Impaired TRPM3-dependent calcium influx and restoration using Naltrexone in natural killer cells of myalgic encephalomyelitis/chronic fatigue syndrome patients

Natalie Eaton-Fitch1,2,3*, Stanley Du Preez1,2,3, Hélène Cabanas3,4, Katsuhiko Muraki3,5, Donald Staines2,3 and Sonya Marshall-Gradisnik2,3

Abstract

Background: Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a serious disorder of unknown aetiology. While the pathomechanism of ME/CFS remains elusive, reduced natural killer (NK) cell cytotoxic function is a consistent immunological feature. NK cell effector functions rely on long-term sustained calcium ($Ca^{2+}$) influx. In recent years evidence of transient receptor potential melastatin 3 (TRPM3) dysfunction supports the hypothesis that ME/CFS is potentially an ion channel disorder. Specifically, reports of single nucleotide polymorphisms, low surface expression and impaired function of TRPM3 have been reported in NK cells of ME/CFS patients. It has been reported that mu (µ)-opioid receptor (µOR) agonists, known collectively as opioids, inhibit TRPM3. Naltrexone hydrochloride (NTX), a µOR antagonist, negates the inhibitory action of µOR on TRPM3 function. Importantly, it has recently been reported that NTX restores impaired TRPM3 function in NK cells of ME/CFS patients.

Methods: Live cell immunofluorescent imaging was used to measure TRPM3-dependent $Ca^{2+}$ influx in NK cells isolated from n = 10 ME/CFS patients and n = 10 age- and sex-matched healthy controls (HC) following modulation with TRPM3-agonist, pregnenolone sulfate (PregS) and TRPM3-antagonist, ononetin. The effect of overnight (24 h) NTX in vitro treatment on TRPM3-dependent $Ca^{2+}$ influx was determined.

Results: The amplitude (p < 0.0001) and half-time of $Ca^{2+}$ response (p < 0.0001) was significantly reduced at baseline in NK cells of ME/CFS patients compared with HC. Overnight treatment of NK cells with NTX significantly improved TRPM3-dependent $Ca^{2+}$ influx in ME/CFS patients. Specifically, there was no significance between HC and ME/CFS patients for half-time response, and the amplitude of $Ca^{2+}$ influx was significantly increased in ME/CFS patients (p < 0.0001).

Conclusion: TRPM3-dependent $Ca^{2+}$ influx was restored in ME/CFS patients following overnight treatment of isolated NK cells with NTX in vitro. Collectively, these findings validate that TRPM3 loss of function results in altered $Ca^{2+}$ influx supporting the growing evidence that ME/CFS is a TRP ion channel disorder and that NTX provides a potential therapeutic intervention for ME/CFS.

*Correspondence: ncned@griffith.edu.au
1 School of Pharmacy and Medical Sciences, Griffith University, Gold Coast, Australia
Full list of author information is available at the end of the article

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Keywords: Myalgic encephalomyelitis, Chronic fatigue syndrome, Natural killer cells, Transient receptor potential melastatin 3, Calcium, Naltrexone

Background

Transient receptor potential (TRP) channels are a superfamily of polymodal sensors present in many tissue and cell types, and their involvement in sensory reception is well known [1]. The TRPM3 (melastatin) channel acts as a non-selective cation channel that possesses high permeability for calcium (Ca$^{2+}$) [2, 3]. The activation of TRPM3 channels leads to a cascade of intracellular pathways to facilitate cell function in both excitable and non-excitable cells [4]. TRPM3 shares the typical structure of all TRP channels with six transmembrane domains with both the N-terminal and C-terminal domains located within the cytosol. The endogenous neurosteroid, pregnenolone sulfate (PregS) (EC$_{50}$ = 12–32 µM) is commonly used in research to activate TRPM3 ion channel activity to induce an increase in intracellular Ca$^{2+}$ concentration [5] while ononetin (IC$_{50}$ = 0.2–2 µM) rapidly and reversibly inhibits PregS-evoked ionic currents [6]. Changes in the function or activation of TRPM3 would result in changes to Ca$^{2+}$ and as a consequence impacts cell function.

Ca$^{2+}$ is a versatile, universal secondary messenger that facilitates vital biological processes in all cell types. Ca$^{2+}$ ions are in part responsible for promoting intracellular signalling pathways, cell differentiation and proliferation, programmed cell death and translational events in many cell types [7]. The intracellular concentration of Ca$^{2+}$ ([Ca$^{2+}$]$_{cytosol}$) is tightly controlled in homeostatic conditions. Growing evidence suggests that the expression of TRPM3 on natural killer (NK) cells indicates a role of TRPM3 in the regulation of Ca$^{2+}$ in immune cells [8–11]. In addition to the processes described above, the influx of Ca$^{2+}$ in NK cells is important for microtubule rearrangement resulting in granule polarisation, formation of the immune synapse, release of cytolytic granules and granzyme dependent target cell death [12, 13]. Therefore, disturbances in Ca$^{2+}$ homeostasis in lymphocytes can adversely impact immune cell functions and consequently lead to immune diseases and immunodeficiencies [14].

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a severe multisystemic illness hallmarked by post-exertional neuroimmune exhaustion accompanied by a range of symptoms that are broadly categorised as neurological, immunological, cardiovascular, gastrointestinal and endocrinological [15, 16]. The pathomechanism underlying ME/CFS is unknown, however immunological disturbances have been well reported with emphasis on NK cell dysfunction [17]. In 2016, five single nucleotide polymorphisms (SNPs) were identified in the TRPM3 gene (rs6560200, rs1106948, rs12350232, rs11142822, rs1891301) in NK cells from ME/CFS patient [18]. Subsequently, flow cytometry revealed a significant reduction in surface expression of TRPM3 and preliminary data reported significantly reduced Ca$^{2+}$ mobilisation in NK cells of ME/CFS patients compared with healthy controls (HC) [8, 9]. Electrophysiological experiments have demonstrated a loss of TRPM3 ion channel function in NK cells of ME/CFS patients compared with HC [10, 11]. Thus, there is a growing body of evidence suggesting the pathomechanism of ME/CFS may be a channelopathy [8, 10, 19]. Therefore, a working hypothesis for the pathophysiology of ME/CFS is that impaired TRPM3 ion channel function limits the influx of Ca$^{2+}$ and downstream signalling pathways, and consequentially impedes cell function.

Currently, no universal treatment exists for ME/CFS, instead treatment is aimed at alleviating targeted symptoms by administering central nervous system (CNS) stimulants, sleeping medications and analgesics [20]. Importantly, TRPM3 has been identified as a thermosensitive and nociceptive channel implicated in detecting pain and heat produced during inflammation with emphasis on heat hyperalgesia and general pain transmission in the CNS [21]. Moreover, the wide spread expression of TRPM3 in areas of the CNS including the cerebrum, brain stem, hypothalamus and hippocampus suggests a role in muscle coordination, cognitive behaviour and memory [8, 22]. These functional properties overlap considerably with symptoms of ME/CFS as epidemiological data has shown ME/CFS patients experience sensitivity for pain with up to 94% of patients reporting muscle aches and pains while 84% report multi-joint pain [23]. Therefore, TRPM3 dysfunction provides a potential therapeutic target for the treatment of ME/CFS.

Recent research has found that naltrexone (NTX) at low doses (LDN) improves the symptoms of ME/CFS [24]. Electrophysiology experiments in ME/CFS patients reported a potential benefit in restoring impaired TRPM3 ion channel activity following treatment of isolated NK cells with NTX [25]. Interestingly, NTX is a long-lasting antagonist to a subfamily of receptors known as opioid receptors (OR) used to treat opioid and alcohol dependence [26]. Mu-OR (µOR) belong to a large and diverse group of membrane receptors known as G protein coupled receptors (GPCRs). GPCRs are widely distributed...
in the human body where activated G proteins can interact and regulate many effectors or molecules such as Ca2+ sensors, ion channels and protein kinases [27]. The activity of TRPM3 can be inhibited by the activation of G protein subunits and direct binding of these subunits to the channel [28]. NTX specifically inhibits the μOR, thus negating these inhibitory effects on TRPM3 [28–30]. Whole-cell patch clamp electrophysiology has been used to investigate the function of TRPM3 ion channels in ME/CFS patients who regularly administered LDN. TRPM3 function yielded similar results between ME/CFS patients taking LDN and HC [31]. As NTX restored TRPM3 ion channel function, it may in turn re-establish Ca2+ influx in NK cells leading to normalised downstream signalling pathways and immune functions.

While the function of TRPM3 ion channels have been determined in NK cells of ME/CFS patients using the whole-cell patch clamp technique, this current investigation was designed as a complementary study to evaluate Ca2+ influx in order to validate the loss of TRPM3 channel function using an additional method to ensure full potential of research. We aimed, for the first time, to investigate the speed and maximum response of TRPM3-dependent Ca2+ influx in ME/CFS patients compared with HC using an immunofluorescent technique. This current investigation provides further insights of the potential therapeutic role of NTX by examining the effect of overnight in vitro treatment on TRPM3-dependent Ca2+ influx.

**Methods**

**Recruitment**

ME/CFS patients and HC were contacted using the National Centre for Neuroimmunology and Emerging Diseases (NCNED) patient database. Participants were screened in accordance to the Canadian Consensus Criteria (CCC) and International Consensus Criteria (ICC) case definitions for ME/CFS using a comprehensive online questionnaire. ME/CFS patients were included if they met the CCC or ICC case definitions for diagnosis and reported being diagnosed by a physician. Potential eligible participants were invited to volunteer in this project. Of those contacted using the NCNED database, 10 ME/CFS patients from South-East Queensland were invited to volunteer in this project. ME/CFS patients were age- and sex-matched with HC. The HC group was defined as those who have not been diagnosed with any underlying illness and are non-fatigued. All participants were aged between 18 and 60 years, had a BMI between 18.5 and 29.9 (kg/m2) and were non-smokers.

Participants were excluded if they reported a history of alcohol abuse, cardiovascular disease, diabetes, metabolic syndrome, thyroid disease, malignancies, insomnia, chronic fatigue, and if they were pregnant or breastfeeding. Furthermore, all participants were excluded if they reported the use of pharmacological agents that directly or indirectly interfere with TRPM3 ion channel function as well as Ca2+ signalling and immune cell activity. This investigation was approved by the Gold Coast Human Research Ethics Committee (HREC/2019/QGC/56469) and Griffith University Human Research Ethics Committee (GU/2019/1005).

**Participant data collection and sample collection**

All participants completed an online questionnaire to provide sociodemographic background, medical history, medications, and symptom history for ME/CFS patients. The 36-item short form health survey (SF-36) and World Health Organization (WHO) Disability Assessment Schedule (DAS) were used to determine level of disability and quality of life (QoL) [32, 33].

Between 7:00 a.m. and 11:00 a.m. at collection locations including Griffith University, Royal Brisbane and Women’s Hospital, Robina Hospital, Toowoomba Base Hospital, Sunshine Coast University Hospital and Tweed Hospital, a total of 84 ml of whole, non-fasted blood was collected from consenting participants into ethylenediaminetetraacetic acid (EDTA) tubes via venepuncture by a qualified phlebotomist. Four ml of EDTA whole blood was used for red blood cell count, white blood cell count and granulocyte cell count within four hours of blood collection for each participant.

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

Samples were deidentified using a unique code and delivered to the laboratory. Eighty ml of blood was used for peripheral blood mononuclear cells (PBMC) isolation by density gradient centrifugation using Ficoll (GE Healthcare, Uppsala, Sweden) as previously described [34]. PBMCs were stained with trypan blue (Invitrogen, Carlsbad, 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 the EasySep Negative Human NK cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada). Approximately 2.5–4 × 10^6 cells NK cells were isolated and used for Ca2+ imaging experiments. NK cell purity was defined by CD3−CD56+ surface expression using flow cytometry (Additional file 1: Figure S1). Specifically, NK cells were incubated for 20 min at room temperature in the presence of CD3 PE-Cy7 (5 μl/test) and CD56 APC (20 μl/test) monoclonal antibodies (Becton Dickinson [BD] Biosciences, San Jose, CA, USA). Cells were acquired at 10,000 events using the Accuri C6 flow
cytometer (BD Biosciences, San Diego, CA, USA). The average NK cell purity (%) for this study was 86.73±9.71 (Additional file 1: Figure S2).

Interleukin-2 stimulation and in vitro drug treatment
Freshly isolated NK cells (~4.5×10^6 cells) were stimulated with 20 IU/ml of recombinant human IL-2 (Miltenyi Biotec, BG, Germany) and treated with 200µM NTX (Sigma-Aldrich, St. Louis, MO, USA) for 24 h at 37 °C with 5% CO2 in Roswell Park Memorial Institute Medium (RPMI)-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA, USA). The use of IL-2 is designed to support the culture of NK cells overnight.

Calcium imaging
NK cells (2.0×10^5 NK cells/well) were immobilised using Corning Cell-Tak™ Cell and Tissue Adhesive (BD Biosciences, San Jose, CA, USA) coated 24 well plate (Ibidi, Lochhamer Schlag, Germany). NK cells were incubated for 30 min at 37 °C with 1 µM Fluo-8 (Abcam, Cambridge, UK) with 0.02% Pluronic F127 (Thermofisher, Waltham, Massachusetts, United States). Cells were then washed in indicator free solution and incubated for a further 30 min at room temperature to allow for de-esterification of Fluo-8 AM. All experiments were performed at room temperature (23±2 °C) and in a 1.8 mM Ca^{2+} solution. The Ca^{2+} solution was prepared in milliQ water and contained: NaCl 140 mM, KCl 5.4 mM, CaCl2 1.8 mM, MgCl2 1.0 mM, HEPES 10 mM, NaOH was used to adjust pH to 7.4 and osmolality was adjusted to 300 mOsM/L using D-Glucose.

NK cells were imaged using the Nikon A1R microscope, and fluorescence emissions were monitored using a iXon Life 888 Electron Multiplying CCD camera. Baseline Ca^{2+} was imaged for 2 min before cells were stimulated with 50 µM PregS followed by 1 µM ionomycin for 3 min each. Alternatively, due to the natural decline in fluorescence following peak PregS-dependent Ca^{2+} flux, the effect of ononetin was measured using a separate protocol. In order to desensitize TRPM3 channels, baseline Ca^{2+} was imaged for 3 min in the presence of 4 µM of ononetin followed by 4 µM ononetin + 50 µM PregS, followed by 50 µM PregS and lastly 1 µM ionomycin. Ca^{2+} flux measurements in response to ononetin was added as supplementary material to demonstrate effective inhibition of 50 µM of PregS (Additional file 1: Figure S3). The use of 1 µM of ionomycin aligns with previous literature [9, 35, 36]. The concentration of PregS and ononetin was determined using dose response analysis (Additional file 1: Figure S4).

Variations in fluorescence were measured using NIS Research Elements (Nikon, NIS-Elements V5.2, Tokyo, Japan). Three measurements were reported to represent Ca^{2+} influx. Amplitude value represents the peak of Ca^{2+} influx curve upon activation by PregS and from this the half-time of maximum (T1/2) response was also determined. The rate of Ca^{2+} influx was determined using the initial slope of the curve as calculated using OriginLabs. PregS dependent Ca^{2+} influx measurements were normalised against ionomycin response curves to give the proportion of maximum response.

Chemicals and reagents
PregS (product code: RDS537650) and ononetin (product code: RDS514350) were purchased from In Vitro Technologies and stock solutions were prepared at 100 mM in 100% DMSO and stored according to the suppliers' instructions. NTX (product code: N3136-100MG) was prepared fresh prior to each experiment and constituted at 100 mM in distilled water. IL-2 was purchased from Miltenyi Biotec (product code: 130-097-744) stored at 100,000 IU stock in distilled water for up to 1 month. Ionomycin was purchased from Sigma Aldrich (product code: I9657-1MG) and resuspended at 10 mg/ml in 100% DMSO and stored for up to 1-month at −20 °C. Fluo-8 was purchased from abcam (product code: ab142773) and stored at 1 mM aliquots for up one month in 100% DMSO. Flow cytometric antibodies were purchased from BD Biosciences, CD3 PE-Cy7 (product code: 563423) and CD56 APC (product code: 555518).

Statistical analysis
Shapiro–Wilk test was used to assess normality of distribution of investigated parameters. Additional visual observation of histogram plots was completed. Data presented as mean±standard deviation (SD) unless otherwise stated. Differences were tested using the Mann–Whitney U non-parametric T test. Flow cytometry data were exported from Accuri C6 software. Fluorescence and time-course data were exported from NIS-Elements Advanced Research version 5.2. Statistical analysis was done using GraphPad Prism V8 (Graphpad Software Inc., Version 8, La Jolla, CA, USA) and OriginLabs (OriginLab Corporation, Northampton, MA, USA). Significance was set at p<0.05.

Results
Participants and disease characteristics
During the study period of July 2021 to October 2021, 10 ME/CFS patients and 10 age- and sex-matched HC participated in this project. All ME/CFS patients reported symptoms fulfilling the CCC case definition and no other fatigue-related illness that may account
for their symptoms. Table 1 includes demographic data of the participants. The average age of participants was 44.10 ± 10.39 and 43.90 ± 10.71 for HC and ME/CFS patients, respectively. All participants who volunteered in this project were female except n=1. The average BMI of HC were within normal range (18.5−24.9) measuring at 23.68 ± 3.96. While the average BMI of ME/CFS patients were above normal range at 25.74 ± 5.49. There was a significant difference in employment status between HC and ME/CFS patients (p=0.009). Six of the included ME/CFS patients reported unemployment due to illness and/or disability.

The SF-36 and WHO DAS surveys were used to assess QoL in ME/CFS patients compared with HC. As reported in Table 2, means scores were significantly reduced in ME/CFS patients across all SF-36 domains compared with HC. Lowest SF-36 scores in ME/CFS patients were observed in limitations due to physical role (24.38 ± 19.19). Mean domain scores for the WHO DAS show a significant increase in disability in ME/CFS patients compared with HC. ME/CFS patients reported greatest difficulty in ability to maintain life activities (66.87 ± 24.66). All blood parameters were within normal range according to Queensland Health Pathology.

All ME/CFS patients successfully completed the NCNED registry questionnaire. Data extracted from relevant questionnaire responses are presented Table 3. On average, patients were 26.7 years of age at the time of diagnosis and experienced symptoms of ME/CFS for 17.7 years. Eight of the ten ME/CFS patients (80.0%) included in this present study reported an infection prior to onset of symptoms. All ME/CFS patients (100%) reported experiencing key symptoms of ME/CFS including cognitive difficulties, body pain and sleep disturbances.

**Effect of PregS on calcium influx**

Ca^{2+} influx images were obtained from isolated human NK cells prior to overnight incubation with IL-2 and NTX (Fig. 1). TRPM3-dependent Ca^{2+} influx was stimulated by 50 µM of PregS. While there was no significant difference reported for the slope of the Ca^{2+} influx curve between groups, the T1/2 response (p<0.0001) and amplitude (p<0.0001) were significantly reduced in ME/CFS patients compared with HC. The inhibition of 50 µM PregS by ononetin was also determined at baseline. Ononetin at a concentration of 4 µM effectively blocked PregS stimulation of TRPM3, however there was no statistical significance between groups (Additional file 1: Figure S3.1).

**Effect of PregS on calcium influx after NTX treatment**

Human NK cells were incubated overnight supplemented with IL-2 with and without NTX (Fig. 2). Post-24 h, Ca^{2+} influx images were obtained from both control IL-2 stimulated cells and NTX treated cells. There was a significant reduction in slope (p<0.0001), T1/2 response (p=0.0123) and amplitude (p<0.0001) in ME/CFS patients compared with HC. There was no significant difference for NTX treated cells between groups for slope and T1/2 response. A significant increase in amplitude (p<0.0001) was reported in ME/CFS patients compared with HC following NTX treatment. The inhibition of 50 µM PregS by ononetin was also determined following stimulation of NK cells with IL-2 and treatment using NTX. Ononetin at a concentration of 4 µM effectively blocked PregS stimulation of TRPM3, however there was no statistical significance between groups (Additional file 1: Figure S3.2).

**Discussion**

We report, for the first time the significant reduction in Ca^{2+} influx via TRPM3 in NK cells in ME/CFS patients compared with HC using Ca^{2+} imaging technique. This current investigation provides novel findings for the rate, or speed, and insight into the maximum TRPM3-dependent Ca^{2+} influx response in ME/CFS patients. Previous investigations have demonstrated TRPM3 channel dysfunction in isolated NK cells of ME/CFS patients compared with HC using the whole-cell patch clamp technique [10, 11]. While patch-clamp is regarded as a gold standard technique for ion channel research,

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**Table 1** Participant demographics

|                      | HC (n=10) | ME/CFS (n=10) | P-value |
|----------------------|-----------|---------------|---------|
| Age (years)          | 44.10 ± 10.39 | 43.90 ± 10.71 | 0.853   |
| Gender n (%)         | > 0.9999   |               |         |
| Female               | 9 (90.0%)  | 9 (90.0%)     |         |
| Male                 | 1 (10.0%)  | 1 (10.0%)     |         |
| BMI (kg/m²)          | 23.68 ± 3.96 | 25.74 ± 5.49  | 0.280   |
| Employment Status    |           |               |         |
| Full Time            | 6 (60.0%)  | 2 (20.0%)     | 0.0090  |
| Part Time            | 3 (30.0%)  | 1 (10.0%)     |         |
| Casual               | 1 (10.0%)  | 1 (10.0%)     |         |
| Unemployed           | 0 (0.0%)   | 0 (0.0%)      |         |
| Illness/disability   | 0 (0.0%)   | 6 (60.0%)     |         |
| Education            |           |               |         |
| Primary education    | 0 (0.0%)   | 0 (0.0%)      | 0.9985  |
| High school          | 0 (0.0%)   | 1 (10.0%)     |         |
| Undergraduate        | 5 (50.0%)  | 3 (30.0%)     |         |
| Postgraduate/doctoral| 2 (20.0%)  | 4 (40.0%)     |         |
| Other                | 3 (30.0%)  | 2 (20.0%)     |         |

Values in bold are statistically significant

HC healthy controls, ME Myalgic encephalomyelitis, CFS chronic fatigue syndrome, BMI body mass index.
TRPM3-dependent \( \text{Ca}^{2+} \) influx was not clear in our previous studies due to the small inward currents activated by PregS. Therefore, our data supports the characterisation of \( \text{Ca}^{2+} \) influx and TRPM3 activity for the pathomechanism of ME/CFS and the role for NTX as a potential intervention. The combination of electrophysiology and \( \text{Ca}^{2+} \) imaging protocols provides a comprehensive and complete analysis of TRPM3-dependent \( \text{Ca}^{2+} \) changes in NK cells of ME/CFS patients.

This current investigation reports a significant reduction in the \( T1/2 \) and amplitude of maximum TRPM3-dependent \( \text{Ca}^{2+} \) responses in ME/CFS patients compared with HC at baseline. The reduction in TRPM3-dependent \( \text{Ca}^{2+} \) influx in ME/CFS patients compared with HC validates previous research which demonstrate significant loss in TRPM3 ion channel function [10, 11, 25]. Alterations in the profile of \( \text{Ca}^{2+} \) influx are strongly associated with significant physiological consequences. Changes in \( \text{Ca}^{2+} \) influx profiles play a critical role in determining the magnitude of \( \text{Ca}^{2+} \)-dependent responses and are associated with physiological consequences [37]. It is hypothesised that TRPM3 ion channel dysfunction results in reduced \( \text{Ca}^{2+} \) influx in NK cells which has negative consequences on cytotoxic function in ME/CFS patients. Therefore, simultaneous reductions in amplitude and rate may augment effects of reduced \( \text{Ca}^{2+} \) concentration on cellular function and further research into \( \text{Ca}^{2+} \) mobilisation may elucidate the pathomechanism of ME/CFS.

Subsequently, the effect of IL-2 alone on \( \text{Ca}^{2+} \) influx was determined. The culturing of NK cells with the addition of IL-2 supports viability, preactivation, proliferation and development of cells; however, as IL-2 primes NK cells for activation this condition was used as a control for NTX treated cells [38]. The amplitude and \( T1/2 \) response were significantly reduced in IL-2 stimulated NK cells from ME/CFS patients compared with HC. Additionally, a significant reduction was reported for slope of \( \text{Ca}^{2+} \) influx in ME/CFS patients compared with HC. It is interesting that significance was reported for

| Table 2  | Participant Quality of Life, disability scores and serology |
|----------|-----------------------------------------------------------|
|          | HC                                   | ME/CFS                  | P-value   |
| SF-36 (%)|                                       |                         |
| Physical functioning | 94.0±17.29 | 37.5±30.93 | <0.0001 |
| Physical role      | 95.0±11.71 | 24.38±19.19 | <0.0001 |
| Pain              | 90.25±20.83 | 400±27.99 | 0.003    |
| General Health     | 78.75±11.69 | 29.59±18.37 | <0.0001 |
| Social functioning | 98.75±3.95 | 27.5±26.22 | <0.0001 |
| Emotional role     | 99.17±2.63 | 69.99±23.31 | 0.002    |
| Emotional wellbeing| 76.96±11.04 | 39.17±12.23 | <0.0001 |
| WHO DAS (%)        |                                       |                         |
| Communication & understanding | 1.62±2.09 | 45.83±24.29 | <0.0001 |
| Mobility           | 2.50±6.35 | 560±30.26 | <0.0001 |
| Self-care          | 0.0±0.0 | 33.13±29.47 | <0.0001 |
| Interpersonal relationships | 1.25±10.62 | 33.13±31.6 | 0.009    |
| Life activities    | 6.25±10.62 | 66.87±24.66 | <0.0001 |
| Participation in Society | 2.19±3.62 | 57.49±23.11 | <0.0001 |
| Full blood count   |                                       |                         |
| White Cell Count (×10⁹/L) | 5.97±0.79 | 5.58±0.84 | 0.247    |
| Lymphocytes (×10⁹/L) | 2.02±0.69 | 1.60±0.42 | 0.315    |
| Neutrophils (×10⁹/L) | 3.37±0.69 | 3.29±0.86 | 0.684    |
| Monocytes (×10⁹/L) | 0.44±0.10 | 0.41±0.07 | 0.436    |
| Eosinophils (×10⁹/L) | 0.14±0.11 | 0.16±0.09 | 0.579    |
| Basophils (×10⁹/L) | 0.03±0.01 | 0.04±0.01 | 0.075    |
| Platelets (×10¹²/L) | 250.40±55.41 | 255.4±31.49 | 0.684    |
| Red Cell Count (×10¹²/L) | 4.47±0.45 | 4.47±0.34 | 0.912    |
| Haematocrit         | 0.41±0.04 | 0.41±0.03 | 0.631    |
| Haemoglobin (g/L)   | 134.40±16.34 | 135.4±11.71 | 0.853    |

Values in bold are statistically significant

HC healthy controls, ME Myalgic encephalomyelitis, CFS chronic fatigue syndrome, SF-36 36-item short form survey, WHO world health organization, DAS disability assessment schedule.
Table 3  ME/CFS symptom characteristics

| Symptom                          | Yes | No  | Percentage |
|----------------------------------|-----|-----|------------|
| Age of diagnosis (Years [Mean ± SD]) | 26.70 ± 12.75 |
| Disease duration (Years [Mean ± SD]) | 17.70 ± 14.95 |
| Infectious onset, n (%)          | 8 (80.0%) |
| Cognitive difficulties           | 10 (100%) | 0 (0%) |
| Pain                             | 10 (100%) | 0 (0%) |
| Sleep disturbances               | 10 (100%) | 0 (0%) |
| Sensory disturbances             | 9 (90.0%) | 1 (10.0%) |
| Immune disturbances              | 9 (90.0%) | 1 (10.0%) |
| Gastrointestinal disturbances    | 9 (90.0%) | 1 (10.0%) |
| Cardiovascular disturbances      | 8 (80.0%) | 2 (20.0%) |
| Respiratory disturbances         | 8 (80.0%) | 2 (20.0%) |
| Thermostatic instability         | 10 (100%) | 0 (0%) |
| Urinary disturbances             | 5 (50.0%) | 5 (50.0%) |

ME Myalgic encephalomyelitis, CFS chronic fatigue syndrome, SD standard deviation, n number

The consistent reduction in slope of Ca²⁺ influx following IL-2 stimulation, but not at baseline. Ca²⁺ measurements differed between baseline and IL-2 stimulation conditions. Stimulation of NK cells with IL-2 is known to enhance NK cell cytotoxic function through Ca²⁺-dependent pathways [39, 40]. In a recent publication, the authors suggested a cross-talk between TRPM3- and IL-2-dependent cellular pathways leading to enhanced NK cell function in vitro [41]. It is therefore likely that changes in slope reported between baseline and IL-2 stimulation that are reflected in HC indicate that pathways involved in TRPM3 and IL-2 signalling may promote Ca²⁺ mobilisation in NK cells. The consistent reduction in amplitude and T1/2 of response reported in this current investigation suggests that TRPM3 dysfunction reported in ME/CFS patients impairs sufficient Ca²⁺ entry in NK cells. Changes in ion channel function may be a consequence of an unstable open channel [42] and channel stability may be a component to consider in future research.

Ca²⁺ has a critical and beneficial role in promoting the activation of NK cell function as previous investigations using Ca²⁺ blockers have led to a significant reduction in NK cell cytotoxicity [43, 44]. The coupling between receptor and ligand initiates a Ca²⁺-dependent cascade that relies on sustained and long-term influx of the cation. Ca²⁺ facilitates the phosphorylation and activation of protein kinases such as Ras, P38, phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) and mitogen activation protein kinases (MAPK) by enabling protein–protein and protein-phosphatase interactions [45–47]. The significant reduction in phosphorylation of protein kinases has been reported in activated NK cells of ME/CFS patients [48]. Therefore, changes in Ca²⁺ can either enhance, or in the case of ME/CFS, impair protein kinase phosphorylation, thus impacting functional outcomes such as cytokine production and cytotoxicity. Moreover, phosphatidylinositol 4,5-bisphosphate (PIP₂) is a critical component of numerous signalling pathways including PI3K signalling and Ca²⁺ mobilisation. The function of TRPM3 also relies on the presence and binding of PIP₂ [49]. A recent publication reported a potential association between TRPM3 dysfunction and the involvement of PIP₂ in the pathomechanism of ME/CFS [41]. Therefore, TRPM3 dysfunction may contribute to ME/CFS pathomechanism due to consequences of impaired Ca²⁺ signalling, including impeding Ca²⁺-dependent cellular pathways that result in impaired NK cells function that is consistently reported in literature.

Under homeostatic conditions, the activation of TRPM3 leads to an increase in cytosolic Ca²⁺ that is important for regulating numerous biological processes including but not limited to temperature and pain sensation, insulin secretion and vascular smooth muscle contraction [50]. Furthermore, ion channels are located on different organelles throughout cells, and mitochondrial dysfunction reported in ME/CFS patients may be attributable to ion channel dysfunction, namely TRPM3 [51]. In NK cells, store-operated Ca²⁺ entry (SOCE) is a primary form of Ca²⁺ regulation [52]. However, it is possible that TRPM3 monitors intracellular Ca²⁺ levels in lymphocytes in addition to SOCE, as observed in oligodendrocytes [53, 54]. This current investigation did not aim to assess the activity of SOCE and therefore further investigation on the relationship between TRPM3 and SOCE in NK cells is proposed. It is important to outline that within the N-terminal of TRPM3 there are two calmodulin (CaM) binding sites. CaM senses changes in [Ca²⁺] to either up- or down-regulate TRPM3 activity [55, 56]. The interaction of CaM and TRPM3 suggests strong activity in Ca²⁺ sensing that may be augmented by Ca²⁺ store depletion or by stimulation of GPCRs [57]. The activity of CaM in TRPM3 function has not been investigated in ME/CFS patients but provides an interesting target for future research to determine any problems in TRPM3-dependent Ca²⁺ homeostasis.

Currently, there is no universal treatment to improve symptoms of ME/CFS. However, new technologies may provide future avenues to further assist in characterising
TRPM3 dysfunction and impaired Ca$^{2+}$ mobilisation in ME/CFS research. Recent electrophysiology investigations have reported the restoration of TRPM3 function following in vitro treatment of isolated NK cells with NTX [25]. In this current investigation we report the restoration of TRPM3-dependent Ca$^{2+}$ influx following in vitro treatment of isolated NK cells overnight with NTX. The cellular mechanism involves the inhibition of µOR which would otherwise inhibit the activation of TRPM3 channels [28, 58]. Therefore, by negating the inhibitory action of µOR, it is hypothesised that this restores TRPM3 function, thus reinstating TRPM3-dependent Ca$^{2+}$ influx in NK cells from ME/CFS patients.

The expression of µOR has been reported in lymphocytes where they are important in the inflammatory pain response [59]. The activation of µOR by certain endogenous and synthetic opioids leads to immunosuppression [60, 61, 61, 62]. Administration of the synthetic opioid, morphine, has resulted in a significant reduction in NK cell cytotoxic activity in rats, mice and humans [63–65]. Interestingly, NTX inhibits the suppressive activity of morphine on NK cells through the µOR [66]. Research has reported that ME/CFS patients secrete insufficient opioid peptides that regulate pain and release of
cytokines [31]. Beta-endorphins (β-EP) are an endogenous opioid neuropeptide produced by many cell types including lymphocytes. The release of β-EP from lymphocytes are regulated by the activity of µOR resulting in changes to cell proliferation and immune function [67]. Interestingly, within a specific dosage window of 1–5 mg/day, it is reported that LDN increases β-EP release in NK cells resulting in increased cytotoxic activity [68, 69]. An investigation by Conti and colleagues found significantly reduced β-EP in ME/CFS patients reflecting chronic immune activation [69]. Therefore, targeting OR, with NTX, on immune cells may resolve NK cell dysfunction through ion channel regulation such as the indirect effects seen with TRPM3. Future research may aim to investigate NK cell cytotoxicity in ME/CFS patients following in vitro treatment with NTX.

TRPM3 function has previously been assessed in a group of ME/CFS patients who routinely administer

![Fig. 2](image-url)
LDN daily [31]. ME/CFS patients who take LDN yielded similar TRPM3 function to HC and no significant differences between groups were observed [31]. Medical data from a cohort of 218 ME/CFS patients administering LDN reported a positive treatment response in 73.9% of responders [24]. In a separate investigation, self-reported retrospective comparison of ME/CFS symptoms before and after LDN commencement suggested an improvement in cognitive symptoms and immune disturbances [31]. Moreover, studies with fibromyalgia patients have found that LDN significantly reduced pain, fatigue, sleep disturbances, headaches and gastrointestinal issues [70]. Collectively, these results suggest there is potential therapeutic benefit of LDN to treat TRPM3 dysfunction in ME/CFS patients.

Conclusion

ME/CFS is a serious and chronic condition that affects multiple organ systems of the human body. TRPM3 dysfunction has been consistently found in NK cells of ME/CFS, with consequential implications for Ca\(^{2+}\) signalling and cell function. TRPM3-dependent Ca\(^{2+}\) influx was significantly reduced in NK cells of ME/CFS patients compared with HC. In this current investigation the treatment of isolated NK cells with NTX restored TRPM3-dependent Ca\(^{2+}\) influx in ME/CFS patients to the extent that Ca\(^{2+}\) influx measurements were either similar to HC or significantly increased. This investigation provides additional evidence for the potential therapeutic benefit of NTX for ME/CFS.

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Authors’ contributions

NE-F performed all experiments, data analysis and writing of the manuscript. NE-F, SDP, HC, DS and SM-G designed the project. HC, DS, SM-G and SDP participated in study coordination and critically reviewed this manuscript. KM oversaw data analysis and critically reviewed study design. All authors read and approved the final manuscript.

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Data availability

Datasets analysed and/or generated during the current study are not publicly available due to confidentiality agreements but are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This project was approved by Griffith University Human Research Ethics Committee (GU:2019/1005) and Gold Coast University Hospital Human Research Ethics Committee (HREC/2019/QGC/56469). All participants provided written consent prior to participation.

Competing interests

The authors declare that they have no competing interests.

Author details

1 School of Pharmacy and Medical Sciences, Griffith University, Gold Coast, Australia. 2 National Centre for Neuroimmunology and Emerging Diseases, Menzies Health Institute Queensland, Griffith University, Gold Coast, Australia. 3 Consortium Health International for Myalgic Encephalomyelitis, Griffith University, Gold Coast, Australia. 4 Université de Paris, INSERM U944 and CNRS UMR 7212, Institut de Recherche Saint Louis, Hôpital Saint Louis, APHP, 75010 Paris, France. 5 Laboratory of Cellular Pharmacology, School of Pharmacy, Aichi-Gakuin University, NAGOY, Japan.

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1 Additional file 1. Supplementary figures.

References

1. Flockerzi V, Nilius B. TRPs: Truly Remarkable Proteins. In: Nilius B, Flockerzi V, editors. Mamm. Transient Recept. Potential TRP Cation Channels Vol. 1. Berlin, Heidelberg: Springer; 2014. p. 1–12. https://doi.org/10.1007/978-3-642-54215-2_1.

2. Oberwinkler I, Lis A, Gieleh KM, Flockerzi V, Philipp SE. Alternative splicing switches the divalent cation selectivity of TRPM3 channels. J Biol Chem. 2005;280:22540–8. https://doi.org/10.1074/jbc.M503092200.

3. Grimm C, Kraft R, Sauерbruch S, Schultz G, Harteneck C. Molecular and functional characterization of the melastatin-related cation channel TRPM3. J Biol Chem. 2003;278:21493–501. https://doi.org/10.1074/jbc.M300945200.

4. Owiszcz G, Talavera K, Voets T, Nilius B. Permeation and selectivity of TRP channels. Annu Rev Physiol. 2006;68:685–717. https://doi.org/10.1146/annurev.physiol.68.040204.101406.

5. Wagner TFJ, Loch S, Lambert S, Straub I, Manneckbach S, Mathar I, et al. Transient receptor potential M3 channels are ionotropic steroid receptors
in pancreatic beta cells. Nat Cell Biol. 2008;10:1421–30. https://doi.org/10.1038/ncl.1801.

6. Straub B, Mohr F, Stab J, Konrad M, Philipp SE, Oberwinkler J, et al. Citrus fruit and fabacea secondary metabolites potently and selectively block TRPM3. Br J Pharmacol. 2013;168:1835–50. https://doi.org/10.1111/bph.12076.

7. Berridge MJ, Bootman MD, Roderick HL. Calcium: Signal transduction: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol. 2003;4:517–29. https://doi.org/10.1038/nrm1155.

8. Nguyen T, Staines D, Nilius B, Smith P, Marshall-Gradisnik S. Novel identification and characterisation of Transient receptor potential melastatin 3 ion channels in natural killer cells and B lymphocytes: effects on cell signalling in Chronic fatigue syndrome/Myalgic encephalomyelitis patients. Biol Res. 2016;49:27. https://doi.org/10.1186/s40559-016-0087-2.

9. 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 melastatin 3 ion channels. Clin Exp Immunol. 2017;187:284–93. https://doi.org/10.1111/cei.12882.

10. Cabanas H, Muraki K, Eaton N, Balinas C, Staines D, Marshall-Gradisnik S. Loss of Transient Receptor Potential Melastatin 3 ion channel function in natural killer cells from Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. Mol Med. 2018;24:44. https://doi.org/10.1186/s12020-018-0046-x.

11. Cabanas H, Muraki K, Balinas C, Eaton-Fitch N, Staines D, Marshall-Gradisnik S. Validation of impaired Transient Receptor Potential Melastatin 3 ion channel activity in natural killer cells from Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. Mol Med. 2019;25:14. https://doi.org/10.1186/s12020-019-0083-4.

12. Kloc M, Kubiak JZ, Li XC, Gobrial RM. The newly found functions of MTOC in immunological response. J Leukoc Biol. 2014;95:417–30. https://doi.org/10.1189/jlb.0813468.

13. Orenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol. 2003;4:532–65. https://doi.org/10.1038/nrm1150.

14. Berridge MJ. Calcium signalling remodelling and disease. Biochem Soc Trans. 2012;40:297–309. https://doi.org/10.1042/BST20110766.

15. Carruthers BM, Jain AK, Meirleir KLD, Peterson DL, Klimas NG, Lerner AM, et al. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. J Chronic Fatigue Syndr. 2003;11:7–115. https://doi.org/10.1300/J092v11n01_02.

16. Carruthers BM, van de Sande MJ, Meirleir KLD, Klimas NG, Broderick GJ, Mitchell T, et al. Myalgic encephalomyelitis: International Consensus Criteria. J Intern Med. 2011;270:327–38. https://doi.org/10.1111/j.1365-2796.2011.02428.x.

17. Eaton-Fitch N, du Preez S, Cabanas H, Staines D, Marshall-Gradisnik S. A systematic review of natural killer cell profiles and cytotoxic function in myalgic encephalomyelitis/chronic fatigue syndrome. Syst Rev. 2019;8:279. https://doi.org/10.1186/s13643-019-1202-6.

18. Marshall-Gradisnik S. Impaired calcium channel activity in natural killer cells from Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. Mol Med. 2019;25:14. https://doi.org/10.1186/s12967-021-02974-4.

19. Balinas C, Cabanas H, Staines D, Marshall-Gradisnik S. Transient receptor potential melastatin 2 channels are overexpressed in myalgic encephalomyelitis/myalgic encephalomyelitis/chronic fatigue syndrome patients. J Transl Med. 2019;17:1629-019-02154-0.

20. Larkins RG, Molesworth SR. Chronic fatigue syndrome clinical practice guidelines. Med J Aust. 2002;177:51–2. https://doi.org/10.5694/mja1.13643-190-1202-6.

21. 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. https://doi.org/10.2147/TACG.S99405.

22. Mills GB, Cheung RK, Grinstein S, Gelfand EW. Interleukin 2-induced lymphocyte proliferation is independent of increases in cytosolic-free calcium concentrations. J Immunol. 1985;134:2431–5.

23. Eaton-Fitch N, Cabanas H, du Preez S, Staines D, Marshall-Gradisnik S. The effect of IL-2 stimulation and treatment of TRPM3 on channel co-localisation with PIP2 and NK cell function in myalgic encephalomyelitis/chronic fatigue syndrome patients. J Transl Med. 2021;19:306. https://doi.org/10.1186/s12967-021-02974-4.

24. Zhao S, Yudin Y, Rohacs T. Disease-associated mutations in the human TRP3 gene render the channel overactive via two distinct mechanisms. Elife. 9:e5634. Doi: https://doi.org/10.7554/elif.9.e5634.

25. Lanier LL. Natural killer cell receptor signaling. Curr Opin Immunol. 2003;15:308–14. https://doi.org/10.1016/S0952-7915(03)00395-6.
47. Chuderland D, Seger R. Calcium regulates ERK signaling by modulating its protein–protein interactions. Commun Integr Biol. 2008;1:4–5.
48. Huth TK, Staines D, Marshall-Gradyinsk S. ERK1/2, MEK1/2 and p38 downstream signaling molecules impaired in CD56brightCD16−/ CD56brightCD16+ – natural killer cells in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. J Transl Med. 2016. https://doi.org/10.1186/s12976-016-0859-z.
49. Tóth BI, Konrad M, Ghosh D, Mohr F, Halaszovich CR, Leitner MG, et al. Regulation of the transient receptor potential potential channel TRPM3 by phosphoinositides. J Gen Physiol. 2015;146:51–63. https://doi.org/10.1085/jgp.201411339.
50. Thiel G, Rubli S, Lesch A, Guetherlein LA, Rössler OG. Transient receptor potential channel TRPM3 channels. Pharmacology, signaling, and biological functions. Pharmacol Res. 2017;124:92–9. https://doi.org/10.1016/j.phrs.2017.07.014.
51. Holden S, Maksovud R, Eaton-Fitch N, Cabanas H, Staines D, Marshall-Gradyinsk S. A systematic review of mitochondrial abnormalities in myalgic encephalomyelitis/chronic fatigue syndrome/systemic exertion intolerance disease. J Transl Med. 2020;18:290. https://doi.org/10.1186/s12976-020-0452-3.
52. Oh-hora M, Rao A. Calcium signaling in lymphocytes. Curr Opin Immunol. 2008;20:250–8. https://doi.org/10.1016/j.coi.2008.04.004.
53. Papanikolaou M, Lewis A, Butt AM. Store-operated calcium entry is essential for glial calcium signalling in CNS white matter. Brain Struct Funct. 2017;222:2993–3005. https://doi.org/10.1007/s00429-017-1380-8.
54. Verkhratsky A, Parpura V. Store-operated calcium entry in neuroglia. Neurosci Bull. 2014;30:125–33. https://doi.org/10.1007/s12264-013-1343-x.
55. Przibilla J, Dembila S, Rizun O, Lis A, Jung M, Oberwinkler J, et al. Ca2+-dependent regulation and binding of calmodulin to multiple sites of Transient Receptor Potential Melastatin 3 (TRPM3) ion channels. Cell Calcium. 2018;73:40–52. https://doi.org/10.1016/j.ceca.2018.03.005.
56. Holendova B, Grycova L, Jirku M, Teisenger J. PtdIns(4,5)P2 interacts with CaM binding domains on TRPM3 N-terminus. Channels. 2012;6:479–82. https://doi.org/10.4161/chan.22177.
57. Lee N, Chen J, Sun I, Wu S, Gray KR, Rich A, et al. Expression and characterization of human transient receptor potential melastatin 3 (hTRPM3): J Biol Chem. 2003;278:20890–7. https://doi.org/10.1074/jbc.M211232200.
58. Badheka D, Yudin Y, Borbíro I, Hartle CM, Yasici A, Mirshahi T, et al. Inhibition of Transient Receptor Potential Melastatin 3 ion channels by G-protein βγ subunits. ELife. 2017. https://doi.org/10.7554/elif.26147.
59. Eisenstein TK. The role of opioid receptors in immune system function. Front Immunol. 2019. https://doi.org/10.3389/fimmu.2019.02904.
60. McCarthy L, Szabo I, Brindle JF, Pintar JE, Rogers TJ. Expression of functional mu-opioid receptors during T cell development. J Neuroimmunol. 2001;114:73–80. https://doi.org/10.1016/S0165-5728(01)00248-x.
61. Maher DP, Walia D, Heller NM. Suppression of human natural killer cells by different classes of opioids. Anesth Analg. 2019;128:1013–21. https://doi.org/10.1213/ANE.0000000000004598.
62. Fuggiotta MP, Di Francesco P, Falcetti R, Cottarelli A, Rossi L, Tricarico M, et al. Effect of morphine on cell-mediated immune responses of human lymphocytes against allogeneic malignant cells. J Exp Clin Cancer Res CR. 2005;24:255–63.
63. Franchi S, Panerai AE, Sacerdote P. Buprenorphine ameliorates the effect of surgery on hypothalamic-pituitary-adrenal axis, natural killer cell activity and metastatic colonization in rats in comparison with morphine or fentanyl treatment. Brain Behav Immun. 2007;21:767–74. https://doi.org/10.1016/j.bbi.2007.01.001.
64. Sacerdote P, Manfredi B, Mantegazza P, Panerai AE. Antinociceptive and immunosuppressive effects of opiate drugs: a structure-related activity study. Br J Pharmacol. 1997;121:834–40. https://doi.org/10.1038/ bj.1997.138.
65. Veager MP, Colacchio TA, Yu CT, Hildebrandt L, Howell AL, Weiss J, et al. Morphine inhibits spontaneous and cytokine-enhanced natural killer cell cytotoxicity in volunteers. Anesthesiology. 1995;83:500–8. https://doi.org/10.1095/0000542-199509000-00008.
66. Shavit Y, Martin FC, Yirmiya R, Ben-Eliyahu S, Teman GW, Weiner H, et al. Effects of a single administration of morphine or footshock stress on natural killer cell cytotoxicity. Brain Behav Immun. 1987;1:318–28. https://doi.org/10.1016/0891-5896(87)90034-1.

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