Loss of Transient Receptor Potential Melastatin 3 ion channel function in natural killer cells from Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients

Hélène Cabanas¹²*, Katsuhiko Muraki³, Natalie Eaton¹², Cassandra Balinas¹², Donald Staines¹² and Sonya Marshall-Gradisnik¹²

Abstract

Background: Chronic Fatigue Syndrome (CFS)/Myalgic Encephalomyelitis (ME) is a debilitating disorder that is accompanied by reduced cytotoxic activity in natural killer (NK) cells. NK cells are an essential innate immune cell, responsible for recognising and inducing apoptosis of tumour and virus infected cells. Calcium is an essential component in mediating this cellular function. Transient Receptor Potential Melastatin 3 (TRPM3) cation channels have an important regulatory role in mediating calcium influx to help maintain cellular homeostasis. Several single nucleotide polymorphisms have been reported in TRPM3 genes from isolated peripheral blood mononuclear cells, NK and B cells in patients with CFS/ME and have been proposed to correlate with illness presentation. Moreover, a significant reduction in both TRPM3 surface expression and intracellular calcium mobilisation in NK cells has been found in CFS/ME patients compared with healthy controls. Despite the functional importance of TRPM3, little is known about the ion channel function in NK cells and the epiphenomenon of CFS/ME. The objective of the present study was to characterise the TRPM3 ion channel function in NK cells from CFS/ME patients in comparison with healthy controls using whole cell patch-clamp techniques.

Methods: NK cells were isolated from 12 age- and sex-matched healthy controls and CFS patients. Whole cell electrophysiology recording has been used to assess TRPM3 ion channel activity after modulation with pregnenolone sulfate and ononetin.

Results: We report a significant reduction in amplitude of TRPM3 current after pregnenolone sulfate stimulation in isolated NK cells from CFS/ME patients compared with healthy controls. In addition, we found pregnenolone sulfate-evoked ionic currents through TRPM3 channels were significantly modulated by ononetin in isolated NK cells from healthy controls compared with CFS/ME patients.

Conclusions: TRPM3 activity is impaired in CFS/ME patients suggesting changes in intracellular Ca²⁺ concentration, which may impact NK cellular functions. This investigation further helps to understand the intracellular-mediated roles in NK cells and confirm the potential role of TRPM3 ion channels in the aetiology and pathomechanism of CFS/ME.

Keywords: Transient receptor potential Melastatin 3, Calcium, Chronic fatigue syndrome/Myalgic encephalomyelitis, Natural killer cells, Flow cytometry, Patch-clamp

* Correspondence: h.cabanas@griffith.edu.au

¹School of Medical Science, Griffith University, Gold Coast, QLD, Australia
²The National Centre for Neuroimmunology and Emerging Diseases, Menzies Health Institute Queensland, Griffith University, Gold Coast, QLD, Australia

© The Author(s). 2018 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is a complex and debilitating disorder hallmarkcd by persistent or relapsing chronic fatigue that is inadequately alleviated by rest (Fukuda et al., 1994). At least four concurrent symptoms related to multiple systems including the immune, neurological, musculoskeletal, gastrointestinal, and cardiovascular systems accompany this unexplained fatigue (Carruthers et al., 2011). Without a pathology test, diagnosis is complex and relies on different case definitions that address the characteristics of CFS/ME (Carruthers et al., 2011; Jason et al., 2015). Although the Centers for Disease Control and Prevention (CDC) criteria are widely employed, this definition has been considered too broad in its symptom requirements (Johnston et al., 2013). Thus, the definition of CFS/ME was revised producing the Canadian Consensus Criteria (CCC) and finally in 2011 a more specific definition was established known as the International Consensus Criteria (ICC) to assist diagnosis (Carruthers et al., 2011). The underlying aetiology of CFS/ME remains unknown. However, a consistent feature of CFS/ME in the literature is immune dysfunction, and more precisely a significant reduction in Natural Killer (NK) cell cytotoxicity, a hallmark of NK cell function (Brenu et al., 2012; Brenu et al., 2011; Curriu et al., 2013; Hardcastle et al., 2015; Huth et al., 2016a; Klimas et al., 1990; Maher et al., 2005; Natelson et al., 2002; Nijs & Frémon, 2008; Sharpe et al., 1991; Siegel et al., 2006; Stanietsky & Mandelboim, 2010).

NK cells are effector lymphocytes of the innate immune system that eliminate pathogens and tumour cells, in addition to immune cell activation and cytokine production (Vivier et al., 2008). NK cells can be divided into five different phenotypes determined by their expression of cell-surface molecules including CD56 and CD16. The two main mature NK cell subtypes are CD56^bright^CD16^dim^ and CD56^dim^CD16^bright^. CD56^bright^CD16^dim^ NK cells exist as the minority in peripheral blood as efficient cytokine producers (Cooper et al., 2001). The CD56^dim^CD16^bright^ subset constitutes 90% of human peripheral NK cells with significantly higher cytotoxic activity than CD56^bright^ NK cells as they contain an abundance of cytolytic proteins. Additionally, the presence of the low-affinity Fc-γ receptor CD16 facilitates the activation of antibody-dependent cellular cytotoxicity (ADCC) (Moretta, 2010; Stabile et al., 2015). Differences in NK cell phenotypes and significantly reduced peripheral NK cell numbers resulting in significant reduction in NK cell cytotoxicity, have been reported in CFS/ME and implicated in disease severity (Brenu et al., 2013; Hardcastle et al., 2015; Lanier, 2003; Maher et al., 2005; Nijs & Frémon, 2008). Importantly, NK cells require calcium (Ca^{2+}) to regulate cellular functions including NK cell cytotoxicity. Indeed, numerous steps including adhesion to the target cell, activation of surface receptors, microtubule reorganisation, polarisation of secretory granules and release of lytic proteins, including granzyme A and granzyme B, creation of the immune synapse, formation of perforin pores, and finally granzyme-induced target cell apoptosis require tight regulation of Ca^{2+} signalling (Anasetti et al., 1987; Henkart, 1985; Kass & Orrenius, 1999; Schwarz et al., 2013).

Transient Receptor Potential (TRP) ion channels can trigger specific Ca^{2+}-dependent signal transduction pathways regulating many biological processes in both excitable and nonexcitable cells (Gees et al., 2010). TRP channels represent a large and diverse family of nonselective cation channels that are widely expressed and respond to a wide range of chemical and physical stimuli (Voets et al., 2005). Moreover, genetic variations in TRP genes and noxious stimuli have been implicated in several pain-related pathological conditions/modalities, including inflammatory, neuropathic, visceral and dental pain, as well as pain associated with cancer (Mickle et al., 2016; Nilius, 2007). The mammalian TRP channels are divided into subgroups according to amino acid sequence similarities: TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPML (mucolipin), and TRPP (polycystin) (Clapham et al., 2001). TRP channel subfamily M member 3 (TRPM3) is a Ca^{2+}-permeable nonselective cation channel, expressing a calmodulin binding region on the N-terminus which plays a role in activation of Ca^{2+} dependent signalling pathways (Holakoska et al., 2012; Lee et al., 2003; Oberwinkler & Philipp, 2014). The human TRPM3 gene encodes for many different TRPM3 isoforms due to alternative splicing and exon usage, leading to channels with divergent pore and gating properties (Frühwald et al., 2012; Oberwinkler et al., 2005; Thiel et al., 2013). In particular, the TRPM3α2 isoform has been characterized as being highly permeable for Ca^{2+} and other divalent cations (Frühwald et al., 2012). TRPM3 ion channels are highly expressed in neurons of dorsal root ganglia, where they serve as thermosensitive channels implicated in the detection of noxious heat (Vriens et al., 2011). Furthermore, TRPM3 has been identified in a number of tissues and cell types, including pancreatic beta cells, brain, pituitary gland, eye, kidney, and adipose tissue, that serve many different functions (Hoffmann et al., 2010; Oberwinkler & Philipp, 2014; Wagner et al., 2008). While expressed ubiquitously in mammalian cells, the roles and functions of TRPM3 have yet to be determined in immune cells and more particularly in NK cells, where TRPM3 has been previously identified without electrophysiological evaluation (Nguyen et al., 2017; Nguyen et al., 2016). TRPM3 ion channels are quickly (< 100 ms) and reversibly activated by a neuronal steroid, Pregnenolone...
sulfate (PregS) (Wagner et al., 2008). The precursor, pregnenolone is derived from cholesterol and sulphated in vivo for biological actions in the immune and central nervous systems. It is associated with memory and cognition, neuronal myelination, activation of neurotransmitter-gated channels, modulation of glutamate–nitric oxide–guanosine 3′,5′-(cyclic) phosphate pathways, maintenance of glucose and insulin homeostasis and the management of noxious stimuli (Harteneck, 2013; Nilius & Voets, 2008). Stimulation of TRPM3 with PregS in pancreatic beta-cells induces an intracellular signalling cascade, involving a rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), activation of the protein kinases Raf and extracellular signal-regulated kinases (ERK), resulting in the regulation of different cellular processes and a change in gene expression pattern (Thiel et al., 2013). However, it is notable that concentrations of PregS were sometimes high (30–100 μM), causing non-specific effects without TRPM3. On the other hand, a natural compound, deoxybezoain ononetin, has been identified as a selective and potent blocker of PregS-induced TRPM3 currents in TRPM3-expressing dorsal root ganglia neurones and TRPM3 transfected HEK293 cells (Straub et al., 2013). Therefore, both use of TRPM3 agonist and blocker are critical to identify the activity of TRPM3 currents in native cells.

Regulation and importance of TRPM3 channels in NK cells and the epiphenomenon of CFS/ME is relatively unknown. Five single nucleotide polymorphisms (SNPs) (rs65660200, rs1106948, rs12350232, rs11142822, rs1891301) have been identified in TRPM3 genes in CFS/ME patients (Marshall-Gradisnik et al., 2016). A recent investigation characterising TRPM3 related responses in NK cells and B lymphocytes found a significant reduction in expression of TRPM3 on the NK cell surface in CFS/ME patients compared with healthy controls (HC) (Nguyen et al., 2016). Moreover, isolated NK cells from CFS/ME patients have impaired TRPM3 activity following PregS stimulation, resulting in impaired Ca\(^{2+}\) mobilisation and reduced NK cell cytotoxicity (Nguyen et al., 2017). These results strongly suggest the importance of TRPM3 in the pathophysiology of CFS/ME. However, as the electrophysiological characterisation of endogenous TRPM3 channels on isolated NK cells is lacking, we aimed to characterise TRPM3 channel currents using whole cell patch-clamp measurements in HC and CFS/ME patients after modulation with PregS and ononetin. This novel approach may help to understand the clinical importance of TRPM3 in the pathomechanism of CFS/ME.

**Methods**

**Participant recruitment**

Six CFS/ME patients and six age- and sex-matched HC were recruited using the National Centre for Neuroimmunology and Emerging Diseases (NCNED) research database. Participants were screened using a comprehensive questionnaire corresponding with the CDC, CCC and ICC case definitions. All six CFS/ME patients were defined by the CCC. HC reported no incidence of fatigue and were in good health without evidence of illness. Participants were excluded from this study if they reported history of smoking, autoimmune diseases, cardiac disease, diabetes or other co-morbidities.

Two of the six CFS/ME patients reported regular administration of non-steroidal anti-inflammatory for pain relief. No participants reported use of pharmacological agents that directly or indirectly influence TRPM3 or Ca\(^{2+}\) signalling.

This investigation was approved by the Griffith University Human Research Ethics Committee (HREC/15/QGC/63).

**Peripheral blood mononuclear cell isolation and natural killer cell isolation**

A total of 85 ml of whole blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes between 8:00 am and 12:00 am. Routine full blood analysis was performed within 4h of collection for red blood cell count, white blood cell count and granulocyte cell count. Peripheral blood mononuclear cells (PBMCs) were isolated from 80 ml of whole blood by centrifugation over a density gradient medium (Ficoll-Paque Premium; GE Healthcare, Uppsala, Sweden) as previously described (Brenu et al., 2011; Munoz & Leff, 2006). PBMCs were stained with trypan blue (Invitrogen, Carlsband, CA, USA) to determine cell count and cell viability. PBMCs were adjusted to a final concentration of 5 × 10^7 cells/ml for NK cell isolation.

NK cells were isolated by immunomagnetic selection using an EasySep Negative Human NK Cell Isolation Kit (Stem Cell Technologies, Vancouver, BC, Canada). NK Cell purification was determined using flow cytometry. NK cells were incubated for 20 min at room temperature in the presence of CD56 FITC (0.25 μg/5 μl) and CD3 PE Cy7 (0.25 μg/20 μl) monoclonal antibodies (BD Bioscience, San Jose, CA, USA) as previously described (Nguyen et al., 2017).

**Whole cell electrophysiology recording**

Borosilicate glass capillaries with an outside diameter of 1.5 mm and inside diameter of 0.86 mm (Harvard Apparatus, Holliston, MA, USA) were used as patch pipettes. Pipette resistance when filled with pipette solution was 8–12 MΩ. The pipettes were mounted on a CV203BU head-stage (Molecular Devices, Sunnyvale, CA, USA) connected to a 3-way coarse manipulator and a micro-manipulator (Narishige, Tokyo, Japan). Electrical signals were amplified and recorded using an Axopatch 200B amplifier and PCclamp 10.7 software (Molecular Devices, Sunnyvale, CA, USA). Data were 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 s). The liquid junction potential between the pipette and bath solutions (−10 mV) was corrected. A leak current component was not subtracted from the recorded currents. Electrode was filled with the intracellular pipette solution containing 30 mM CsCl, 2 mM MgCl₂, 110 mM L-Aspartic acid, 1 mM EGTA, 10 mM HEPES, 4 mM ATP, 0.1 mM GTP, adjusted pH to 7.2 with CsOH and osmolality of 290 mOsm/L with D-mannitol. The pipette solution was filtered using a 0.22 μm membrane filter (Sigma-Aldrich, St. Louise, MO, USA), divided into aliquots and stored at −20 °C. Bath solution contained: 130 mM NaCl, 10 mM CsCl, 1 mM MgCl₂, 1.5 mM CaCl₂·2H₂O, 10 mM HEPES, adjusted pH to 7.4 with NaOH and osmolality 300 mOsm/L with D-glucose. All reagents were purchased from Sigma-Aldrich, except for ATP and GTP that were purchased from Sapphire Bioscience. TRPM3 currents were stimulated by adding 100 μM PregS (Tocris Bioscience, Bristol, UK) to the bath solution, whereas PregS-induced TRPM3 currents were blocked by adding 10 μM ononetin (Tocris Bioscience, Bristol, UK). All measurements were performed at room temperature. The authors removed the possibility of chloride current involvement in TRPM3 assessment by using L-Aspartic acid in the intracellular pipette solution. Unstable currents were also removed from the analysis. This ensured only TRPM3 function was assessed.

Statistical analysis
Cytometry data was exported from FacsDiva v8.1 and analysed using SPSS v24 (IBM Corp, Version 24, Armonk, NY, USA) and GraphPad Prism v7 (GraphPad Software Inc., Version 7, La Jolla, CA, USA). Electrophysiological data were analysed using pCLAMP 10.7 software (Molecular Devices, Sunnyvale, CA, USA). Origin 2018 (OriginLab Corporation, Northampton, MA, USA) and GraphPad Prism v7 (GraphPad Software Inc., Version 7, La Jolla, CA, USA) were used for statistical analysis and data presentation. The Shapiro-Wilk test was used to determine population normality. Statistical comparison was performed using the Mann-Whitney U test (Table 1, Fig. 1 and Fig. 3e), and Fishers exact test (Fig. 3g), to determine any significant differences. Significance was set at p < 0.05 and the data are presented as mean ± SEM unless otherwise stated.

Results
Participant characteristics and blood parameters
A total of twelve age- and sex-matched participants were included for this investigation. Demographic and clinical

| Table 1 Blood parameters and patient demographic |
|-----------------|--------|--------|-----|
| Age (years)     | CFS/ME | HC     | P Value |
| Gender n(%)     |        |        |       |
| Male            | 2 (20%)| 2 (20%)| 1.000 |
| Female          | 4 (80%)| 4 (80%)|       |
| BMI (kg/m²)     | 23.54 ± 0.54 | 23.35 ± 1.37 | 0.240 |
| SF-36 Fatigue (%)| 30.42 ± 8.91 | 77.92 ± 6.25 | 0.009 |
| SF-36 General Health (%)| 31.25 ± 5.97 | 74.31 ± 4.50 | 0.004 |
| SF-36 Physical Functioning (%)| 70.83 ± 6.64 | 93.33 ± 4.77 | 0.015 |
| SF-36 Role Physical (%)| 20.83 ± 9.64 | 87.50 ± 12.5 | 0.009 |
| SF-36 Role Emotional (%)| 59.38 ± 9.24 | 96.88 ± 3.13 | 0.004 |
| SF-36 Social Functioning (%)| 37.5 ± 5.59 | 95.83 ± 4.17 | 0.002 |
| SF-36 Body Pain (%)| 49.17 ± 5.27 | 84.17 ± 8.41 | 0.015 |
| White Cell Count (x10⁹/L) | 5.58 ± 0.30 | 7.13 ± 0.49 | 0.03 |
| Lymphocytes (x10⁹/L) | 1.99 ± 0.2 | 1.75 ± 0.22 | 0.423 |
| Neutrophils (x10⁹/L) | 3.03 ± 0.23 | 4.78 ± 0.42 | 0.01 |
| Monocytes (x10⁹/L) | 0.41 ± 0.05 | 0.39 ± 0.06 | 0.748 |
| Eosinophils (x10⁹/L) | 0.12 ± 0.02 | 0.18 ± 0.06 | 0.936 |
| Basophils (x10⁹/L) | 0.04 ± 0.01 | 0.04 ± 0.002 | 0.799 |
| Platelet (x10⁹/L) | 265.0 ± 23.36 | 266.17 ± 15.70 | 1.00 |
| Red Cell Count (x10¹²/L) | 4.63 ± 0.09 | 4.69 ± 0.13 | 0.63 |
| Haematocrit | 0.41 ± 0.01 | 0.42 ± 0.01 | 0.624 |
| Haemoglobin (g/L) | 135.0 ± 3.52 | 139.67 ± 5.21 | 0.687 |

SF-36 scores were analysed using participant questionnaire responses. Results from routine full blood analysis in CFS/ME patients and HC. Data presented as mean ± SD. Abbreviations: CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; HC, healthy controls; BMI, body mass index.

Fig. 1 Natural Killer cell purity. Bar graphs representing isolated NK cell purity for HC and CFS/ME patients. Data presented as mean ± SEM. HC = 98.07% ± 0.80 and CFS/ME = 98.28% ± 0.58. Abbreviations: CFS/ME, chronic fatigue syndrome/myalgic encephalomyelitis; HC, healthy controls; NK cell, natural killer cell.
data for patients are summarised in Table 1. There was no significant difference in age or gender between patients and HC. The 36-Item Short Form Survey (SF-36) was used to determine participant health-related-quality of life. As expected, there was a significant difference in SF-36 scores between CFS/ME patients and HC. Moreover, while there was a significant difference in white cell count and neutrophils, all results remained within normal range for age and sex as provided by the Gold Coast University Hospital Pathology Unit, NATA accredited laboratory.

**(Natural killer cell purity)**

NK cell purity (CD3+/CD56−) was 98.07% ± 0.80 for HC and 98.28% ± 0.58 for CFS/ME patients (Fig. 1) as determined by flow cytometry. There was no significant difference in NK cell purity in CFS/ME patients compared with HC.

**TRPM3 activity after Pregnenolone sulfate stimulation**

A whole-cell patch-clamp technique was used to measure endogenous TRPM3 activity, enabling a small size of current recordings under voltage-clamp conditions and observation of the typical shape of the TRPM3 current–voltage relationship (I–V) (Fig. 2). We used 100 μM PregS to stimulate the channels as TRPM3 is minimally expressed in NK cells (Nguyen et al., 2017; Nguyen et al., 2016). As expected, the ionic current evoked by PregS was relatively small (Fig. 2a and e) in NK cells isolated from HC and we report a typical I–V of TRPM3, which had a clear outward rectification (Fig. 2b). In contrast, the amplitude of ionic current after PregS stimulation was significantly smaller (Fig. 2c, d) in NK cells from CFS/ME patients than that from HC (Fig. 2e, p < 0.0001), suggesting impaired TRPM3 channel activity after PregS stimulation in CFS/ME patients.

**Discussion**

Our previous investigations have proposed that NK cells from CFS/ME patients have significantly reduced expression of TRPM3 and subsequent reduction in intracellular Ca2+ mobilisation compared with HC (Nguyen et al., 2017; Nguyen et al., 2016). This present study used electrophysiological methods to characterise endogenous TRPM3 activity in peripheral NK cells from HC and CFS/ME patients. We provide evidence suggesting PregS-dependent channel activity for TRPM3 is significantly lower in CFS/ME patients compared with HC. Moreover, ionic currents in CFS/ME patients were resistant to ononentin in the presence of PregS.

The patch clamp technique is regarded as a gold standard for ion channel research and offers direct insight into ion channel properties through the characterization of ion channel activity. In this study, we characterised, for the first time, the TRPM3 ion channel current in isolated human NK cells. We report a significant reduction amplitude of TRPM3 current after PregS stimulation in isolated NK cells from CFS/ME patients compared with HC. This is consistent with our previous findings showing significantly reduced TRPM3 expression as well as significantly reduced intracellular Ca2+ mobilisation in isolated NK cells from CFS/ME patients compared with HC (Nguyen et al., 2017; Nguyen et al., 2016). In addition, we found PregS-evoked ionic currents through TRPM3 channels were significantly modulated by ononentin in isolated NK cells from HC compared with CFS/ME patients. Indeed, isolated NK cells from CFS/ME were resistant to ononentin suggesting that PregS may activate non-TRPM3 cationic currents in CFS/ME patients. Alternatively, CFS/ME patients may express different spliced isoforms of TRPM3 that are non-sensitive to ononentin. Although we demonstrate TRPM3 channel activity dysfunction in CFS/ME patients, further investigations are required to elucidate the mechanisms involved in the impaired TRPM3 channel activity as well as the different TRPM3 isoform types that are expressed in NK cells.

Previous electrophysiological investigations have shown that TRPM3 forms an ion channel permeable to Ca2+, sodium (Na+), magnesium (Mg2+), and manganese (Mn2+) (Grimm et al., 2005; Oberwinkler et al., 2005). Ca2+ plays an important role in intracellular signalling pathways, cell differentiation and division, apoptosis, and transcriptional events. In non-excitable cells, such as immune cells, a main Ca2+ entry pathway is known as store-operated Ca2+ entry (SOCE) and some TRP ion channels are associated with this pathway. While the sub-family TRPC have been traditionally associated with this important cellular mechanism (Cheng et al., 2013; Ong et al., 2016; Salido et al., 2009), recent research has also identified TRPM3 as a component for SOCE in...
white matter of the central nervous system (CNS) (Papanikolaou et al., 2017). Upon TRPM3 channel activation, changes in \([\text{Ca}^{2+}]\)i occur, resulting in the regulation of many biological processes that correspond to an array of cells expressing this channel. TRPM3 is located and linked to vascular smooth muscle contraction, modulation of glucose-induced insulin release from pancreatic islets, detection of noxious heat in dorsal root ganglia and development of epithelial cells of the choroid plexus, as well as function of oligodendrocytes and neurons (Hoffmann et al., 2010; Oberwinkler et al., 2005; Vriens et al., 2011; Wagner et al., 2008). Therefore, dysregulation of TRPM3 family, affecting SOCE and more generally, \(\text{Ca}^{2+}\) signalling has significant implications for cell regulatory machinery.

Significant reduction in NK cell cytotoxicity is a consistent feature reported in CFS/ME patients (Brenu et al., 2012; Brenu et al., 2011; Curriu et al., 2013; Hardcastle et al., 2015; Huth et al., 2016a; Klimas et al., 1990; Maher et al., 2005; Natelson et al., 2002; Nijs & Frémont, 2008; Sharpe et al., 1991; Siegel et al., 2006; Stanietsky & Mandelboim, 2010). NK cell cytotoxic activity is a \(\text{Ca}^{2+}\)-dependent process, which drives the intracellular microtubule reorganisation, polarisation of cytoplasmic granules, release of lytic proteins and the creation of the immune synapse (Anasetti et al., 1987; Henkart, 1985). Moreover, \(\text{Ca}^{2+}\)-dependent cytotoxic processes allow for the production and recruitment of lytic proteins (Voskoboinik et al., 2015). Following cytotoxic granule
### Data analysed

|               | Sensitive | Insensitive | Total |
|---------------|-----------|-------------|-------|
| HC            | 16        | 17          | 33    |
| CFS/ME        | 2         | 26          | 28    |
| **Total**     | **18**    | **43**      | **61**|

| Percentage of row total | Sensitive | Insensitive |
|-------------------------|-----------|-------------|
| HC                      | 48.48%    | 51.52%      |
| CFS/ME                  | 7.14%     | 92.86%      |

**Fig. 3** (See legend on next page.)
delivery to the immune synapse, the formation of perforin pores and granzyme-induced cell apoptosis are highly dependent on Ca$^{2+}$ (Orrenius et al., 2003; Voskoboinik et al., 2005). Previous studies have reported impaired Ca$^{2+}$ signalling in NK cells from CFS/ME patients demonstrated through changes to ERK1/2 and mitogen-activated protein kinase (MAPK) pathways (Chacko et al., 2016; Huth et al., 2016b). CFS/ME patients have significantly decreased ERK1/2 following incubation with K562 cells (Huth et al., 2016b). ERK1/2 is activated in a Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-dependent manner that may also be associated with cytoplasmic Ca$^{2+}$ ion levels through activation of TRPM3 (Lee et al., 2003). In the absence of phosphatidylinositol 4,5-bisphosphate (PIP$_2$), TRPM3 is not activated, resulting in reduced cytosolic Ca$^{2+}$ (Tóth et al., 2015). Previous research completed by Nguyen and colleagues reported that the expression of TRPM3 ion channel in and [Ca$^{2+}$], were significantly reduced in NK cells from CFS/ME patients (Nguyen et al., 2017). In addition, ERK1/2 requires Ca$^{2+}$ as the final activator to initiate NK cell lysis (Huth et al., 2016b). Changes in Ca$^{2+}$ signalling may impair cytokine production, including Interferon (IFN)-γ and Tumor Necrosis Factor (TNF), therefore interfering with systemic inflammation and anti-tumour responses (Romee et al., 2013). Previous investigations have reported both an increased and decreased inflammatory profile of CFS/ME patients along with reduced IFN-γ (Klimas et al., 1990; Lorusso et al., 2009). TRPM3-related Ca$^{2+}$ dysfunction may then result in a reduction of [Ca$^{2+}$], which may lower the function and cytotoxic capacity of the NK cells in CFS/ME patients.

Importantly, TRPM3 ion channels have a role in the detection of heat and in pain transmission in the CNS (Held et al., 2015). TRPM3 has been previously identified as a nociceptor channel involved in acute heat sensing and inflammatory heat hyperalgesia, and thus as a potential target for analgesic treatments (Vriens et al., 2011). Dysregulation of thermoregulatory responses has been reported in CFS/ME patients (Wyler et al., 2007). Generalised pain is a characteristic of CFS/ME and occurs in the absence of overt tissue damage, and this is suggestive of potential CNS impairments (Barnden et al., 2015; Barnden et al., 2016; Shan et al., 2016; Shan et al., 2017). Our present findings suggest TRPM3 ion channels may be involved in the pathomechanism of CFS/ME and hence have a possible role in nociception and thermoregulation.

While this study provides evidence of TRPM3 channel activity dysfunction in CFS/ME patients, this study is not without limitations with the low sample numbers. Indeed, these findings need to be validated in a larger cohort of patients. Additionally, the use of high and single doses of PregS and Ononetin may reduce the drugs specificity and selectivity and consequently modulate other non-TRPM3 cationic currents. Further investigations are also required to assess the impact of PregS and Ononetin on the different TRPM3 isoforms. This could explain the differences observed between HC and CFS/ME patients as well as within groups. Finally, TRPM4, TRPM5, TRPM2 and TRPM7 surface expression has been reported on B cells, bone marrow cells, splenic cells, lymph node B cells and T and mast cells (Zierler et al., 2017), suggesting our recent findings may also be pertinent to other TRPM channel functions and Ca$^{2+}$-mediated roles, such as SOCE.

Conclusions

We have demonstrated impaired TRPM3 activity in CFS/ME patients through electrophysiological investigations in NK cells after modulation with PregS and ononetin. As TRPM3 is widely distributed in the body, particularly brain, eye, cardiovascular system, gastrointestinal system and pancreas, we assert that widespread changes in TRPM3 function in key body systems may contribute to CFS/ME. We suggest changes to Ca$^{2+}$ ion concentration in the cytosol and intracellular stores result from changes in TRPM3 function, which may impact NK cellular functions. Therefore, Ca$^{2+}$ signalling pathways could be an alternative therapeutic target in CFS/ME because of their importance in various cellular processes. This investigation confirms the potential role of TRPM3 ion channels in the aetiology and pathomechanism of CFS/ME, and could suggest potential therapeutic targets and/or prognostic markers.
Abbreviations
[Ca2+]i: Intracellular Ca2+ concentration; ADCC: Antibody-dependent cellular cytotoxicity; BMI: Body Mass Index; Ca2+: Calcium; CCC: Canadian Consensus Criteria; CDC: Centers for Disease Control and Prevention; CFS/ME: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis; CNS: Central nervous system; EDTA: Ethylenediaminetetraacetic acid; ERK: Extracellular signal-regulated kinases; HC: Healthy controls; ICC: International Consensus Criteria; IFN: Interferon; MAPK: Mitogen-activated protein kinase; Mg2+: Magnesium; Mn2+: Manganese; Na+: Sodium; NCNED: National Centre for Neuroimmunology and Emerging Diseases; NK: Natural killer; PBMCs: Peripheral blood mononuclear cells; PI, K: Phosphatidylinositol-4, 5-bisphosphate 3-kinase; PIP2: Phosphatidylinositol 4,5-bisphosphate; PregS: Pregnenolone sulfate; SF-36: 36-Item Short Form Survey; SNPs: Single Nucleotide polymorphisms; SOCE: Store-operated Ca2+ entry; TNF: Tumour necrosis factor; TRP: Transient Receptor Potential; TRPA: Transient Receptor Potential Ankyrin; TRPC: Transient Receptor Potential Canonical; TRPM: Transient Receptor Potential Melastatin; TRPML: Transient Receptor Potential Mucolipin; TRPP: Transient Receptor Potential Polycystin; TRPV: Transient Receptor Potential Vanilloid.

Funding
This study was supported by the Mason Foundation, McCusker Charitable Foundation, Stafford Fox Medical Research Foundation, Mr. Douglas Stutt, Alison Hunter Memorial Foundation, Buxton Foundation, Blake Beckett Trust, Henty Donation, and the ME Charity.

Availability of data and materials
No data sets were generated for this investigation. All relevant data are present within the manuscript. Data sharing is not applicable to this article. No data sets were generated for this investigation. All relevant data are present within the manuscript.

Authors' contributions
HC, KM, SM-G and DS designed the study and wrote the manuscript with contributions from other authors. HC, NE and CB performed experiments. HC and KM performed data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All participants gave written consent to participate. Ethics approval was under Human Research Ethics Committee Griffith University.

Consent for publication
All participants gave written consent to publish.

Competing interests
The authors declare that they have no competing interest.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1 School of Medical Science, Griffith University, Gold Coast, QLD, Australia
2 The National Centre for Neuroimmunology and Emerging Diseases, Menzies Health Institute Queensland, Griffith University, Gold Coast, QLD, Australia.
3 Laboratory of Cellular Pharmacology, School of Pharmacy, Aichi-Gakuin University, Chikusa, Nagoya, Japan.

Received: 21 May 2018 Accepted: 1 August 2018
Published online: 14 August 2018

References
Anasetti C, Martin PJ, June CH, Hellstrom KE, Ledbetter JA, Rabinovitch PS, et al. Induction of calcium flux and enhancement of cytolytic activity in natural killer cells by cross-linking of the sheep erythrocyte binding protein (CD2) and the fce-receptor (CD16). J Immunol. 1987;139:1772–9.
Bamden LR, Crouch B, Kwiatek R, Burnet R, Del Fante P. Evidence in chronic fatigue syndrome for severity-dependent upregulation of prefrontal myelination that is independent of anxiety and depression. NMR Biomed. 2015;28:404–13.

Bamden LR, Kwiatek R, Crouch B, Burnet R, Del Fante P. Autonomic correlations with MRI are abnormal in the brainstem vasomotor Centre in chronic fatigue syndrome. Neuroimaging Clin. 2016;11:530–7.
Brenu EW, Hardcastle SL, Atkinson GM, van Driel ML, Kreijkamp-Kaspers S, Ashton KJ, et al. Natural killer cells in patients with severe chronic fatigue syndrome. Auto Immun Highlights. 2013;4:669–80.
Brenu EW, van Driel ML, Staines DR, Ashton KJ, Hardcastle SL, Keane J, et al. Longitudinal investigation of natural killer cells and cytokines in chronic fatigue syndrome/myalgic encephalomyelitis. J Transl Med. 2012;10:88.
Brenu EW, van Driel ML, Staines DR, Ashton KJ, Ramos SB, Keane J, et al. Immunological abnormalities as potential biomarkers in chronic fatigue syndrome/myalgic encephalomyelitis. J Transl Med. 2011;9:81.
Carruthers BM, van de Sande MJ, De Meirleir KL, Nalmas NG, Broderick G, Mitchell T, et al. Myalgic encephalomyelitis: international consensus criteria. J Intern Med. 2012;270:327–38.
Chacko A, Staines DR, Johnston SC, Marshall-Gradisnik SM. Dysregulation of protein kinase gene expression in NK cells from chronic fatigue syndrome/myalgic encephalomyelitis patients. Gene Regul Syst Biol. 2016;10:85–93.
Cheng KT, Ong HL, Liu X, Ambatkatar IS. Contribution and regulation of TRPC channels in store-operated Ca2+ entry. Curr Top Membr. 2013;71:169–79.
Clapham DE, Runnels LW, Strubing C. The TRP ion channel family. Nat Rev Neurosci. 2012;12:387–96.
Cooper MA, Fehniger TA, Jagiura MA. The biology of normal human killer-cell subsets. Trends Immunol. 2001;22:633–40.
Curiu M, Carrillo J, Massanella M, Riguà J, Alegre J, Poujé J, et al. Screening NK-, B- and T-cell phenotype and function in patients suffering from Chronic Fatigue Syndrome. J Transl Med. 2013;11:68.
Fruhwald J, Camacho Londoño J, Dembla S, Mannebach S, Lis A, Drews A, et al. Alternative splicing of a protein domain indispensable for function of transient receptor potential melastatin 3 (TRPM3) ion channels. J Biol Chem. 2012;287:36663–72.
Fukuda K, Strauss SE, Hickie I, Sharpe MC, Dobbs JJ, Komaroff A. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International chronic fatigue syndrome study group. Ann Intern Med. 1994;121:1593–9.
Gees M, Colsoot B, Nilius B. The Role of Transient Receptor Potential Cation Channels in Ca2+ Signalling. Cold Spring Harb Perspect Biol. 2010 [cited 2018 May 3];2. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2944357/
Grimm C, Kraft R, Schütz G, Harteneck C. Activation of the melanin-related cation channel TRPML3 by D-erythro-sphingosine (corrected). Mol Pharmacol. 2005;67:798–805.
Hardcastle SL, Brenu EW, Johnston S, Nguyen T, Huth T, Wong N, et al. Characterisation of cell functions and receptors in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME). BMC Immunol. 2015 [cited 2018 May 3];14. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4650911/
Harteneck C. Pregnenolone sulfate: from steroid metabolite to TRP channel ligand. Molecules. 2013;18:12012–28.
Held K, Voets T, Vriens J. TRP3M3 in temperature sensing and beyond. Temperature (Austin). 2015;2:201.
Held K, Voets T, Vriens J. TRP3M3 in temperature sensing and beyond. Temperature (Austin). 2015;2:201–13.
Henkart PA. Mechanism of lymphocyte-mediated cytotoxicity. Annu Rev Immunol. 1985;3:31–58.
Hoffmann A, Grimm C, Kraft R, Goldbaum O, Wrede A, Nolte C, et al. TRPM3 is expressed in sphingosine-responsive myelinating oligodendrocytes. J Neurochem. 2010;114:654–65.
Holakova B, Grycova L, Jirku M, Sulc M, Bumba L, Teisinger J, Cimbalov and S100A1 protein interact with N terminus of TRPM3 channel. J Biol Chem. 2012;287:16645–55.
Huth TK, Brenu EW, Ramos S, Nguyen T, Broadley S, Staines D, et al. Pilot study of natural killer cells in chronic fatigue syndrome/Myalgic encephalomyelitis and multiple sclerosis. Scand J Immunol. 2016a;83:44–51.
Huth TK, Staines D, Marshall-Gradisnik S. ERK1/2, MEK1/2 and p38 downstream signalling molecules impaired in CD55dimCD16+ and CD55brightCD16dim/− natural killer cells in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. J Transl Med. 2016b [cited 2018 May 10];14. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4859077/
Janson LA, Kot B, Surnqvist M, Brown A, Evans M, Jantke R, et al. Chronic fatigue syndrome and Myalgic encephalomyelitis: toward an empirical case definition. Health Psychol Behav Med. 2015;3:80–9.
Kothe M, Cocks D, Vorselen D, Chen K, Brehm A, G öek M, et al. Natural killer cell subsets. Trends Immunol. 2001;22:633–40.
Kothe M, Cocks D, Vorselen D, Chen K, Brehm A, G öek M, et al. Natural killer cell subsets. Trends Immunol. 2001;22:633–40.
Kass GE, Orrenius S. Calcium signaling and cytotoxicity. Environ Health Perspect. 1999;107:25–35.
Klimas NG, Salvato FR, Morgan R, Fletcher MA. Immunologic abnormalities in chronic fatigue syndrome. J Clin Microbiol. 1990;28:1403–10.
Lanier LL. Natural killer cell receptor signaling. Curr Opin Immunol. 2003;15:308–14.
Lee N, Chen J, Sun L, Wu S, Gray KB, Rich A, et al. Expression and characterization of human transient receptor potential melastatin 3 (hTRPM3). J Biol Chem. 2003;278:20890–7.
Lorussou L, Mikhaylova SV, Capelli E, Ferrari D, Ngonga GK, Ricevuti G. Immunological aspects of chronic fatigue syndrome. Autoimmun Rev. 2009;8:287–91.
Maher KJ, Klimas NG, Fletcher MA. Chronic fatigue syndrome is associated with diminished intracellular perforin. Clin Exp Immunol. 2003;142:505–11.
Marshall-Gradisnik S, Huth T, Chacko A, Johnston S, Smith P, Staines D. Natural killer cells and single nucleotide polymorphisms of specific ion channels and receptor genes in myalgic encephalomyelitis/chronic fatigue syndrome. Appl Clin Genet. 2016;9:39–47.
Miao AD, Shepherd AJ, Mohapatra DP. Nocturnal TRP Channels: Sensory Detectors and Transducers in Multiple Pain Pathologies. Pharmaceuticals (Basel). 2016 [cited 2018 May 16]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5198047/
Moretta L. Dissecting CD56dim human NK cells. Blood. 2010;116:3689–91.
Munoz NM, Leff AR. Highly purified selective isolation of eosinophils from human peripheral blood by negative immunomagnetic selection. Nat Protoc. 2006;1:16–20.
Natelson BH, Hagghighi MH, Porzo NM. Evidence for the presence of immune dysfunction in chronic fatigue syndrome. Clin Daqgn Lab Immunol. 2002;9:747–52.
Nguyen T, Johnston S, Clarke L, Smith P, Staines D, Marshall-Gradisnik S. Impaired calcium mobilization in natural killer cells from chronic fatigue syndrome/myalgic encephalomyelitis patients is associated with transient receptor potential 3 ion channels. Clin Exp Immunol. 2017;187:284–93.
Nguyen T, Staines D, Nilus B, Smith P, Marshall-Gradisnik S. Novel identification and characterisation of transient receptor potential melastatin 3 ion channels on natural killer cells and B lymphocytes: effects on cell signalling in chronic fatigue syndrome/Myalgic encephalomyelitis patients. Biol Res. 2016;49:27.
Nijs J, Frémont J. Intracellular immune dysfunction in myalgic encephalomyelitis/chronic fatigue syndrome: state of the art and therapeutic implications. Expert Opin Ther Targets. 2008;12:281–9.
Nilus B. TRP channels in disease. Biochim Biophys Acta. 2007;1729:2085–12.
Nilus B, Voets T. A TRP channel-steroid marriage. Nat Cell Biol. 2008;10:1383–9.
Oberwinkler J, Philipp SE. TRPM3. Handb Exp Pharmacol. 2014;222:427–9.
Ong HL, de Souza LB, Ambudkar IS. Role of TRPC channels in store-operated calcium entry. Adv Exp Med Biol. 2016;898:87–109.
Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol. 2003;4:552–66.
Papanikolaou M, Lewis A, Butt AM. Store-operated calcium entry is essential for fibroblast secondary metabolites potently and selectively block TRPM3. Br J Pharmacol. 2013;168:1835–50.
Thiel G, Müller L, Rössler OG. Signal transduction via TRPM3 channels in pancreatic β-cells. J Mol Endocrinol. 2013;50:R75–83.
Thöö BL, Oberwinkler J, Voets T. Phosphoinositide regulation of TRPM channels – TRPM3 joins the club! Channels (Austin). 2015;10:83–5.
Vivier E, Tomassello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008;9:503–10.
Voets T, Talavera K, Owsianik G, Nilus B. Sensing with TRP channels. Nat Chem Biol. 2005;1:85–92.
Voskoboinik I, Thia MC, Fletcher J, Ciccone A, Browne K, Smyth MJ, et al. Calcium-dependent plasma membrane binding and cell lysis by perforin are mediated through its C2 domain: a critical role for aspartate residues 429, 435, 483, and 485 but not 491. J Biol Chem. 2005;280:8426–34.
Voskoboinik I, Whistock JC, Trapani JA. Perforin and granzymes: function, dysregulation and human pathology. Nat Rev Immunol. 2015;15:388–400.
Vriens J, Owsianik G, Hofmann T, Philipp SE, Stab J, Chen X, et al. TRPM3 is a nociceceptor channel involved in the detection of noxious heat. Neuron. 2011;70:482–94.
Wagner TF, Loch S, Lambert S, Straub I, Mannebach S, Mathar I, et al. Transient receptor potential M3 channels are ionotropic steroid receptors in pancreatic β cells. Nat Cell Biol. 2008;10:1421–30.
Wyller VB, Godang K, Marklid K, Saul JP, Thaulow E, Wallé E. Abnormal thermoregulatory responses in adolescents with chronic fatigue syndrome: relation to clinical symptoms. Pediatrics. 2007;120:e29–37.
Zierler S, Hampe S, Nadolni W. TRPM channels as potential therapeutic targets against pro-inflammatory diseases. Cell Calcium. 2017;67:105–15.