI participants.

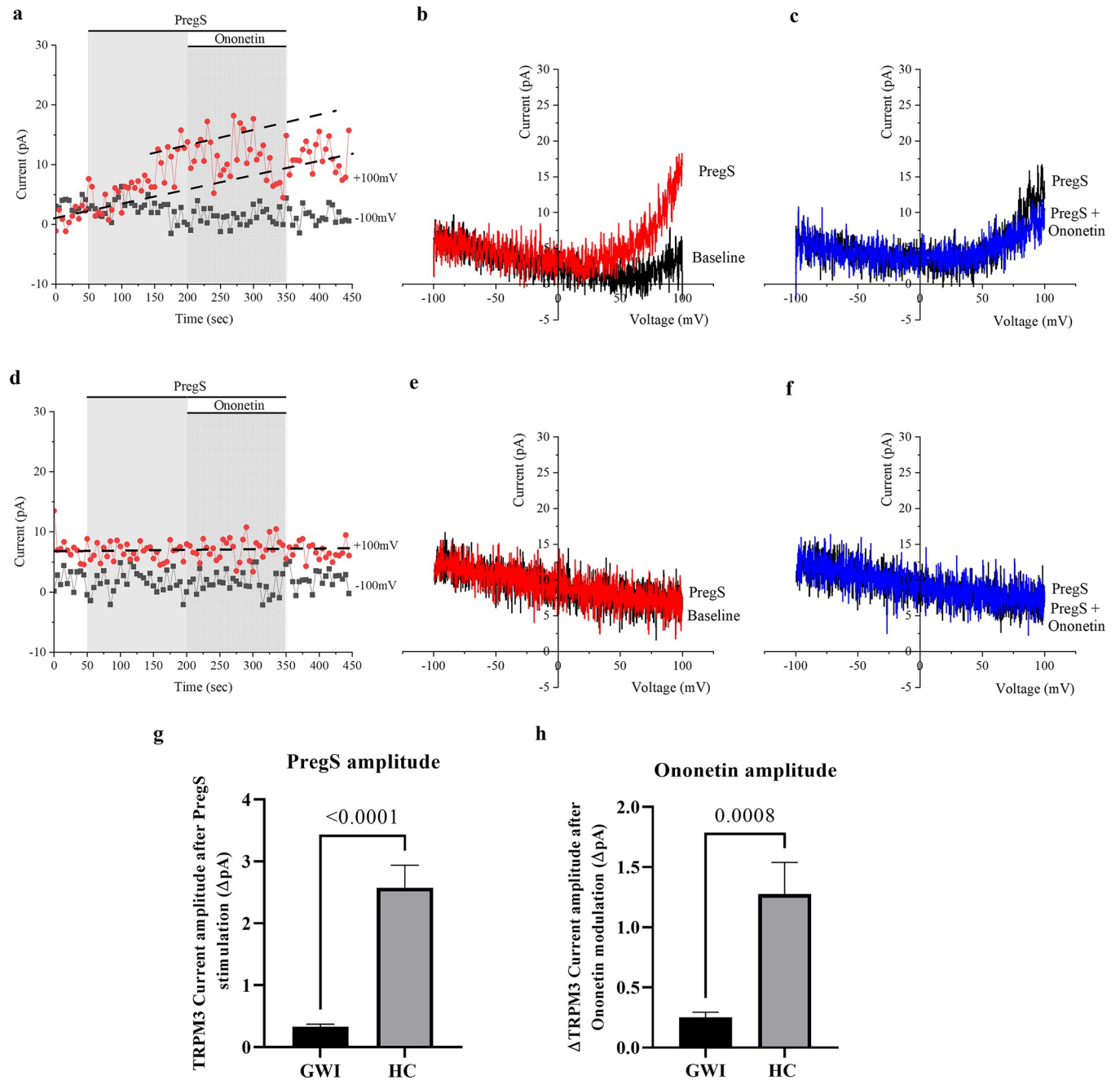


Fig 1. Representation of TRPM3 ion channel activity in NK cells from HC and GWI participants (whole-cell patch-clamp). a, b, c—Current obtained in whole-cell patch-clamp condition in a NK cell from a HC participant. (a) A representative time-series of current amplitude at +100 mV and –100 mV. (b) I–V curve before and after 100 μ M PregS stimulation. (c) I–V curve on PregS stimulation and after modulation with 10 μ M Ononetin in the presence of PregS. d, e, f—Current obtained in whole-cell patch-clamp condition in a NK cell from a GWI participant. (d) A representative time-series of current amplitude at +100 mV and –100 mV. (e) I–V curve before and after 100 μ M PregS stimulation. (f) I–V curve on PregS stimulation and after modulation with 10 μ M Ononetin in the presence of PregS. g–h: Bar graphs representing TRPM3 current amplitude at +100 mV, (g) is regarding TRPM3 stimulation with 100 μ M PregS in GWI (N = 6; n = 47) compared with HC participants (N = 6; n = 48), while (h) is regarding TRPM3 modulation with 10 μ M Ononetin in the presence of PregS in GWI (N = 6; n = 42) compared with HC participants (N = 6; n = 39). Dash-lines in time-series illustrate the baseline and PregS effects. N refers to number of participants and n to number of records analysed. Data presented as mean \pm SEM and determined by Mann-Whitney U test. Abbreviations: GWI, Gulf War Illness; HC, healthy control; NK, natural killer; PregS, pregnenolone sulfate.

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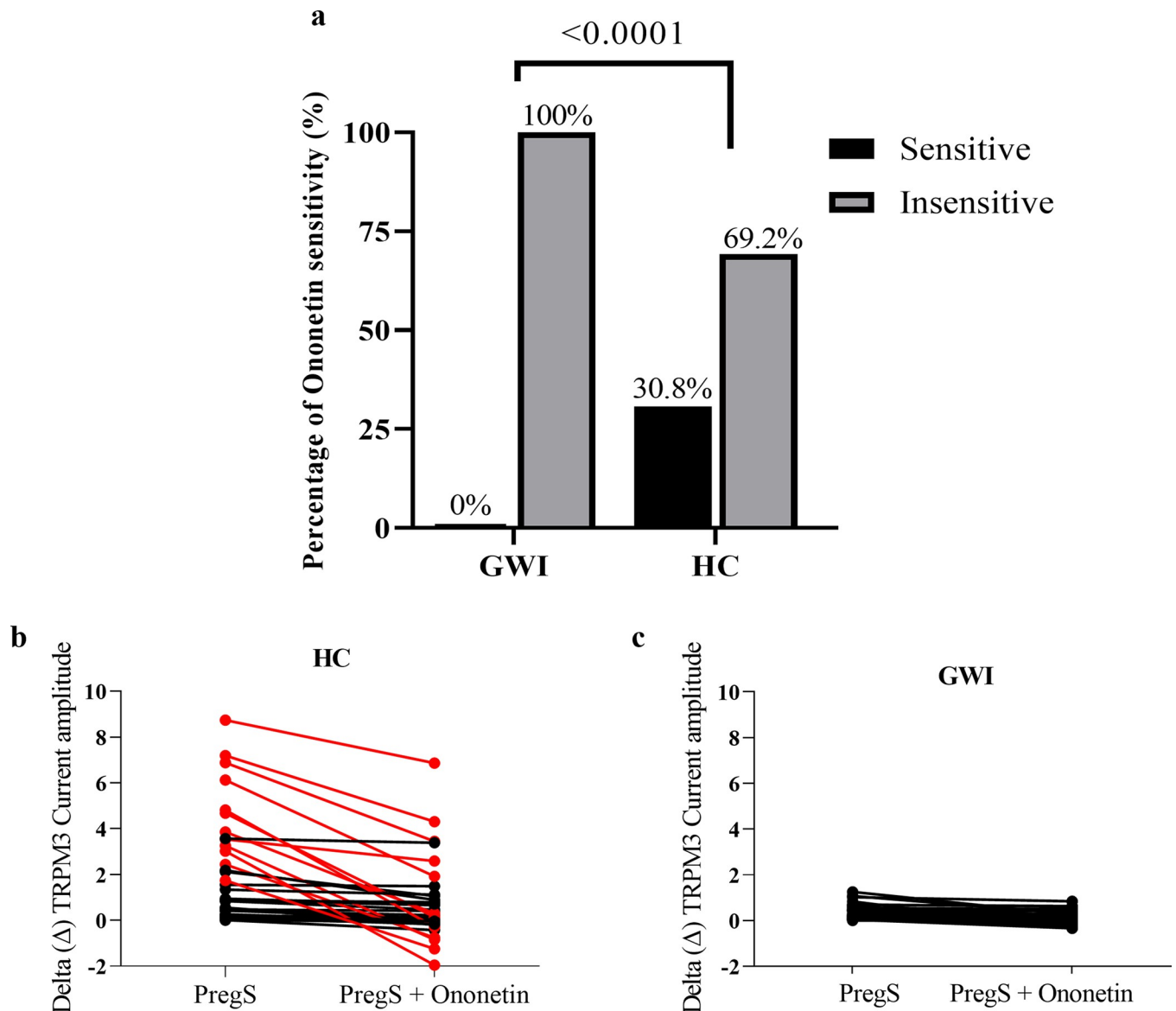


Fig 2. Ononetin results. (a) Bar graphs representing percentage of sensitive and insensitive NK cells to 10 μ M Ononetin in presence of PregS, from HC (N = 6; n = 39), and GWI (N = 6; n = 42). (b–c) Scatter plots representing change of each current amplitude before and after application of Ononetin in presence of PregS in NK cells from HC and GWI participants respectively. Each red line represented a cell sensitive to Ononetin as a reduction in amplitude was recorded. N to number of participants and n to number of records analysed. Abbreviations: GWI, Gulf War Illness; HC, healthy control; NK, natural killer; PregS, pregnenolone sulfate.

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Discussion

Our data is the first to report significant impairment in TRPM3 ion channel function in NK cells from participants with GWI compared to HC. TRPM3 is a non-selective cation channel that highly exhibits permeability to Ca^{2+} and plays a critical role in a variety of biological processes due to its contribution in the Ca^{2+} signalling regulation [24,57,58]. For instance, Ca^{2+} is an essential element for intracellular signalling pathways, immunity activation, proliferation and maintenance of cellular activities, degranulation, release of cytolytic proteins and homeostasis [24,28,37,57–60]. Likewise, abnormal intracellular Ca^{2+} concentration associated with

dysfunctional TRPM3 may cause inadequate cell function and imbalance intracellular signaling pathways [26,27,61].

The design of the present study used a primary NK cell model to investigate TRPM3 function as the immune system plays a substantial role in GWI. Many studies have supported impaired immunological function and inflammation as significant components of GWI pathophysiology [23,62,63]. Whistler and colleagues results showed significant reduction in NK cell cytotoxicity which is indicative of immune disruption in GWI patients [23]. TRP channels are involved in oxidative stress, pain and inflammatory responses, while TRPM3 specifically in peripheral nociceptors have pronociceptive and pro-inflammatory properties [37,64,65]. Elhaj et al recently identified an increase in interleukin 6 (IL-6) and C-reactive protein (CRP) levels in GWI patients compared with other veterans without GWI, which is consistent with previous research [62,66] and corroborate the role of chronic neuroimmune and neuroinflammation disturbances [1].

Recent literature in the immune field, has described similarities among individuals with GWI, long COVID-19 or ME/CFS, including symptoms, impact on quality of life, lack of diagnostic testing and treatment [6]. Notably, our present finding of decreased TRPM3 function in NK cells from GWI is compatible with results reported in ME/CFS and long COVID cohorts [26,61,67], suggesting a common overlap of TRPM3 dysfunction, or TRP ion channels, in the pathomechanism of each of these diseases. Support for this argument is highlighted by TRP ion channels being modulated by numerous stimuli, for example, by environmental (e.g. temperature, light, chemicals), mechanical (e.g. osmolarity, pressure), natural (e.g. herbs or spices, venoms, toxins), endogenous factors, tissue damage, infection and inflammation [40,41,68–71]. In addition, people diagnosed with these diseases report having been exposed to some of these threats/stimuli prior to the symptom's onset. For instance, the precedent factor for long COVID-19 is unequivocally the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection, while ME/CFS patients often report prior infections, stressors, trauma and toxin exposure, with about 75% of cases associated to infection-like episodes before ME/CFS onset [22,72]. The hypothesis of TRP ion channel dysfunction underpinning the potential pathomechanism due to the intense exposure of a wide variety of chemical components, as well as vaccine and prophylactic drugs, is highly plausible [5,6,9,17].

Furthermore, TRP ion channels have been recognized as potentially associated with multi-system diseases and emerging as important drug targets due to their ubiquitous expression in cells from human organs and peripheral tissues [30,40,41,44,73,74]. Fonfria et al. characterized the expression of all TRPM family members in many human tissues, TRPM3 specifically was detected in cells from the brain, pituitary, kidney, adipose tissue, pancreas, prostate, and bone [75]. Other researchers also reported TRPM3 in different parts of the central nervous and cardiovascular systems, immune cells, liver, urinary and genital systems [24,26,50,73,76,77]. Despite the present study having assessed only TRPM3 ion channels in NK cells, these findings suggest this ion channel is dysfunctional in other tissue cells in GWI participants. Hence, the ubiquitous expression of TRPM3 ion channels is compatible with the constellation of GWI symptoms.

Interestingly, animal studies have reported that other TRP channels are related to chemical exposure suffered by veterans in the GW that could potentially explain their symptoms. For instance, Ding et al demonstrated through application of Transient Receptor Potential Ankyrin 1 (TRPA1) antagonists that TRPA1 is a principal mediator of organophosphate-induced delayed neuropathy, a condition that occurs due to acute or chronic exposure and is usually correlated to acetylcholinesterase suppression [78]. However, Ding and colleagues reported that tested organophosphates activated TRPA1 (also a Ca²⁺ permeable non-selective

cation channel), increased the influx of Ca^{2+} while TRPA1 antagonists significantly relieved organophosphate-induced neuropathy models [78].

A substantial variety of animal models have been developed to investigate GWI pathology, mainly induced by animal exposure to a single or combination of threats/agents (organophosphates, PB, stressors) to reproduce the GW environment [9]. Although animal studies are crucial tools for health research, results should not be extrapolated to humans. Principal limitations of animal models are genomic differences that studies evaluate on short term effects disregarding decades of GWI, and focusing on symptoms individually [1,77,79]. In contrast, our results are directly based on freshly isolated immune cells from veterans diagnosed with GWI compared to cells from HC. Moreover, these results were obtained evaluating endogenous TRPM3 from human primary cells freshly isolated from blood, through the gold standard technique for studying ion channels.

Notably, the importance of identifying TRPM3 as a biomarker for GWI is mainly to facilitate a diagnosis. Currently, due to the absence of a screening or specific diagnostic test, GWI remains diagnosed through case definitions and exclusion of other conditions that would not explain the symptoms expressed by GW veterans [80]. Nevertheless, the novel findings of these studies provide the perspective of developing a test focused on the characterization of ion channel function using an easily acquired biological sample.

Conclusion

In conclusion, the present study creates a rationale for future studies analysing other TRP channels in plasma membrane, as well as organelles. It further provides an opportunity to lead investigations of therapeutic strategies to treat and manage the GWI condition, to alleviate severe symptoms and consequently improve their quality of life. Further, ion channel studies are necessary to investigate the contribution of other TRP channels in the pathophysiology of GWI.

Supporting information

S1 Fig. NK cell purity. NK cell purity was acquired at 10,000 events using the BD LSRFortessa™ X-20. NK cells, defined as $\text{CD3}^{\text{CD56}^+}$ lymphocytes were $96.08\% \pm 0.953$ for HC and $94.33\% \pm 1.144$ for participants with GWI. (a) lymphocytes were gated based on Side Scatter (SSC) and versus Forward Scatter (FSC). (b) CD3^+ cell population was gated from selected lymphocyte population. Gating was identified through isotype controls. (c) NK cell purity was based on CD56^+ population from the CD3^+ population. (d) Bar graphs illustrating percentage of NK cell population. Data presented as mean \pm SEM and determined by Mann-Whitney U test. Abbreviation: GWI, Gulf War Illness; HC, healthy controls; NK, natural killer. (TIF)

S2 Fig. Individual recording analysis. Two representative time-series of current amplitude at +100 mV and -100 mV showing the effect of PregS and Ononetin in the presence of PregS. Baseline = blue dash-lines; PregS baseline = green dash-lines. (A) Baseline; (B) PregS points; (C) PregS baseline; (D) Ononetin points. PregS amplitude = (B)-(A) and Ononetin amplitude = (C)-(D). PregS was effective when presented with an increase at +100 mV current and Ononetin was effective when there is a decrease at +100 mV current. No difference means drugs were not effective to stimulate agonist or antagonist effect on TRPM3 ion channels. On (a), PregS and Ononetin in the presence of PregS were effective, however, on (b) only PregS was effective. (TIF)

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