Endoplasmic reticulum stress in the dorsal root ganglia regulates large-conductance potassium channels and contributes to pain in a model of multiple sclerosis

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Abstract

Neuropathic pain is a common symptom of multiple sclerosis (MS) and current treatment options are ineffective. In this study, we investigated whether endoplasmic reticulum (ER) stress in dorsal root ganglia (DRG) contributes to pain hypersensitivity in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS. Inflammatory cells and increased levels of ER stress markers are evident in post-mortem DRGs from MS patients. Similarly, we observed ER stress in the DRG of mice with EAE and relieving ER stress with a chemical chaperone, 4-phenylbutyric acid (4-PBA), reduced pain hypersensitivity. In vitro, 4-PBA and the selective PERK inhibitor, AMG44, normalize cytosolic Ca2+ transients in putative DRG nociceptors. We went on to assess disease-mediated changes in the functional properties of Ca2+-sensitive BK-type K+ channels in DRG neurons. We found that the conductance-voltage (GV) relationship of BK channels was shifted to a more positive voltage, together with a more depolarized resting membrane potential in EAE cells. Our results suggest that ER stress in sensory neurons of MS patients and mice with EAE is a source of pain and that ER stress modulators can effectively counteract this phenotype.

Abbreviations: 4-PBA, 4-phenylbutyric acid; AMG44, Amgen compound 44, PERK inhibitor (also known as AMG PERK 44); ATF, activating transcription factor; BiP, binding immunoglobulin protein (also known as glucose-regulated protein 78 (GRP78) or heat shock protein 5 (HSPA5); BK channel, calcium-sensitive large conductance (BK) potassium channel; CFA, complete Freund’s adjuvant; CHOP, C/EBP homologous protein (also known as DNA damage-inducible transcript 3 (Ddit3)); CICR, calcium-induced calcium release; CNS, central nervous system; DRG, Dorsal root ganglia; EAE, experimental autoimmune encephalomyelitis; eIF2, eukaryotic initiation factor 2; eIF2α, α-subunit of eIF2 complex; ER, endoplasmic reticulum; GCN2, general control nonderepressible 2; HRI, heme-regulated inhibitor kinase; IB4, isolectin B4; Iba1, ionized calcium-binding adapter molecule 1; IP3, inositol trisphosphate; IP3R, IP3 receptor; IRE1, inositol-requiring enzyme 1; ISR, integrated stress response; ISRIB, ISR inhibitor; MOG35-55, myelin oligodendrocyte glycoprotein peptide 35-55; mRNA, messenger RNA; MS, multiple sclerosis; NFH, neurofilament heavy; PERK, PKR-like ER kinase; PKR, protein kinase RNA-activated; PNS, peripheral nervous system; RNA, ribonucleic acid; RyR, ryanodine receptor; SC, spinal cord; SERCA, Sarco/ endoplasmic reticulum Ca2+-ATPase; siRNA, silencing RNA; uORF, upstream open reading frame; UPR, unfolded protein response; XBP1, X-box binding protein 1.
INTRODUCTION

Multiple sclerosis (MS) is a chronic, neurodegenerative disorder characterized by immune activation, and loss of myelin in the central nervous system (CNS). Among the many sensory abnormalities associated with MS, pain is common and often debilitating. Pain is experienced by one-third to half of the population with MS at some point during their disease course and a significant percentage are diagnosed with neuropathic pain.\(^1,2\) Current pharmacological approaches to alleviate this pain have been largely ineffective with low patient confidence in prevailing treatment approaches.\(^3\) To investigate the pathophysiology of pain in MS, we employed a commonly used animal model, experimental autoimmune encephalomyelitis (EAE).

Neuropathic pain is thought to arise from increased excitability of neurons along the pain axis, comprising sensory neurons in the peripheral dorsal root and trigeminal ganglia and the integrative central processes of the spinal cord and the brain.\(^4-6\) The role of the CNS as a modulator of pain in MS/EAE has been widely studied.\(^7\) However, only a handful of studies to date has investigated the contribution of the peripheral branch of the somatosensory nervous system to pain pathophysiology in EAE and MS.\(^8-14\)

In response to MS/EAE, the central projections of the DRG neuronal somata may sustain indirect injury through chronic neuroinflammatory processes occurring in the CNS. These injuries at the spinal terminal may evoke a retrograde stress response in the cell bodies of the DRG. When cells are subject to chronic stressors, such as prolonged inflammation and cytoskeletal disruption, they may undergo endoplasmic reticulum (ER) stress. The ER is an important organelle required for lipid biosynthesis, calcium ion (Ca\(^{2+}\)) storage, and protein folding and processing.\(^15\) Stress can impair protein folding thus triggering a cascade of events that are collectively known as the unfolded protein response (UPR).\(^16,17\)

The ER-based signalling mechanism initially functions to mitigate cellular damage. Activation of the UPR is mediated by three ER stress sensor proteins, IRE1, PERK, and ATF6.\(^15\) Signaling initiated through these three independent pathways promotes cell survival by ultimately reducing misfolded protein levels both via reducing mRNA translation and via enhancing the ER’s folding capacity. If the stressor is particularly severe or prolonged, however, the UPR can drive the cell into an apoptotic program of regulated cell death.\(^15,17\) Emerging evidence is now demonstrating that ER stress may also be a significant factor for developing pain hypersensitivity in various animal models across a variety of cell types.\(^18-25\) In this study, we investigated whether ER stress in DRG neurons contributes to the well characterized pain hypersensitivity that occurs in the EAE model and by extension in MS.\(^7,26\)

MATERIALS AND METHODS

2.1 Human tissue

Human tissue was obtained from the Netherlands Brain Bank (NBB; http://www.brainbank.nl). Subjects or their next of kin provided written informed consent for the use of their tissue and clinical information for research purposes to the NBB. All MS individuals (n = 9) experienced the progressive phase of the disease and presented evidence of chronic pain such as trigeminal neuralgia, migraine, extremity pain, and back pain. The average disease duration was 21.0 ± 4.61 years. The average age at death was 78.6 ± 4.32 years for the nondemented controls (n = 7) and 59.7 ± 4.04 years for individuals with MS. The majority of the donors elected to be euthanized with a combination of barbiturates (thiopental, pentobarbital) and muscle relaxant (Rocuronium bromide). Only female human tissue was examined. Patient demographics are further summarized in Table 1.

Snap frozen human DRGs were sectioned onto Superfrost Plus glass slides (VWR International, Leuven, Belgium) at 10 µm thickness. Ten micrometer thick sections were harvested for RNA and protein analysis. Tissue was lysed in 600 µL of Buffer RLT (Qiagen, 79216) with β-mercaptoethanol (Sigma, M3148, 10 µL/mL of Buffer RLT) using a 2 mL of Potter-Elvehjem homogenizer. The homogenate was then centrifuged at full speed for 2 minutes in QIAshredder column (Qiagen, 79656). RNA was extracted from the flow-through using the RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer’s instructions. RNA was quantified using a NanoDrop 2000. Reverse transcription and PCR analysis were performed as described later.

The flow-through after the RNeasy spin column centrifugation step was stored at 4°C for protein extraction. The protein was precipitated using ice-cold acetone (500 µL of sample + 1500 µL of acetone). The acetone laden sample was stored at −20°C for 2 hours and then centrifuged at 15 000g for 10 minutes at 4°C. The supernatant was discarded and the pellet was redissolved in 100 µL of 5% SDS. Protein was quantified using the DC Protein Assay (Bio-Rad, 5000112). Western blots using 10 µg of protein were performed as described below.
Immunohistochemical analysis of human DRG sections was performed a similar to previously described. In brief, slides were fixed in acetone for 10 minutes, followed by three 5-min gentle PBS washes, after which the slides were air-dried in a fume hood for 30 minutes. The sections were blocked at room temperature with 10% NGS in PBS. Primary antibody dissolved in antibody solution (1% BSA and 0.2% NGS in PBS) was placed over the DRG sections overnight. The slides were then washed in PBS (3x–5 minutes each) followed by 1-hour incubation in secondary antibody (1:200) dissolved in antibody solution. Another set of washes in PBS (3x–5 minutes each) was performed and then the slides were mounted using VECTASHIELD with DAPI (Vector Labs, H-1200).

The use of tissue and access to medical records was approved by the Ethics Committee of the VU University Medical Center, Amsterdam, The Netherlands.

2.2 EAE induction and behavioral assessment

As previously described, experimental autoimmune encephalomyelitis (EAE) was induced in female C57BL/6 mice (8-10 weeks old; Charles River) by subcutaneously injecting 50 μg myelin oligodendrocyte glycoprotein (MOG35-55; Stanford University Peptide Synthesis Facility) emulsified in complete Freund's adjuvant (CFA; 1.5 mg/mL) followed by inoculations with 300 ng of pertussis toxin, Bordetella pertussis, (List Biological Labs) on the day of induction and 48 hours later. All animal experiments were performed according to the Canadian Council on Animal Care's Guidelines and Policies with approval from the University of Alberta Health Sciences Animal Care and Use Committee.

Mechanical hypersensitivity, facial sensitivity, and gross locomotor ability were assessed as previously described. Data were compared to the baseline threshold of each individual mouse in order to control for individual differences and experimenter variability.

2.3 4-PBA administration in vivo

4-PBA (200 mg/kg; Tocris, 2682) was completely dissolved in 1X sterile PBS and injected intraperitoneally (n = 6) daily, beginning at disease onset until day 7-10 post-onset. PBS was administered as a vehicle control (n = 5). Von Frey testing was performed one-hour after injection on the day of disease onset. Facial sensitivity was assessed using the air puff assay one-hour after the final injection 7-10 days postonset.

2.4 Immunohistochemistry

The mouse tissue was fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) overnight at 4°C followed by

| Patient ID | Sex | Age | Post-mortem delay (hh:mm) | Disease subtype | Chronic pain | Spinal lesion | MS duration (years) | Cause of death |
|------------|-----|-----|---------------------------|-----------------|--------------|--------------|--------------------|---------------|
| S02        | F   | 85  | 6:25                      | NDC             | Y            | N            | –                  | Euthanasia     |
| S05        | F   | 78  | 7:10                      | NDC             | Y            | N            | –                  | Euthanasia     |
| S07        | F   | 60  | 8:10                      | NDC             | Y            | N            | –                  | Euthanasia     |
| S11        | F   | 95  | 7:05                      | NDC             | N            | N            | –                  | Cancer         |
| S15        | F   | 75  | 9:10                      | NDC             | Y            | N            | –                  | Euthanasia     |
| S19        | F   | 88  | 6:20                      | NDC             | N            | N            | –                  | Euthanasia     |
| S20        | F   | 60  | 5:30                      | NDC             | Y            | N            | –                  | Euthanasia     |
| S01        | F   | 65  | 10:45                     | SPMS            | Y            | Y            | 16                 | Euthanasia     |
| S04        | F   | 56  | 10:30                     | SPMS            | N            | Y            | 21                 | Euthanasia     |
| S06        | F   | 35  | 10:20                     | SPMS            | N            | N            | 10                 | Euthanasia     |
| S08        | F   | 61  | 10:00                     | SPMS            | Y            | Y            | 2                  | Euthanasia     |
| S09        | F   | 74  | 7:50                      | SPMS            | Y            | Y            | 50                 | Euthanasia     |
| S10        | F   | 57  | 10:40                     | SPMS            | Y            | Y            | 25                 | Euthanasia     |
| S13        | F   | 50  | 9:05                      | SPMS            | Y            | Y            | 12                 | Euthanasia     |
| S17        | F   | 66  | 9:45                      | PPMS            | Y            | Y            | 23                 | Pneumonia      |
| S18        | F   | 73  | 7:05                      | PPMS            | Y            | N/A          | 30                 | Euthanasia     |

Abbreviations: CVA, cardiovascular accident; NDC, non-demented control; PPMS, primary progressive MS; SPMS, secondary progressive MS.

*Euthanasia was typically performed with barbiturate (thiopental, pentobarbital) overdose coupled with a muscle relaxant (rocuronium bromide).

bMetastasized mamma carcinoma.

Table 1: Demographics of MS patients and non-demented controls
two 30% sucrose washes, each overnight at 4°C. After removing excess sucrose, the tissue was embedded in TissueTek OCT (Sakura Finetek, 4583). DRGs were cryosectioned with 10 µm thickness, while the spinal cords were sectioned at 20 µm onto glass slides. The remaining staining protocol was identical to a previously established protocol.¹⁰

### 2.5 Western blotting

Protein lysates were diluted in RIPA (25 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, 1% NP-40) with protease (cOmplete EDTA-free, Roche, 04693159001) and phosphatase inhibitors (PhosSTOP, Roche, 04906837001) and 5X sample buffer was added. Directly before loading onto 10% SDS-PAGE gels, samples were boiled at 100°C for 10 minutes. Gels were run at 150 V for 60 minutes and transferred onto nitrocellulose membranes with 400 mA over 120 minutes.

Membranes were stained with REVERT Total Protein Stain (LI-COR) according to manufacturer's instructions and then blocked in 2% BSA in 1X DPBS for 1 hour at room temperature followed by overnight incubation at 4°C with a primary antibody dissolved in blocking solution. Membranes were then washed three times (3 minutes each) in TBS-T and incubated in 2% milk in TBS-T with Alexa Fluor-coupled secondary antibodies for 1 hour at room temperature. After another wash step, membranes were scanned using an Odyssey infrared imager (LI-COR).

Western blots using human tissue were performed as previously described.¹⁰ Stain-free gels (Bio-Rad, 456-8093, 4%-20%) were transferred onto low-fluorescence PVDF blots (Bio-Rad, 1704274) using the Trans-Blot Turbo transfer system (Bio-Rad). Total protein was quantified using Stain-Free technology (Bio-Rad) according to the manufacturer’s instructions. Blots were imaged with Bio-Rad ChemiDoc XRS + system and quantified using Image Lab 6.0 (Bio-Rad) with total protein as a loading control. Antibodies are summarized in Table 2.

| Antibody          | Host | Source                          | Dilution factor  |
|-------------------|------|---------------------------------|------------------|
| BiP               | Ms   | BD Biosciences, 610979          | 1:1000 (WB)      |
| BiP               | Rb   | Novus, NBP1-06274               | 1:2000 (WB), 1:200 (IHC) |
| CHOP              | Ms   | Enzo Life Sciences, ALX-804-551-C100 | 1:1000          |
| XBP1              | Rb   | Abcam, ab37152                 | 1:1000 (WB), 1:200 (IHC) |
| eIF2α             | Rb   | Cell Signaling, 9722            | 1:1000           |
| p-eIF2α (Ser51)   | Rb   | Cell Signaling, 3597            | 1:1000           |
| PERK              | Rb   | Cell Signaling, 3192            | 1:1000           |
| p-PERK (Thr980)   | Rb   | Cell Signaling, 3179            | 1:500            |
| Iba1              | Rb   | Wako, 019-19741                 | 1:500            |
| CD4               | Rt   | BioRad, MCA2691                 | 1:200            |
| cFOS              | Rb   | Cell Signalling, 2250           | 1:1000           |
| CD88 (or C5aR1)   | Rt   | BioRad, MCA2456                 | 1:200            |
| CD3               | Rt   | BioRad, MCA1477                 | 1:200            |
| NFH               | Ck   | ThermoFisher, PA1-10002         | 1:5000           |
| IB4-AF488         | –    | ThermoFisher, I21411            | 1:100            |
| Goat anti-Mouse IgG, AF750 | Gt   | Abcam, ab175733                | 1:10 000         |
| Goat anti-Rabbit IgG, AF680 | Gt   | ThermoFisher, A21057           | 1:10 000         |
| Goat anti-Chicken IgG AF594 | Gt   | ThermoFisher, A11042           | 1:200            |
| Goat anti-Rabbit IgG AF488 | Gt   | ThermoFisher, A11008           | 1:200            |
| Goat anti-Rat IgG AF594 | Gt   | ThermoFisher, A11007           | 1:200            |
| Goat anti-Rabbit IgG HRP | Gt   | Jackson Laboratories, 111-035-144 | 1:10 000         |

**TABLE 2** Antibodies used in this study
2.6 | Quantitative real-time PCR

Reverse transcription and PCR analyses were performed as previously described.\textsuperscript{10} Reverse transcription on human samples was performed using 160 ng of total RNA. PCRs were performed on StepOnePlus thermocycler using Rpl5 (mouse) or RPLP0 (human) as housekeeping genes. Primers used in this study were obtained from Qiagen: Rpl5 (PPM25102A), Xbp1 (PPM05627A), Ddit3 (PPM03736A), Hspa5 (PPM03586B), Kcnmb1 (PPM04055A), Kcnmb4 (PPM36505B), Kcnma1 (PPM04054G), RPLP0 (PPH21138F), C3AR1 (PPH02514A), C5AR1 (PPH06063F), CD3E (PPH01486B), CD4 (PPH01629C), XBP1 (PPH02850A), KCNMA1 (PPH01663A), KCNMB1 (PPH01417A), KCNMB4 (PPH17370A). Xbp1 primers targeted both spliced and unspliced mRNA.

2.7 | Dissociated DRG cultures

Dissociated DRG cultures for calcium imaging were prepared from freshly excised DRGs according to our previously published protocol (see “Dissociated dorsal root ganglia cultures”,\textsuperscript{11}) with STEMzyme I (2 mg/mL; Worthington, LS004106) replacing collagenase IV. For electrophysiology experiments, DRG neurons were dissociated with a mix of STEMzyme I (1 mg/mL) and trypsin (0.5 mg/mL; HyClone, SV3003101).

In vitro application of 4-PBA was prepared as a stock solution of 100 mM in Hank’s balanced salt solution (HBSS) (HyClone, SH30030.02). Stock solution of AMG44 (Tocris, 5517, 3 mM) was prepared in sterile 60% dimethyl sulfoxide (DMSO; Sigma, D2650). Dissociated cells received diluted 4-PBA (final concentration: 10 mM) and AMG44 (final concentration: 5 µM) treatment one-hour after plating in cell media (DMEM/F12 [Gibco, 10565018], 1% N2 [Gibco, 17502048], 1% penicillin/streptomycin [Gibco, 1570063]). Vehicle treatment in each experiment consisted of an equal volume of either HBSS or DMSO (final concentration: 0.1%). Cells were incubated in their respective treatment conditions for 20-24 hours prior to Ca\textsuperscript{2+} imaging and electrophysiology.

Gene knockdown experiments were performed with the HiPerFect transfection system (Qiagen, 301705) using FlexiTube siRNA (Qiagen; XBP1: GS22433, Ddit3: GS13198, AllStars Negative Control siRNA: 1027284). The FlexiTube siRNA contains a cocktail of multiple siRNA targeting multiple regions of the mRNA. The siRNA cocktail was prepared according to the manufacturer’s instructions. The final total siRNA concentration was 0.04 µM (4 individual siRNAs, each at 0.01 µM). About 100 µL of siRNA mixture was added to cells (100 µL droplet) one hour after plating them onto glass coverslips. The cells were incubated for 10 minutes at 37°C and then topped up to 1 mL in cell media followed by incubation for 20-24 hours prior to Ca\textsuperscript{2+} imaging.

2.8 | Live cell Ca\textsuperscript{2+} imaging in DRG neurons

Confocal imaging of cytosolic Ca\textsuperscript{2+} transients was performed as previously described\textsuperscript{11} with the addition of caffeine (Sigma, C0750) dissolved in superfusate (in mM) (120 NaCl, 3 KCl, 1 CaCl\textsubscript{2}, 2 MgSO\textsubscript{4}, 20 glucose). After 5 minutes of equilibration in the optical recording chamber with superfusate, administered with a peristaltic pump at 4 mL/min, the imaging paradigm was as follows: 30 seconds superfusate perfusion (baseline), 30 seconds caffeine (20 mM), 4 minutes superfusate perfusion, 5 minutes washout period, 30 seconds superfusate perfusion (baseline), 30 seconds KCl (30 mM), 4 minutes superfusate perfusion. The imaging data were analyzed using Olympus FV10-ASW software with the first 30 seconds as a baseline for each caffeine and KCl application. The remaining recording was divided by the baseline to obtain a ratio of change in fluorescence (Fluo-4 F/F). These data were further normalized to an internal control of the particular experiment (eg, Ca\textsuperscript{2+} transients were normalized to CFA in the AMG44 experiment, EAE vehicle in the 4-PBA and siRNA experiments). As such, the average amplitude of all cells in the control group was used to normalize the Ca\textsuperscript{2+} response of the treated group. Once the imaging was completed, the 15 mm diameter coverslips were placed in 12-well plate with 600 µL of Buffer RLT (Qiagen). These plates were stored at −80°C until RNA extraction. Total RNA was extracted from individual coverslips using RNeasy Micro Kit (Qiagen, 74004) according to the manufacturer’s instructions.

2.9 | Perforated patch whole-cell recordings

Solutions: The extracellular bath solution contained (in mM): 135 NMDG, 5 KCl, 2.8 Na\textsubscript{2}CH\textsubscript{3}CO\textsubscript{2}, 1CaCl\textsubscript{2}, 1MgCl\textsubscript{2}, 10 HEPES and was adjusted to pH 7.4 with HCl. The intracellular (pipette) solution contained (in mM): 135 KCl, 5 EGTA, 10 HEPES and was adjusted to pH 7.2 with KOH. Amphotericin B was used to perforate the patch and solutions were made fresh before use. About 6 mg amphotericin powder (Sigma) was added to 100 µL of DMSO and solubilized in a 1.5 mL centrifuge tube. From the 60 mg/mL of stock solution, 20 µL was added to 5 mL of pipette solution for a final concentration of 240 µg/mL. Paxilline (Tocris), used to inhibit BK channels, was dissolved in EtOH at a stock concentration of 1 mM. Paxilline was added to 40 mL of bath solution for a desired working concentration of 1 µM and perfused into the chamber when appropriate.

Data acquisition and analysis: Prior to experiments, 5 µL of Alexa 488-conjugated IB4-antibody (Invitrogen, 1 mg/
mL) was added for 10 minutes then removed, to differentiate between IB4 + and IB4- DRG neurons. Glass coverslips containing cells were removed from the incubator (37°C) and placed in a superfusion chamber containing the bath solution at ambient temperature (22-23°C). IB4 + neurons were observed with epifluorescence illumination. Patch pipettes were manufactured from soda-lime glass (Fisher), using a Sutter P-97 puller (Sutter Instrument). When filled with internal solution, patch pipettes had a tip resistance of 2-4 MΩ. After the formation of a gigahm seal between pipette tip and cell, currents were recorded through amphotericin B-induced pores. Whole-cell perforated patch-clamp recordings were acquired and analyzed using a Digidata 1440 digitizer, an Axopatch 200B amplifier, and a Clampex 10 software (Molecular Devices). Recordings were sampled at 10 kHz and filtered at 5 kHz, with manual capacitance compensation and series resistance compensation at (80%). In voltage-clamp mode, total IK was measured by stepping between −130 and 200 mV (100 ms in 10 mV increments) from a −100 mV holding potential followed by a 100 ms tail current voltage at −30 mV. Bath solution containing 1 µM paxilline was perfused in the chamber for 2 minutes to inhibit BK channels. During perfusion, IK was recorded with a +60 mV depolarizing pulse for 150 ms with 5 seconds interpulses from a −80 mV holding voltage to observe paxilline-induced current reduction. To isolate BK currents, currents were subtracted immediately before and after the application of 1 µM Paxilline from paxilline-sensitive (IB4+) DRG neurons. BK channel conductance-voltage (G/V) relationships were generated by analyzing the tail current amplitudes and fit with a Boltzmann function. BK channel current density was measured by dividing the current amplitude (pA) at +60 mV by cell capacitance (pF). Resting membrane potentials were recorded using current-clamp mode from IB4 + DRG neurons using the perforated-patch clamp. Vehicle treatment with HBSS (vehicle for 4-PBA) and 0.01% DMSO (vehicle for AMG44) resulted in identical recordings and hence, two vehicle treatments were collapsed into a single group. DRG neurons from EAE animals were obtained at disease onset and the chronic time point (7-10 days postonset).

2.10 | Experimental design and statistical analysis

Statistical analyses were performed using GraphPad Prism 6 with appropriate statistical tests. Detailed statistical analyses have been mentioned in the Results section. Animals were assigned to each experimental group randomly. Western blot and PCR data were log-transformed prior to statistical testing in order to ensure the data fit the homogeneity of variance assumptions for each statistical test. The data presented in the figures are back-transformed onto a linear scale for the ease of the reader. Statistical annotations represent the output of tests performed on log-transformed data. Significance was set at P < .05. Graphics were generated using Biorender.com.

3 | RESULTS

3.1 | Inflammation and ER stress in the post-mortem MS DRG

Although MS is classically identified as a CNS targeted disease, we sought to investigate whether sensory neurons residing in the DRG of the PNS are also affected by the disease. We first examined post-mortem DRG tissue from MS patients for the activation of innate and adaptive immune responses. At the transcript level, the complement component 3a receptor 1 (C3AR1), and complement component 5a receptor 1 (C5AR1), were upregulated in MS tissue compared to non-demented controls (tC3aR1(10) = 2.019, P = .0711; tC5aR1(11) = 3.928, P = .0024, unpaired t-test) (Figure 1A,B). We also noted a significant increase in the T-cell marker transcripts, CD3 and CD4, further suggesting that the adaptive immune response was engaged in the DRG during the disease (tCD3E(11) = 4.358, P = .0011; tCD4(11) = 3.466, P = .0053, unpaired t-test) (Figure 1C,D). Similar activation at the level of the DRG in the EAE animal model has been previously described by our group.10 Further analysis revealed a significant increase in specific markers of ER stress at the level of the DRG in MS. XBP1 mRNA as well as BiP and XBP1 protein levels were increased in MS tissue, suggesting that the DRG in MS undergoes ER stress (PCR: tXBP1(9.119) = 3.482, P = .0068, unpaired t-test with Welch's correction; WB: tBiP(14) = 2.579, P = .0219; tXBP1(14) = 2.290, P = .0523, unpaired t-test) (Figure 1E-H).

We next performed immunofluorescence experiments to identify the source of inflammation and ER stress. C5aR1 (CD88), and CD3 immunopositive immune cells were increased in MS samples as compared to non-demented controls (Figure 1I). Furthermore, BiP expression was observed to be increased in MS DRGs and largely localized to sensory neurons (Figure 1I). This data indicates that peripheral sensory neurons in the DRGs of MS patients are subjected to immune activation and ER stress.

3.2 | EAE mice develop pain hypersensitivity

We next generated EAE in female C57BL/6 mice using myelin oligodendrocyte glycoprotein (MOG)35,53. The median day for the onset of EAE clinical signs was day 10 postimmunization (Figure 2A,B). Behavioral testing was carried out at this time-point. In our model of EAE, mice
**FIGURE 1**  Human DRGs undergo inflammation, immune activation, and ER stress in MS. A and B, PCR analysis of post-mortem human DRGs revealed that the complement component C3a receptor 1 (C3AR1) and component C5a receptor 1 (C5AR1) genes are upregulated in chronic MS tissue (n = 8) as compared to DRGs obtained from non-demented controls (NDC; n = 5). C and D, Similarly, we observed an increase in T-cell enriched CD3E and CD4 mRNA expression in MS tissue as compared to NDC. E, mRNA transcripts of X-box binding protein 1 (XBP1) were also found to be elevated in MS DRG tissue with respect to levels in NDC samples. F-H, Western blotting revealed an increase in binding immunoglobulin protein (BiP) and XBP1 protein levels in MS DRGs (n = 9) compared to NDCs (n = 7). H, Immunofluorescence experiments localized expression of C5ar1 and CD3 in immune cells; however, BiP expression was restricted to be elevated in neurons. Bars indicate mean ± standard error of mean (SEM). *P < .05, **P < .01, unpaired t-test
exhibit a characteristic mechanical hypersensitivity at disease onset that is reflected by a reduced threshold to von Frey hair stimulation as expressed as a percentage of their own baseline threshold \(F_{\text{interaction}}(1,11) = 28.43, P = .0002, F_{\text{timepoint}}(1,11) = 29.40, P = .0002, F_{\text{disease}}(1,11) = 7.932, P = .0168, F_{\text{subjects}}(11,11) = 3.584, P = .0224, \text{RM two-way ANOVA}\) (Figure 2C). At this time point, we do not observe any significant change in locomotor abilities (time spent on the rotarod) \(F_{\text{interaction}}(1,8) = 1.090, P = .3269, F_{\text{timepoint}}(1,8) = 0.7348, P = .4163, F_{\text{disease}}(1,8) = 0.6992, P = .4273, F_{\text{subjects}}(8,8) = 1.035, P = .4811, \text{RM two-way ANOVA}\) (Figure 2D). Hence, mechanical hypersensitivity...
observed in EAE mice at disease onset was not confounded by paralysis or lack of motor coordination.

### 3.3 ER stress in the DRG of EAE mice

We next assessed the expression of ER proteins in the DRG of mice with EAE over the course of the disease. The levels of BiP, a luminal ER chaperone, were elevated in the DRG at the chronic stage of the disease (F_{BiP}(2,9) = 7.950, P = .0103, One-way ANOVA) (Figure 2E). Levels of phosphorylated eIF2α (p-eIF2α) were significantly upregulated at the chronic time point whereas total eIF2α (t-eIF2α) levels only trended toward an increase with the progression of disease (F_{t-eIF2α}(2,9) = 3.808, P = .0633, F_{p-eIF2α}(2,9) = 7.030, P = .0145; One-way ANOVA) (Figure 2F,G). Hence, phosphorylation of eIF2α, as measured by the ratio of p-eIF2α to t-eIF2α, was found to be increased at the chronic timepoint (F_{p-eIF2α/t-eIF2α}(2,9) = 12.75, P = .0024; One-way ANOVA) (Figure 2H).

Interestingly, levels of the UPR transcription factors XBP1 and CHOP were upregulated at disease onset and remained elevated into the chronic phase (F_{XBP1}(2,9) = 18.59, P = .0006; F_{CHOP}(2,9) = 12.08, P = .0028, One-way ANOVA) (Figure 2L). A key feature of the UPR involves IRE1α splicing of XBP1 mRNA to generate a spliced isoform of XBP1 which acts as a potent transcription factor.\(^{16,29}\) To further elucidate the cellular origin of XBP1, we performed an IHC experiment and discovered that the proportion of neurons with nuclear staining of XBP1 (nXBP1) was increased significantly in the DRG of mice with EAE (F_{total cells}(2,12) = 9.910, P = .0029, One-way ANOVA) (Figure 2L,M). On closer inspection, smaller (<30 µm) diameter neurons demonstrated a significant increase in nXBP1 expression with EAE, while larger (≥30 µm) diameter cells showed minimal change in nXBP1 levels. (F_{<30um}(2,12) = 12.12, P = .0013; F_{≥30um}(2,12) = 0.3210, P = .7314, One-way ANOVA) (Figure 2N,O). These results suggest that the ER stress-pertinent spliced isoform of XBP1 is upregulated in small diameter, putative nociceptors in the DRG.

### 3.4 4-PBA treatment alleviates mechanical and facial hypersensitivity

Recently, the chemical chaperone, 4-phenylbutyric acid (4-PBA), has been shown to ameliorate neuropathic pain by reducing ER stress.\(^{18,22,30}\) We wanted to investigate if daily systemic treatment with 4-PBA (200 mg/kg) starting at the onset of disease (Figure 3A) would alter the EAE disease course and relieve mechanical and orofacial hypersensitivity in mice with EAE. DRGs were then harvested after 7-10 days of treatment with either 4-PBA or vehicle (1x PBS). We did not observe any change in disease course following 4-PBA treatment (F(10,183) = 0.2834, P = .9842) (Figure 3B). At the onset of clinical signs and before 4-PBA administration, we observed the characteristic reduction in von Frey thresholds in mice with EAE. One-hour after 4-PBA injection, the von Frey threshold recovered close to baseline levels. Vehicle administration did not have any behavioral effects (F_{interaction}(2,42) = 4.358, P = .0191, F_{treatment}(2,42) = 39.72, P < .0001, F_{group}(1,21) = 1.746, P = .2006, F_{subject}(21,42) = 1.713, P = .0681, RM two-way ANOVA) (Figure 3C). Due to the ensuing paralysis of the hind limbs at later stages in the disease, we could not assess hindpaw mechanical hypersensitivity 7-10 days postonset at the time of tissue harvest. Instead, we analyzed orofacial pain behaviors (headshakes, single swipe, and continuous swipes) using an air puff assay.\(^8\) As compared to vehicle-treated animals, daily 4-PBA treatment dampened total facial pain behaviors by 50%, headshakes by 66%, single swipe by 50%, and continuous swipes by 33% (Figure 3D).
CNS inflammation has been linked to pain hypersensitivity in EAE mice. To assess whether the antinociceptive effects of 4-PBA were mediated by altered inflammatory responses in the superficial dorsal horn of the spinal cord (SDH), we quantified the levels of Iba1 + microglia/macrophages and CD4 + T-cells in this region (Figure 4A,B).

The levels of Iba1 and CD4 immunoreactivity were elevated in the SDH of both vehicle and 4-PBA-treated animals and were not significantly different between treatments ($F_{\text{Iba1}}(2,11) = 17.38, P = .0004; F_{\text{CD4}}(2,11) = 32.88, P < .0001$, one-way ANOVA). Interestingly, XBP1 immunoreactivity was also increased in the SDH with disease but its expression was not affected with 4-PBA treatment ($F_{\text{XBP1}}(2,11) = 7.845, P = .0076$, one-way ANOVA) (Figure 4C). cFOS, a commonly used marker of cellular activity, was elevated in the SDH of EAE animals.
as shown previously. 4-PBA treatment was not able to significantly reduce Iba1, CD4, and XBP1 immunoreactivity in the dorsal horn of these animals. D, EAE enhanced cFOS expression in the dorsal horn of EAE mice. 4-PBA administration was able to rescue some of the disease-mediated increase in cFOS + cells. *P < .05, **P < .01, ***P < .001, one-way ANOVAs with Tukey’s post hoc analysis across all three groups.

3.6 4-PBA treatment reduces ER stress in the DRG

To further investigate the mechanism of 4-PBA’s beneficial effects on pain behaviors in EAE, levels of UPR-related proteins in the DRG were assessed after 4-PBA treatment (Figure 5). The levels of the ER stress proteins, BiP and t-eIF2α, remained unchanged with 4-PBA treatment ($t_{\text{BiP}}(8) = 0.4978$, $P = .6320$; $t_{\text{t-eIF2α}}(8) = 1.110$, $P = .1495$; unpaired $t$-test) (Figure 5A,B). However, we observed significant reductions
in the phosphorylation of eIF2α, XBP1, and CHOP levels in DRG samples of mice treated with 4-PBA as compared to vehicle administration ($t_{\text{p-eIF2}\alpha}(8) = 3.282, P = .0112$; $t_{\text{p-eIF2}\alpha/\text{t-eIF2}\alpha}(8) = 3.816, P = .0051$; $t_{\text{XBP1}}(8) = 2.517, P = .0360$; $t_{\text{CHOP}}(8) = 2.697, P = .0272$; unpaired $t$-test) (Figure 5C-F). Taken together, these findings suggest that 4-PBA’s antinociceptive effects were due to its ability to dampen ER stress in the DRG, particularly the levels of p-eIF2α, XBP1, and CHOP, each of which represent different pathways of ER stress-induced UPR.

### 3.7 | 4-PBA dampens Ca\(^{2+}\) responses in small-diameter neurons

4-PBA, or its salt sodium 4-phenylbutyrate, can alleviate ER stress primarily as a chemical chaperone\(^3\) (Figure 6A). To further investigate the impact of 4-PBA on neuronal function, we imaged cytosolic Ca\(^{2+}\) transients in dissociated DRG neurons from EAE animals upon caffeine (20 mM) and KCl (30 mM) stimulation (Figure 6B). Caffeine is known to sensitize ryanodine receptors to cytosolic Ca\(^{2+}\) leading to a Ca\(^{2+}\)-induced calcium release (CICR) from the ER. Consistent with a reduction of ER stress-mediated hyperactivation of Ca\(^{2+}\) signaling, 4-PBA treatment reduced the amplitude of caffeine and KCl-mediated Ca\(^{2+}\) rises in small (<30 µm), diameter cells ($t_{\text{Caffeine}}(149) = 3.236, P = .0015$; $t_{\text{KCl}}(185) = 2.238, P = .0264$, unpaired $t$-test) (Figure 6C-F). 4-PBA reduced mRNA levels of Ddit3, Xbp1, and Hspa5, indicating a reduction in ER stress ($t_{\text{Ddit3}}(6) = 2.165, P = .0735$; $t_{\text{Xbp1}}(6) = 3.511, P = .0127$, $t_{\text{Hspa5}}(6) = 3.511, P = .0155$, unpaired $t$-test) (Supplementary Figure S1). These results suggest that 4-PBA reduces CICR and KCl mediated excitability in small diameter cells of the DRG from EAE mice by dampening ER stress.

### 3.8 | Knockdown of Ddit3 and Xbp1 mRNA does not alter evoked Ca\(^{2+}\) rises

Earlier, we observed a reduction in CHOP and XBP1 in vivo after treatment with 4-PBA. To ascertain the contribution of these transcription factors to enhanced Ca\(^{2+}\) signaling observed in EAE cells (Figure 6; Ref. [11]), we silenced...
gene expression of CHOP (encoded by Ddit3) and Xbp1 in dissociated DRG neurons from EAE animals (Figure 7A). Neither XBP1 nor CHOP siRNA treatment changed cytosolic Ca2+ rises upon caffeine and KCl stimulation (FCaffeine(2,221) = 0.5593, P = .5724, FKCl(2,268) = 1.119, P = .3282, one-way ANOVA) (Figure 7B-F). As confirmation of the siRNA efficacy, XBP1 and CHOP siRNA reduced the expression of their respective genes, Xbp1 and Ddit3, as well as Hspa5 (BiP) which may be induced by both XBP1 and CHOP (FXbp1(2,12) = 32.13, P < .0001, FDdit3(2,12) = 25.67, P < .0001, FHspa5(2,12) = 5.020, P = .0260, one-way ANOVA) (Figure 7G-I). Moreover, in order to further assess the efficacy of the transfection system, we transfected dissociated neurons with Alexa Fluor 488-conjugated nonsilencing siRNA which has no known homology to any mammalian gene (Figure 7J). The presence of this siRNA in our neurons suggested that our experimental siRNAs were, indeed, being delivered into the neurons. Altogether, these data indicate that a selective reduction in either CHOP or XBP1 is not sufficient to reduce CICR or KCl excitability in dissociated DRG neurons from EAE animals.

3.9 The PERK inhibitor, AMG44, reduces Ca2+ signalling in small-diameter neurons

Upon ER stress, PERK autophosphorylates, oligomerizes, and subsequently phosphorylates eIF2α. Western blotting of DRGs obtained from EAE mice at disease onset show increased phosphorylation of PERK (t(7) = 4.554, P = .0026, unpaired t-test) (Figure 8A). To investigate the contribution of ER stress-mediated and PERK-induced eIF2α activation, we treated DRG cells from EAE animals with a novel PERK inhibitor, AMG PERK 44 (AMG44)33 (Figure 8B). CICR amplitude, as measured by caffeine stimulation, and KCl depolarization is enhanced in the vehicle (0.1% DMSO)-treated EAE cells as compared to DRG neurons obtained from CFA mice. AMG44 treatment normalizes both caffeine and KCl-induced Ca2+ rises in small (<30 µm) diameter neurons (FCaffeine(2,171) = 3.391, P = .0360, FKCl(2,209) = 4.146, P = .0171, one-way ANOVA) (Figure 8C-G). PCR analysis of dissociated DRG cells revealed that Ddit3 or CHOP transcripts were reduced with AMG44 treatment as compared to vehicle-treated EAE cells (t(8) = 7.013, P = .0001, unpaired t-test). Furthermore, AMG44 treatment increased the expression
of Xbp1 and Hspa5 transcripts ($t_{Xbp1}(8) = 2.865, P = .0210; t_{Hspa5}(8) = 2.738, P = .0255$, unpaired t-test) (Supplementary Figure S1), suggesting that intervening with the PERK arm of UPR allows for a shift toward a more protective IRE1-XBP1-BiP branch of the UPR.

### 3.10 | BK channel current is rescued by 4-PBA and AMG44 treatment

In a previous study,\textsuperscript{10} we noted a reduction in the after-hyperpolarization amplitude of small diameter, putative

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FIGURE 7  Gene knockdown of XBP1 and CHOP did not alter Ca\textsuperscript{2+} transients in small-diameter DRG neurons. A, siRNA transfection knocked down the expression of CHOP and XBP1 mRNA. B-E, Dissociated EAE cells were transfected with XBP1 (n = 77) and CHOP (n = 100) siRNA 20-24 hours prior to Ca\textsuperscript{2+} imaging. We observed no change in the Ca\textsuperscript{2+} transients of EAE DRG neurons during stimulation with caffeine (20 mM) or KCl (30 mM) after siRNA knockdown of XBP1 and CHOP as compared to vehicle (transfection reagent HiPerFect)-treated EAE neurons (n = 94). G and H, As confirmation, PCR analysis of transfected DRG neurons demonstrated a drastic reduction in their respective gene. XBP1 transcript expression was dampened in XBP1 siRNA-treated cells (n = 5) and similarly, CHOP mRNA levels were diminished upon CHOP siRNA treatment (n = 5) as compared to vehicle-treated cells (n = 5). I, We observed that BiP (Hspa5) expression was also reduced upon knockdown of XBP1 and CHOP mRNA. J, To further validate our siRNA delivery system, we transfected cells with a negative siRNA tagged with Alexa Fluor 488. We found that dissociated neurons were transfected with (-)-siRNA using our delivery system further suggesting that XBP1 and CHOP siRNA were successfully delivered in our primary neurons. ### $P < .001$; ****, #### $P < .0001$, one-way ANOVAs with Tukey’s post hoc analysis.
nociceptive neurons from mice with EAE. Ca\(^{2+}\) is known to alter the function of Ca\(^{2+}\)-sensitive K\(^{+}\) channels and hence, we asked whether BK channel properties are modified with EAE disease considering the altered Ca\(^{2+}\) dynamics we observed earlier. In order to prevent dialyzing intracellular calcium, we performed amphotericin B perforated patch-clamp recordings. We found paxilline-sensitive BK channel current almost exclusively in small-diameter IB4 \(^{+}\) neurons similar to previous reports (Figure 9A,D).\(^{34}\) We also found that the conductance-voltage (GV) curve was right-shifted in IB4 \(^{+}\) EAE neurons as compared to neurons from CFA animals, suggesting that BK channel activity was modified in EAE (Figure 9B,E). 4-PBA and AMG44 treatment normalized the GV relationship of the BK channels (Figure 9C,F). EAE responses (red) are the same in Figure 9B,C. In effect, 0 mV test voltage (red trace) minimally activated paxilline-sensitive BK currents in EAE neurons as compared to other conditions illustrating the strong shift in voltage-dependent gating of BK channels in EAE neurons (red trace; Figure 9E,F). The conductance-voltage relationship was quantified as the voltage required for half the maximum conductance (ie, V\(_{1/2}\)) across the cell membrane (Figure 9G) (F(3,34) = 6.631, \(P = .0012\), one-way ANOVA). The current density was not significantly altered (Figure 9H) (F(3,18) = 1.525, \(P = .2421\), one-way ANOVA). However, the resting membrane potential was more depolarized in IB4 \(^{+}\) neurons from EAE animals as compared to IB4 \(^{+}\) control neurons, suggesting that the inherent resting state of the EAE cell was altered due to the disease. 4-PBA and AMG44 treatment in vitro rectified changes in the resting membrane potential of EAE cells (Figure 9I) (F(3,19) = 11.11, \(P = .0002\), one-way ANOVA).

### 3.11 Auxiliary \(\beta_4\) subunit is affected in MS and EAE

In order to investigate the molecular underpinnings of this phenomenon, we assessed the mRNA expression of the pore-forming \(\alpha_1\) subunit (Kcnma1) and the auxiliary \(\beta_1\) and \(\beta_4\) subunits (Kcnmb1 and Kcnmb4, respectively) of the BK channel in human and mouse DRGs. Transcripts of these BK channel subunits have been found to be enriched in non-peptidergic sensory neurons.\(^{35}\) We found a significant loss of \(\beta_4\) subunit transcripts in human DRG samples...
FIGURE 9  ER stress modulators rescue EAE-mediated changes in Ca\(^2+\)-sensitive BK channel properties. A and D, Perforated patch-clamp recordings of DRG neurons from naïve animals revealed that paxilline-sensitive BK current was almost exclusively present in IB4 + non-peptidergic neurons. D, Exemplar BK currents of IB4- and IB4 + neurons in response to paxilline application at 0 (a), 40 (b), 80 (c), 120 (d) seconds. B and C, Conductance-voltage (G-V) relationship among paxilline-sensitive IB4 + DRG neurons extracted from (B) CFA and EAE mice, and (C) EAE neurons treated with 4-PBA and AMG44. Solid lines represent the Boltzmann fit of the G-V relationship. Intersection of the Boltzmann curve and the dotted line represents the voltage at half conduction (E, F) Representative traces of IB4 + neurons from CFA and EAE animals as well as EAE neurons treated with 4-PBA and AMG44. Red trace indicates BK channel conductance at 0 mV. G, Conductance-voltage relationship was quantified as a voltage at half-activation (V\(_{1/2}\)) of BK channels. Vehicle-treated EAE neurons (n = 15) demonstrated a more positive V\(_{1/2}\) in comparison to CFA neurons (n = 12). This effect was reversed with 4-PBA (n = 7) and AMG44 (n = 4) treatment. H, BK current density in IB4 + neurons was not found to be significantly altered. CFA: n = 5, Veh: n = 6, 4-PBA: n = 7, AMG44: n = 4. I, Resting membrane potential was found to be more depolarized in the vehicle-treated EAE neurons (n = 9) as compared to CFA control neurons (n = 5). This was reversed with treatment with ER stress modulators, 4-PBA (n = 4), and AMG44 (n = 5). *P < .05, **P < .01, ****P < .0001, one-way ANOVAs with Holm-Sidak multiple comparison test.
from MS patients as compared to non-demented controls ($t_{KC
mB4}(8.943) = 2.993, P = .0152$, unpaired $t$-test with Welch's correction) (Figure 10A). Similarly, DRGs from EAE animals also showed a loss of $Kcnmb4$ transcripts ($t_{KC
mB4}(6.985) = 3.034, P = .0191$, unpaired $t$-test with Welch's correction) (Figure 10B). In vitro, 4-PBA and AMG44 treatment increased $Kcnmb4$ expression in EAE neurons ($t_{4-PBA}(6) = 2.731, P = .0341; t_{AMG44}(8) = 2.689, P = .0275$, unpaired $t$-test) (Figure 10C,D). Likewise, the expression of $\beta1$ subunit transcripts ($Kcnmb1$) was reduced in whole DRGs obtained from EAE animals and the loss of $Kcnmb1$ expression was rectified with 4-PBA and AMG44 treatment in vitro ($t_{MS}(11) = 0.7320, P = .4795; t_{EAE}(9) = 3.784, P = .0043; t_{4-PBA}(6) = 3.132, P = .0203; t_{AMG44}(8) = 2.574, P = .0329$, unpaired $t$-test) (Supplementary Figure S2). These data suggest that disease-induced ER stress alters BK channel functioning by modulating auxiliary subunits, particularly the $\beta4$ subunit, in IB4 + sensory neurons.

4 | DISCUSSION

Our results demonstrate that post-mortem DRGs from MS patients show evidence of inflammation and immune activation as well as increased expression of ER stress markers. These observations reveal that MS pathology extends beyond the CNS into the PNS. Several recent reports have implicated ER stress in mediating pain hypersensitivity in various models of neuropathy, including diabetic neuropathy, spinal nerve ligation, vasculitic peripheral neuropathy, and CFA-induced orofacial neuropathy. Although MS, like many other neurodegenerative disorders, has previously been associated with ER stress, prior reports have largely focused on studying ER stress and the integrated stress response (ISR) in oligodendrocytes in EAE in the context of CNS demyelination. In contrast, our study highlights a novel role of ER stress in sensory neurons of the PNS for mediating pain in MS/EAE.

Application of 4-PBA has been shown to improve metabolic syndromes, congenital and genetic protein misfolding disorders, inflammation, and neurological disorders such as Parkinson's disease and ischemic brain injury. Daily administration of 4-PBA in our study ameliorated acute mechanical hypersensitivity as well as chronic facial pain behaviors without altering the clinical signs of the disease (Figure 3). A previous study using 4-PBA in EAE, observed that treatment with 4-PBA (400 mg/kg/day) at the time of disease induction reduced clinical signs of EAE. Our current study employs a different variant of the EAE model. Additionally, we designed our study with 4-PBA administration beginning at the onset of clinical signs of EAE to limit the effects of reduced disease severity and its impact on pain behaviors. We were interested in investigating the role of ER stress on pain hypersensitivity in EAE rather than the effect of 4-PBA on the disease itself. Evidently, once EAE has been initiated, 4-PBA administration does not alter EAE disease course or immune activation in the dorsal spinal cord (Figures 3, 4). In the DRG, however, 4-PBA broadly reduced levels of ER stress-related proteins (Figure 5).

**FIGURE 10** ER stress modulators rescue EAE-mediated changes in Ca$^{2+}$-sensitive BK channel properties. A, PCR analysis of post-mortem DRGs extracted from MS patients ($n = 8$) revealed that the BK $\beta4$ subunit ($KC
mB4$) mRNA expression was reduced in MS as compared to DRGs from non-demented controls ($n = 5$). B, Similarly, we observed a loss of $Kcnmb4$ mRNA in EAE samples ($n = 7$) coinciding with pain behaviors in these animals. C and D, In vitro application of 4-PBA ($n = 4$), and AMG44 ($n = 5$) enhanced the expression $Kcnmb4$ transcripts correlating with the normalization of BK current in diseased neurons. *$P < .05$, unpaired $t$-test
The ISR has previously been implicated in pain pathophysiology, as well as EAE. The ISR pathway involves the phosphorylation of eIF2α by an assortment of kinases—PERK, protein kinase R (PKR), heme-regulated eIF2α kinase (HRI), and general control nonderepressible 2 kinase (GCN2)—each of which is initiated by a variety of stressors. Phosphorylation of eIF2α allows the cell to rapidly respond to a stressor by reducing translation of certain genes and increasing translation of others, especially genes with upstream open reading frame (uORF) including, but not limited to, ATF4 and CHOP. Selective translation of genes that may enhance the excitability of sensory neurons in the context of pain, particularly IB4 + nociceptors, has recently been reported. ISR inhibitor (ISRIB) is a commonly used compound to suppress the effects of p-eIF2α without altering its levels; however, the initiating stressors (e.g., UPR, viral infection, amino acid deprivation, etc) and associated kinase activation remain ambiguous. In this study, we hoped to isolate the functional effects of UPR-mediated PERK signalling using a recently developed inhibitor of PERK phosphorylation, AMG44. We found that AMG44 in vitro could suppress caffeine stimulated CICR and KCl mediated Ca2+ excitability in small diameter cells, while gene knock-down of CHOP and XBP1 had no effect on Ca2+ responses of sensory neurons (Figures 7, 8). AMG44 also normalized BK channel physiology in dissociated DRGs from EAE mice as well as mRNA levels of the β4 and β1 auxiliary subunits of BK channels (Figures 9, 10, Supplementary Figure S2). Taken together, these results suggest that EAE mediated activation of PERK in IB4 + sensory neurons alters ER and cytosolic Ca2+ dynamics as well as BK channel physiology resulting in a hyperexcitable state and a painful phenotype. That said, how PERK activation in EAE/MS exactly influences the expression of BK channels and auxiliary subunits remains to be determined.

The majority of cellular Ca2+ is stored in the ER and is tightly regulated by ER-Ca2+ transporters namely sarco/endoplasmic reticulum Ca2+-ATPase (SERCA), inositol triphosphate receptors (IP3R), and ryanodine receptors (RyR). Caffeine is known to sensitize RyRs to very low concentrations of cytosolic Ca2+ which in turn releases ER Ca2+ into the cytosol. Since caffeine stimulation depletes ER Ca2+ stores, we used caffeine-induced Ca2+ transients to examine the levels of luminal Ca2+ in the ER. The exact effect of increased ER luminal Ca2+ on neuronal function is difficult to predict primarily because Ca2+ plays such a varied role inside the cell. However, aberrant Ca2+ dynamics have been closely linked to painful phenotypes. We found small-diameter sensory neurons in EAE to have increased CICR and KCl mediated cytosolic Ca2+ transients. In vitro application of 4-PBA and AMG44 on dissociated DRG neurons from EAE animals reduced the sensitivity to caffeine and KCl stimulation, suggesting that these drugs restore Ca2+ homeostasis of small-diameter sensory neurons.

BK channels act as “coincidence detectors”, consolidating both cytosolic Ca2+ levels and membrane potential, both of which are important factors in initiating and maintaining sensitization. To this effect, a reduction in BK channel current in the DRG is also associated with nerve injury and inflammation-induced pain. In this study, we found paxilline-sensitive BK channel currents almost exclusively in IB4 + sensory neurons (Figure 9) similar to what has previously been reported. BK channels may be spatially coupled with voltage-gated Ca2+ channels and ryanodine receptors allowing even small increases in cytosolic Ca2+ to immediately influence the excitability of the cell. Moreover, reduced BK channel activity, as observed in this study, has been implicated in increased neurotransmission, hyperexcitability, and a more depolarized/resting membrane potential. Behaviorally, sensory neuron-specific (SNS) knockout of the pore-forming β subunit enhanced chronic inflammatory pain, while a BK channel opener, NS1619, dampened inflammation-induced pain behaviors, suggesting that BK channels inhibit sensory input in inflammatory states. In this regard, we observe a loss of β subunit mRNA expression in the DRG of EAE mice (Supplementary Figure S2). However, 4-PBA and AMG44 treatment of EAE sensory neurons in vitro did not reverse this loss, implying that ER stress mechanisms may affect other modulatory subunits of BK channels in this disease.

The auxiliary subunits of BK channels modulate the channel’s Ca2+ and voltage sensitivity, ultimately modulating the cell’s firing properties. In particular, the β4 subunit slows activation and deactivation kinetics of the BK channel as well as reducing Ca2+ sensitivity of the channel at low intracellular Ca2+ levels, while increasing Ca2+ sensitivity at high intracellular Ca2+ concentrations. Knockout of the β4 subunit in hippocampal dentate gyrus granule cells increased fast afterhyperpolarization amplitude and spike frequency of the cell. Loss of β4 subunits is further correlated with seizure activity and heterozygous β4 knockout mice are particularly prone to kainic acid-induced seizures. Of note, increased neuronal activity is also postulated to reduce levels of β4 subunits in an activity-dependent manner. As such, we observed a loss of β4 mRNA expression in the DRG of EAE mice, corresponding to a depolarizing shift in the V1/2 of BK channels in IB4 + neurons and the onset of pain hypersensitivity in our model. In vitro, 4-PBA and AMG44 treatment on EAE neurons increased the expression of Kcnmb4 and normalized BK channel properties. Interestingly, β4 mRNA was also reduced in the DRGs of MS patients further suggesting that...
BK channel physiology may be affected in MS. Hence, alleviation of ER stress may ameliorate pain in MS by normalizing BK channel physiology.

Current approaches to treat pain in MS generally involve NSAIDs, opioids, antidepressants, and antiepileptics. However, these treatment avenues often offer minimal relief and accompany a host of undesired side-effects. In contrast, ER stress modulators like 4-PBA and AMG44 can alleviate ER stress and normalize BK channel properties, ameliorating pain, and limiting the chances for adverse side-effects (Figure 11A,B).

**FIGURE 11** Proposed mechanism (A, B) Sensory neurons, particularly IB4 + nociceptors, from EAE experience ER stress and consequent activation of the PERK-eIF2α pathway. Subsequent modulation of BK channel physiology and ER-Ca²⁺ dyshomeostasis ultimately contributes to pain hypersensitivity in EAE mice. 4-PBA and AMG44 reduce ER stress and PERK phosphorylation, respectively, to normalize Ca²⁺ homeostasis, BK channel physiology, and alleviate pain.
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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
MSY, HTK, TS, BJK was involved in designing the research study. MSY, SS, SML, TF, AC, KT, MD, GT conducted experiments and analyzed the data. GJS processed and provided human samples. KB provided reagents and equipment. MSY and BJK composed the manuscript. BJK supervised the study.

REFERENCES
1. Drulovic J, Basic-Kes V, Grgic S, et al. The prevalence of pain in adults with multiple sclerosis: a multicenter cross-sectional survey. Pain Med. 2015;16:1597-1602.
2. Solaro Claudio, Cella M, Signori Alessio, et al. Identifying neuropathic pain in patients with multiple sclerosis: a cross-sectional multicenter study using highly specific criteria. J Neurol. 2018;265:828-835.
3. Hadjimichael O, Kerns RD, Rizzo MA, Cutter G, Vollmer T. Persistent pain and uncomfortable sensations in persons with multiple sclerosis. Pain. 2007;127:35-41.
4. Waxman SG, Zamponi GW. Regulating excitability of peripheral afferents: emerging ion channel targets. Nat Neurosci. 2014;17:153-163.
5. Chung JM, Chung K. Importance of hyperexcitability of DRG neurons in neuropathic pain. Pain Pract. 2002;2:87-97.
6. Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain. 2009;10:895-926.
7. Khan N, Smith MT. Multiple sclerosis-induced neuropathic pain: pharmacological management and pathophysiologic insights from rodent EAE models. Inflammopharmacology. 2014;22:1-22.
8. Thorburn KC, Paylor JW, Webber CA, Winship IR, Kerr BJ. Facial hypersensitivity and trigeminal pathology in mice with experimental autoimmune encephalomyelitis. Pain. 2016;157:627-642.
9. Yousuf MS, Zubkov K, Tenorio G, Kerr B. The chloride co-transporters, NKCC1 and KCC2, in experimental autoimmune encephalomyelitis (EAE). Neuroscience. 2017;344:178-186.
10. Yousuf MS, Friedman TN, et al. Sensory neurons of the dorsal root ganglia become hyperexcitable in a T-cell-mediated MOG-EAE model of multiple sclerosis. eNeuro. 2019;6:1–15.
11. Mifflin KA, Yousuf MS, Thorburn KC, et al. Voluntary wheel running reveals sex-specific nociceptive factors in murine experimental autoimmune encephalomyelitis. Pain. 2019;160:870-881.
12. Duffy SS, Perera CJ, Makker PGS, Lees JG, CARRIVE P, Moalem-Taylor G. Peripheral and central neuroinflammatory changes and pain behaviors in an animal model of multiple sclerosis. Front Immunol. 2016;7:369.
13. Frezel N, Sohet F, Daneman R, Basbaum AI, Braz JM. Peripheral and central neuronal ATF3 precedes CD4+ T-cell infiltration in EAE. Exp Neurol. 2016;283:224-234.
14. Lu J, Kurejoiva M, Wirotsang LN, Linker RA, Kuner R, Tappe-Theodor A. Pain in experimental autoimmune encephalitis: a comparative study between different mouse models. J Neuroinflammation. 2012;9:233.
15. Stone S, Lin W. The unfolded protein response in multiple sclerosis. Front Neurosci. 2015;9:264.
29. Vidal RL, Hetz C. Unspliced XBP1 controls autophagy through FoxO1. *Cell Res.* 2013;23:463-464.

30. Ayala P, Montenegro J, Vivar R, et al. Attenuation of endoplasmic reticulum stress using the chemical chaperone 4-phenylbutyric acid prevents cardiac fibrosis induced by isoproterenol. *Exp Mol Pathol.* 2012;92:97-104.

31. Olechowski CJ, Parmar A, Miller B, et al. A diminished response to formalin stimulation reveals a role for the glutamate transporters in the altered pain sensitivity of mice with experimental autoimmune encephalomyelitis (EAE). *Pain.* 2010;149:565-572.

32. Kaur B, Bhat A, Chakraborty R, et al. Proteomic profile of 4-PBA treated human neuronal cells during ER stress. *Mol Omi.* 2018;14:53-63.

33. Smith AL, Andrews KL, Beckmann H, et al. Discovery of 1H-pyrazol-3(2H)-ones as potent and selective inhibitors of protein kinase R-like endoplasmic reticulum kinase (PERK). *J Med Chem.* 2015;58:1426-1441.

34. Zhang XL, Mok LP, Katz EJ, Gold MS. BKCa currents are enriched in a subpopulation of adult rat cutaneous nociceptive dorsal root ganglion neurons. *Eur J Neurosci.* 2010;31:450–462.

35. Usoskin D, Furlan A, Islam S, et al. Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. *Nat Neurosci.* 2015;18:145-153.

36. Barragán-Iglesias P, Kuhn J, Vidal-Cantú GC, et al. Activation of the integrated stress response in nociceptors drives myeloidglycosylated-induced pain. *Pain.* 2019;160:160-171.

37. Zhang E, Yi M-H, Shin N, et al. Endoplasmic reticulum stress impairment in the spinal dorsal horn of a neuropathic pain model. *Sci Rep.* 2015;5:11555.

38. Yang ES, Bae JY, Kim TH, Kim YS, Suk K, Bae YC. Involvement of endoplasmic reticulum stress response in orofacial inflammatory pain. *Exp Neurobiol.* 2014;23:372-380.

39. Andhavarakup S, Mubariz F, Arvas M, Bever C, Makar TK. Interplay between ER stress and autophagy: a possible mechanism in multiple sclerosis pathology. *Exp Mol Pathol.* 2019;108:183-190.

40. Yue Y, Stanojlovic M, Lin Y, Karsenty G, Lin W. Oligodendrocyte-specific ATF4 inactivation does not influence the development of EAE. *J Neuroinflammation.* 2019;16:23.

41. Stone S, Wu S, Jamison S, Durose W, Pallais JP, Lin W. Activating transcription factor 6α deficiency exacerbates oligodendrocyte death and myelin damage in immune-mediated demyelinating diseases. *Glia.* 2018;66:1331-1345.

42. Lin W, Lin Y, Li J, et al. Oligodendrocyte-specific activation of PERK signaling protects mice against experimental autoimmune encephalomyelitis. *J Neurosci.* 2013;33:5980-5991.

43. Chen Y, Podojil JR, Kunjamma RB, et al. Sephin1, which prolongs the integrated stress response, is a promising therapeutic for multiple sclerosis. *Brain.* 2019;142:344-361.

44. Way SW, Podojil JR, Clayton BL, et al. Pharmaceutical integrated stress response enhancement protects oligodendrocytes and provides a potential multiple sclerosis therapeutic. *Nat Commun.* 2015;6:6532.

45. Lin W, Bailey SL, Ho H, et al. The integrated stress response prevents demyelination by protecting oligodendrocytes against immune-mediated damage. *J Clin Invest.* 2007;117:448-456.

46. Kolb PS, Ayaub EA, Zhou W, Yum V, Dickhout JG, Ask K. The therapeutic effects of 4-phenylbutyric acid in maintaining proteostasis. *Int J Biochem Cell Biol.* 2015;61:45-52.

47. Dasgupta S, Zhou Y, Jana M, Banik NL, Pahan K. Sodium pentylnoacetate inhibits adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice at multiple steps. *J Immunol.* 2003;170:3874-3882.

48. Hussien Y, Cavener DR, Popko B. Genetic inactivation of PERK signaling in mouse oligodendrocytes: normal developmental myelination with increased susceptibility to inflammatory demyelination. *Glia.* 2014:62-680-691.

49. Pakos-Zebrucka K, Koryga I, Mnich K, Ljubic M, Samali A, Gorman AM. The integrated stress response. *EMBO Rep.* 2016;17:1374-1395.

50. Khutoryansky A, Price TJ. Translational control mechanisms in persistent pain. *Trends Neurosci.* 2018;41:100-114.

51. Hetz C, Axten JM, Patterson JB. Pharmacological targeting of the unfolded protein response for disease intervention. *Nat Chem Biol.* 2015;19:764-775.

52. Verkhratsky A. Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. *Physiol Rev.* 2005;85:201-279.

53. Hagenston AM, Simonetti M. Neuronal calcium signaling in chronic pain. *Cell Tissue Res.* 2014;357:407-426.

54. Bourinet E, Altier C, Hildebrand ME, Trang T, Salter MW, Zamponi GW. Calcium-permeable ion channels in pain signaling. *Physiol Rev.* 2014;94:81-140.

55. Contet C, Goulding SPP, Kuljis DAA, Barth ALL. BK channels in the central nervous system, In: Contet C, ed. *International Review of Neurobiology.* Vol. 128. Amsterdam, the Netherlands: Elsevier; 2016:281-342.

56. Chen S-R, Cai Y-Q, Pan H-L. Plasticity and emerging role of BKCa channels in nociceptive control in neuropathic pain. *J Neurochem.* 2009;110:352-362.

57. Cao X-H, Chen S-R, Li L, Pan H-L. Nerve injury increases brain-derived neurotrophic factor levels to suppress BK channel activity in primary sensory neurons. *J Neurochem.* 2012;121:94-953.

58. Zhang XL, Mok LP, Lee KY, Charbonnet M, Gold MS. Inflammation-induced changes in BK(Ca) currents in cutaneous dorsal root ganglion neurons from the adult rat. *Mol Pain.* 2012;8:37.

59. Berta T, Park C-K, Xu Z-Z, et al. Extracellular caspase-6 drives murine inflammatory pain via microglial TNF-α secretion. *J Clin Invest.* 2014;124:1173-1186.

60. Lu R, Lukowski R, Sausbier M, et al. BKCa channels expressed in sensory neurons modulate inflammatory pain in mice. *Pain.* 2014;155:556-565.

61. Irie T, Trussell LO. Double-nanodomain coupling of calcium channels, ryanodine receptors, and BK channels controls the generation of burst firing. *Neuron.* 2017;96:856-870.e4.

62. Wang B, Bugay V, Ling L, Chuang H-HH, Jaffe DB, Brenner R. Knockout of the BK β4-subunit promotes a functional coupling of BK channels and ryanodine receptors that mediate a LAMP-induced increase in excitability. *J Neurophysiol.* 2016;116:456-465.

63. Griguoli M, Sgritta M, Cherubini E. Presynaptic BK Ca channels control transmitter release: physiological relevance and potential therapeutic implications. *J Physiol.* 2016;594:3489-3500.

64. Sun X, Hirano AA, Brecha NC, Barnes S. Calcium-activated BKCa channels govern dynamic membrane depolarizations of horizontal cells in rodent retina. *J Physiol.* 2017;595:4449-4465.

65. Behrens R, Nolting A, Reimann F, Schwarz M, Waldschütz R, Pongs O. hKCNMB3 and hKCNMB4, cloning and characterization
of two members of the large-conductance calcium-activated potassium channel β subunit family. *FEBS Lett.* 2000;474: 99-106.

66. Whitmire LE, Ling L, Bugay V, et al. Downregulation of KCNMB4 expression and changes in BK channel subtype in hippocampal granule neurons following seizure activity. *PLoS One.* 2017;12:1–17.

67. Urits I, Adamian L, Fiocchi J, et al. Advances in the understanding and management of chronic pain in multiple sclerosis: a comprehensive review. *Curr Pain Headache Rep.* 2019;23:59.

68. Kshatri AS, Gonzalez-Hernandez A, Giraldez T. Physiological roles and therapeutic potential of Ca²⁺ activated potassium channels in the nervous system. *Front Mol Neurosci.* 2018;11:258.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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