

## RESEARCH ARTICLE

# Progesterone receptor membrane component 1 inhibits tumor necrosis factor alpha induction of gene expression in neural cells

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## Abstract

Progesterone membrane receptor component 1 (Pgrmc1) is a cytochrome b5-related protein with wide-ranging functions studied most extensively in non-neural tissues. We previously demonstrated that Pgrmc1 is widely distributed in the brain with highest expression in the limbic system. To determine Pgrmc1 functions in cells of these regions, we compared transcriptomes of control siRNA-treated and Pgrmc1 siRNA-treated N42 hypothalamic cells using whole genome microarrays. Our bioinformatics analyses suggested that Pgrmc1 plays a role in immune functions and likely regulates proinflammatory cytokine signaling. In follow-up studies, we showed that one of these cytokines, TNF $\alpha$ , increased expression of *rtp4*, *ifit3* and *gbp4*, genes found on microarrays to be among the most highly upregulated by Pgrmc1 depletion. Moreover, either Pgrmc1 depletion or treatment with the Pgrmc1 antagonist, AG-205, increased both basal and TNF $\alpha$ -induced expression of these genes in N42 cells. TNF $\alpha$  had no effect on levels of *Rtp4*, *Ifit3* or *Gbp4* mRNAs in mHippoE-18 hippocampal control cells, but Pgrmc1 knock-down dramatically increased basal and TNF $\alpha$ -stimulated expression of these genes. P<sub>4</sub> had no effect on *gbp4*, *ifit3* or *rtp4* expression or on the ability of Pgrmc1 to inhibit TNF $\alpha$  induction of these genes. However, a majority of the top upstream regulators of Pgrmc1 target genes were related to synthesis or activity of steroids, including P<sub>4</sub>, that exert neuroprotective effects. In addition, one of the identified Pgrmc1 targets was Nr4a1, an orphan receptor important for the synthesis of most steroidogenic molecules. Our findings indicate that Pgrmc1 may exert neuroprotective effects by suppressing TNF $\alpha$ -induced neuroinflammation and by regulating neurosteroid synthesis.

**Competing interests:** The authors have declared that no competing interests exist.

## Introduction

Progesterone receptor membrane component 1 (Pgrmc1) is an ancient and somewhat enigmatic molecule with a diverse range of functions and multiple intracellular locations. Structurally, it is a 28-kDa protein with an N-terminal extracellular region, a single transmembrane domain and a cytoplasmic region. The cytoplasmic region contains a cytochrome b5-like heme-binding domain that allows interaction with a number of steroidogenic and drug-metabolizing cytochrome P450 enzymes [1–4]. As suggested by its name, Pgrmc1 also contains a high-affinity progesterone (P<sub>4</sub>) binding site [5–9], most likely within the transmembrane domain and the initial segment of the C terminus [10] and near the heme-binding site [7]. Finally, the Pgrmc1 molecule contains sites that may allow interaction with SH2- and SH3-domain signaling proteins [11].

In view of these diverse molecular structural features, it is not surprising that Pgrmc1 has been implicated in such wide-ranging functions as steroid synthesis [4], heme sensing and Ssynthesis [12], regulation of fatty acid 2 hydroxylase [13], stabilization of tyrosine kinase receptors in cell membranes [14, 15], suppression of p53 and Wnt/ $\beta$ -catenin pathways [16], inhibition of ovarian granulosa cell apoptosis [17] and promotion of breast cancer cell survival and tumor growth [18]. Most studies delineating Pgrmc1 functions have been performed in non-neural tissues and cell lines, but this molecule is also widely distributed throughout the brain [19–22]. Unfortunately, information about Pgrmc1 in neural cells is rather sparse and no unifying concept regarding its neural functions or signaling pathways has emerged.

To better understand the molecular and cellular functions of Pgrmc1 in neural cells, we compared transcriptomes of hypothalamic N42 cells with and without Pgrmc1 knockdown. We independently verified our findings using QPCR and used several bioinformatics tools to identify pathways and neural processes likely regulated by Pgrmc1. Results of these analyses suggest that Pgrmc1 blocks expression of genes downstream of proinflammatory cytokines in a P<sub>4</sub>-independent manner.

## Materials and methods

### Cells and culturing methods

Immortalized hypothalamic N42 neuronal cells (CELLutions Biosystems, Inc.; Burlington, Ontario, Canada; [23]) and mEH-18 hippocampal neuronal cells (CELLutions Biosystems, Inc., [24]) were used for these studies. We verified with PCR that cells of this line do not express the canonical progesterone receptor (Pgr) (see S1 Fig).

Cells were maintained at 37° C and 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Hyclone FBS; Thermo Fisher Scientific, Rockford, IL), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (PS-Gln; GIBCO-BRL; Gaithersburg, MA). When cells reached 60–70% confluence, they were rinsed with phosphate buffered saline (PBS) and media was replaced with phenol red-free DMEM with 10% charcoal-stripped FBS and PS-Gln. After 24 h, cells were transfected with Pgrmc1 siRNA or control scramble sequence constructs.

### Pgrmc1 knockdown with siRNA

N42 cells were transfected with 2  $\mu$ M negative control or Pgrmc1 siRNA constructs (Cat no. S184526 and SI03650318, ThermoFisher; Waltham, MA), using HiPerfect Transfection Reagent according to the manufacturer's protocol (Qiagen; Valencia, CA). Cells were incubated with siRNA for 24 h before harvesting.

To verify Pgrmc1 knockdown, we performed western blots on protein isolated from N42 cell lysates using radioimmunoprecipitation assay buffer (Boston BioProducts; Worcester, MA) containing 1% protease inhibitor cocktail (Calbiochem; San Diego, CA). Protein concentrations were assessed using Pierce BCA Protein Assay (Thermo Fisher Scientific). Ten  $\mu$ g of protein per lane was electrophoresed on 4–15% Tris-HCl SDS-PAGE gradient gels (Biorad; Hercules, CA) and was transferred electrophoretically to Immobilon-P membranes (EMD Millipore; Billerica, MA). Membranes were blocked in 5% skimmed dry milk in Tris-buffered saline plus 0.05% Tween-20 (TBST; pH 7.4) for 1 h at room temperature on an orbital shaker, and then incubated overnight at 4° C with anti-Pgrmc1 antibody (1:1000; Proteintech Group; Chicago, IL) in TBST. Membranes were washed in TBST for 30 min, followed by incubation with horseradish peroxidase-conjugated secondary antibody (anti-rabbit 1:10,000; Abcam; Cambridge, MA) for 1 h, and developed using Chemiluminescence HRP Substrate (EMD Millipore). Blots were apposed to BioMax MR film (Kodak; Rochester, NY), and band intensities were quantified from developed films using densitometric software (GeneTools ver. 3.07 SynGene; Cambridge, England). Blots were re-probed with anti- $\beta$ -actin (1:2000; Abcam) and anti-mouse antisera (1:2000; Abcam) to provide protein loading controls.

We also used QPCR to examine effects of Pgrmc1 knockdown on Pgrmc1 mRNA levels in N42 cells. We isolated RNA using the RNeasy Mini Kit (Qiagen) and reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). QPCR was performed in a Stratagene Mx3000P thermocycler (Agilent Technologies; Wilmington, DE) programmed as follows: 95° C, 10 min; 40 cycles of 95° C for 15 sec; 60° C for 60 sec; and 72° C for 60 sec. We used the QuantiTect SYBR Green Kit (Qiagen) for QPCR following the manufacturer's protocol.

We determined primary efficiency over a range of cDNA concentrations and included samples with no cDNA as negative controls. Primer specificity was validated by observing a single fluorescence peak in each QPCR reaction and also using 2% agarose gel electrophoresis to verify that single products were obtained following the reaction. Further verification entailed a melting curve analyses in which samples were heated to 95° C and fluorescence measurements recording at incremental increases of 0.5° C for 80 cycles.

Data for each sample and controls were obtained using MxPro QPCR analysis software (Agilent Technologies). We used the  $\Delta\Delta$ Ct method to compare treatment and control samples [25], converting data to percent control in each pairwise comparison and calculating means for treatment groups (Pgrmc1 siRNA vs scramble control siRNA). Means of treatment pairs were compared using student t-tests.

### Affymetrix microarray analysis

For these studies, we chose to use microarray technology rather than RNA-Seq because the former is better suited for detecting genes with relatively low expression levels [26]. RNA from N42 cell siRNA experiments described above was used for Mouse Genome 430 2.0 Gene Chip assays (Affymetrix; Palo Alto, CA) performed by the Keck Microarray Institute at Yale University. RNA quality was assessed using an Agilent 2100 Bioanalyzer and RNA 6000 Nano Lab-Chips (Agilent Technologies).

For each treatment group (cells transfected with Pgrmc1 siRNA or with control scramble siRNA), we used three pools of RNA extracted from three replicate studies of transfected N42 cells. The quality of total RNA in each sample was assessed using a Nanodrop Spectrophotometer (Thermo Scientific; Wilmington, DE) and a 2100 Agilent Bioanalyzer (Agilent Technologies). Samples were accepted for analysis if the 260/280 ratio was at least 1.8 and the RNA Integrity Number was greater than 8.0. Preparation of labeled cRNA for hybridization onto Affymetrix GeneChips followed the recommended Affymetrix protocol.

The Yale Center for the NIH Neuroscience Microarray Consortium carried out microarray analysis. Double-stranded cDNA was synthesized from 1 to 5  $\mu$ g of total RNA using a Superscript Choice System (Life Technologies; Carlsbad, CA) with an HPLC-purified oligo (dT) primer containing a T7 RNA polymerase promoter sequence at the 5'-end (Proligo LLC; Boulder, CO). We synthesized the second cDNA strand using *E. Coli* DNA polymerase I, RNase H and DNA ligase, and then generated labeled cRNA using a GeneChip IVT labeling kit (Affymetrix) following the manufacturer's instructions. The labeling procedure incorporated biotinylated synthetic analogs by using a pseudouridine reagent and MEGAscript T7 RNA Polymerase (Life Technologies). Biotin-labeled cRNA was purified using GeneChip cleanup module (Affymetrix). The cRNA was incubated at 94°C for 35 min in fragmentation buffer and the resulting 35- to 200-base fragments were hybridized to the arrays for 16 h at 45°C.

After hybridization, we washed arrays using an Affymetrix fluidics station and stained them with streptavidin-phycoerythrin (10  $\mu$ g/ml; Life Technologies). Arrays were inspected for hybridization artifacts and then scanned with an Affymetrix GeneChip Scanner 3000. Images were analyzed using Affymetrix Microarray Suite 5.0, scaling to a target average intensity. Quality controls included sense strand probes and three housekeeping genes (*gapdh*, *hexokinase* and  *$\beta$ -actin*). We also evaluated spiked controls, background values, scanner noise (Q value) and scaling factors. The CEL files containing levels of probe intensities were analyzed with Partek Genomics Suite, version 6.15 (Partek; St Louis, MO) with the Robust Multi-array Average method of normalization. *P* values were corrected for multiple hypothesis testing using the Benjamini-Hochberg method to control for false discovery. Finally, we performed hierarchical clustering analyses of genes regulated by at least 1.2-fold with  $p < 0.05$ .

## Interrogation of microarray data

We used multiple bioinformatics strategies to identify  $P_4$ -independent pathways likely regulated by Pgrmc1 in neural cells. Integrating data generated with tools that use different algorithms and databases allowed us to develop a more comprehensive mechanistic model.

**Identification of the most highly regulated genes.** We first identified genes regulated by at least 1.2-fold and with a significance of  $p < 0.05$  in each of the two comparison groups (S2 Dataset). We then used GeneCards ([www.genecards.org](http://www.genecards.org)) and NIH Gene ([www.ncbi.nlm.nih.gov/gene](http://www.ncbi.nlm.nih.gov/gene)) to determine general functions of these most highly regulated genes.

**Pathway analysis.** We compared the transcriptomes of Pgrmc1 siRNA- and scramble siRNA-treated N42 cells with Gene Set Enrichment Analysis (GSEA; <http://www.broadinstitute.org/gsea/index.jsp>), software that allows detection of sets of affected genes overrepresented in specific pathways without consideration of the fold change of genes induced by treatment. We used the Hallmark Gene Set from the Molecular Signatures Database of genes known to be involved in specific biological or biochemical relationships or shown to be co-expressed or co-regulated. The Hallmark Gene Sets include 50 sets of genes that represent well characterized biological processes (<http://www.broadinstitute.org/gsea/msigdb/collections.jsp>).

We also compared transcriptomes of Pgrmc1 siRNA-treated and control cells using Ariadne Pathway Studio ([www.elsevier.com/solutions/pathway-studio-biological-research](http://www.elsevier.com/solutions/pathway-studio-biological-research)). The Pathway Studio program is based on a manually curated database of biological relationships derived from published articles beyond the public domain [27].

**Ingenuity upstream regulator analysis.** We used the Ingenuity Pathway Analysis (Qiagen; <https://www.qiagenbioinformatics.com/blog/discovery/publication-roundup-ingenuity-pathway-analysis-3>) to identify upstream transcriptional regulatory cascades linked to the gene changes observed in response to Pgrmc1 knockdown in the absence or presence of  $P_4$ . For this

analysis, we selected the genes that changed by at least 1.2-fold and at a significance level of  $p < 0.01$ .

**Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis.**

We used DAVID Bioinformatics Resources 6.8 program [28, 29] to functionally categorize genes into clusters and determine the fold-enrichment of genes in these clusters. DAVID uses different databases than GSEA and simplifies the process of determining the likely biological significance of microarray data by integrating gene identifier and functional category terms from multiple bioinformatics databases. DAVID uses different databases than GSEA, including Kyoto Encyclopedia of Genes and Genomes, Clusters of Orthologous Groups, Gene Ontology Consortium and the PANTHER Classification System.

DAVID converted the data to DAVID IDs and removed redundancies so that genes detected with multiple probes were not overrepresented in the analysis. We used Functional Annotation Clustering analysis with default databases and stringency settings except that we increased the Kappa Similarity Overlap to 3, Similarity Threshold to 0.7 and the Final Group Membership to 3 in order to minimize duplication of genes in different clusters.

**QPCR verification of microarray findings**

We used QPCR to verify changes in gene expression of targets identified in our microarray analysis. For QPCR studies, we transfected N42 cells with scrambled control siRNA or Pgrmc1 siRNA as described for microarray studies. We then isolated mRNA, performed reverse transcription, measured mRNA levels and verified Pgrmc1 knockdown using QPCR and western blots as described above. Primer pairs for gene targets (Table 1) were based on sequences from

**Table 1. Primers used for QPCR validation of targets identified on microarrays to be significantly regulated by Pgrmc1 knockdown.**

Gene Target	Primer Pairs
<i>Etv1</i>	3' AATGAGGGCCACCGTTTTGG
	5' CCAAACAGCAGGATGGCAC
<i>Gbp4</i>	3' CTGTGCTGTGGGACCAGATT
	5' TGGCTTCCTGGTTCCTGAC
<i>Gpmb</i>	3' TGTCTGATCTCCATCGGCT
	5' TGGCTTGTACGCCTTGTGTT
<i>Ifit3</i>	3' ACTCTTTGGTCATGTGCCGT
	5' AGGACTTCGCCTCCTCTGAA
<i>Nr4a1</i>	3' TTCCCACCACCAGCCACCCA
	5' CTCGCTGCCACCTGAAGCCC
<i>Pgrmc1</i>	3' CCTCTGCATCTTCCTGCTCTA
	5' CGAGCTGTCTCGTCTTTGG
<i>Rtp4</i>	3' AGGCACGCATGAGGATCTTT
	5' CCAAAGTCACCTTCTCACCCA
<i>Tgtp</i>	3' CCCTAAGAGGAAAGCCATCACA
	5' TGGCTCTGTATGGTAGAAGCTC
<i>Trim5</i>	3' GCTGGTTCAAAACAACAATCCA
	5' GCCATGTTCAAGATTCCTTGCTT
<i>Usp18</i>	3' GCTTGACTCCGTGCTTGAGA
	5' CCCAAACCCCTTGCCCATTA
<i>Utx</i>	3' TTGTCGCCTTTAGAACCTTGG
	5' ATGGTAAGACAAAGGGCACAGA

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the Affymetrix Mouse Genome 430 2.0 Gene Chips and were obtained from Integrated DNA Technologies (Coralville, Iowa). To detect spectrin alpha (Spta1) mRNA, we used pre-validated primers (Cat. No. 32969A; SABiosciences). Primary efficiency and specificity were verified as described above.

Data for each sample and controls were obtained using MxPro QPCR analysis software (Agilent Technologies). We used the  $\Delta\Delta C_t$  method to compare treatment and control samples [25], converting data to percent control in each pairwise comparison and calculating means for treatment groups (Pgrmc1 siRNA vs scramble control siRNA). Means of treatment pairs were compared using student t-tests.

### Examination of Pgrmc1 antagonist and P<sub>4</sub> effects on expression of selected genes

To determine whether administration of the Pgrmc1 antagonist, AG-205, altered expression of genes found to be regulated by Pgrmc1, cells were seeded at a density of 50,000 cells/well in 24-well plates and allowed to grow for 48 h in 5% CO<sub>2</sub> at 37° C. They were treated with 10  $\mu$ M AG-205 (Sigma; optimal dosage determined in preliminary studies) or vehicle (cell culture grade DMSO) for 24 h. To determine whether these genes were also regulated by P<sub>4</sub>, we treated N42 cells with either P<sub>4</sub> (10 or 100 nM) or vehicle for 8 h. RNA was then isolated, reverse transcribed and the cDNA was then used as a template in QPCR studies as described above.

### Examination of Pgrmc1 effects on TNF $\alpha$ induction of genes

**Effects of Pgrmc1 knockdown or AG-205 on TNF $\alpha$ -induced expression of genes.** We tested whether TNF $\alpha$  affects *ifit3*, *gbp4* and *rtp4* gene expression and whether Pgrmc1 depletion or blockade changes the responses to TNF $\alpha$ . We chose these targets because they were among the genes most highly regulated by Pgrmc1 knockdown, and they are known targets of cytokines [30–33]. To deplete cells of Pgrmc1, we transfected N42 cells with 10 nM Pgrmc1 siRNA or negative control siRNA, and 40 h later treated cells with 10 ng/ml (0.59 nM) TNF $\alpha$  for 8 h as described above. In a separate study, we treated N42 cells with 10  $\mu$ M AG-205 as described above, incubating the cells for 24 h. RNA was isolated, reverse-transcribed and cDNA used for measurement of *Ifit3*, *Gbp4* and *Rtp4* mRNAs.

**Effects of Pgrmc1 overexpression on TNF $\alpha$ -induced expression of genes.** To determine the effects of overexpression of Pgrmc1 on TNF $\alpha$ -dependent gene expression, we constructed a Pgrmc1 expression vector with puromycin resistance using PCR. We amplified the mouse Pgrmc1 coding sequence with primers that also contained regions homologous to the PstI restriction enzyme cut site region on the 5' ends. The forward primer was: AAC CGG ATC CTC TAG AGT CGA TGG CTG CCG AGG ATG TGG TGG CG and the reverse primer was: CCC CAA GCT TGC ATG CCT GCT CAT TCA TTC TTC CGA GCT GTC. The pBApo-CMV Pur plasmid (Clontech) was digested with PstI (Promega) and ligated to the Pgrmc1 PCR product using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs; Ipswich, MA). We then transformed competent *E. coli* with the resulting Pgrmc1 expression vectors and isolated the plasmids using a Qiagen Plasmid Midi Kit. We confirmed correct insertion of the full coding sequence of Pgrmc1 by sequencing. To generate the stable cell line, N42 cells were plated at a density of 200,000 cells/well in 6-well plates. The following day, cells were transfected with Pgrmc1 expression vector or empty vector using Genejuice transfection reagent (EMD Millipore) following manufacturer's directions. After 48 h, cells were trypsinized and split 1:4 in DMEM containing 3  $\mu$ g/ml puromycin hydrochloride (Cayman Chemical; Ann Arbor, MI). Puromycin-containing media was changed every three days to allow for selection of stably-expressing cells. We verified overexpression of Pgrmc1 with



western blots using the antibody and procedures described above in studies verifying Pgrmc1 downregulation by siRNA.

The resulting N42 cells stably expressing empty or Pgrmc1-containing vector were plated in 24-well plates at a density of 25,000 cells/well and grown in DMEM containing 1  $\mu$ g/ml puromycin. Forty-eight h later, the cells were treated with 10 ng/ml mouse TNF $\alpha$  (Invitrogen) or vehicle (sterile 0.5% BSA in PBS) for 8 h. After lysing the cells in TRIzol (Invitrogen), RNA was extracted, reverse-transcribed and used in QPCR analyses as described above.

For these studies, means were compared among groups using Two-Way ANOVA with Pgrmc1 and TNF $\alpha$  treatments as main effects. Tukey's multiple comparison test was used for *post hoc* analyses.

**Effects of P<sub>4</sub> on TNF $\alpha$ -induced expression of target genes.** Considering that P<sub>4</sub> is a ligand of Pgrmc1, we tested whether TNF $\alpha$ -induction of *ifit3*, *gbp4* and/or *rtp4* expression was affected by P<sub>4</sub> as it is by Pgrmc1 manipulations. We cultured N42 cells as described above, then treated with either P<sub>4</sub> (10 or 100 nM) or vehicle for 1 h before treating with TNF $\alpha$  or vehicle for 8 h. RNA was then isolated using TRIzol (Invitrogen) using the manufacturer's protocol. RNA was reverse transcribed with MMLV-RT (Promega) in the presence of RNasin (Promega) to prevent RNA degradation. The resulting cDNA was then used as a template in QPCR studies using the appropriate gene-specific primers (Table 1). Data were analyzed as described above.

**Effects of Pgrmc1 on cytokine-induced expression of target genes in a hippocampal cell line.** To determine whether the effects of Pgrmc1 on expression of gene targets of pro-inflammatory cytokines is similar in hippocampal cell lines, we used mHippoE-18 cells (CELLutions Biosystems, Inc.). Cells were grown, transfected with Pgrmc1 or negative control siRNA and treated with TNF $\alpha$  as described above. RNA was extracted, reverse-transcribed and cDNA was used to determine effects of Pgrmc1 on *Ifit3*, *Gbp4* and *Rtp4* mRNA levels. Data were analyzed using Two-Way ANOVA with Pgrmc1 and TNF $\alpha$  as main effects followed by Tukey's multiple comparison test.

## Results

### Microarray analyses identified several Pgrmc1-regulated functions

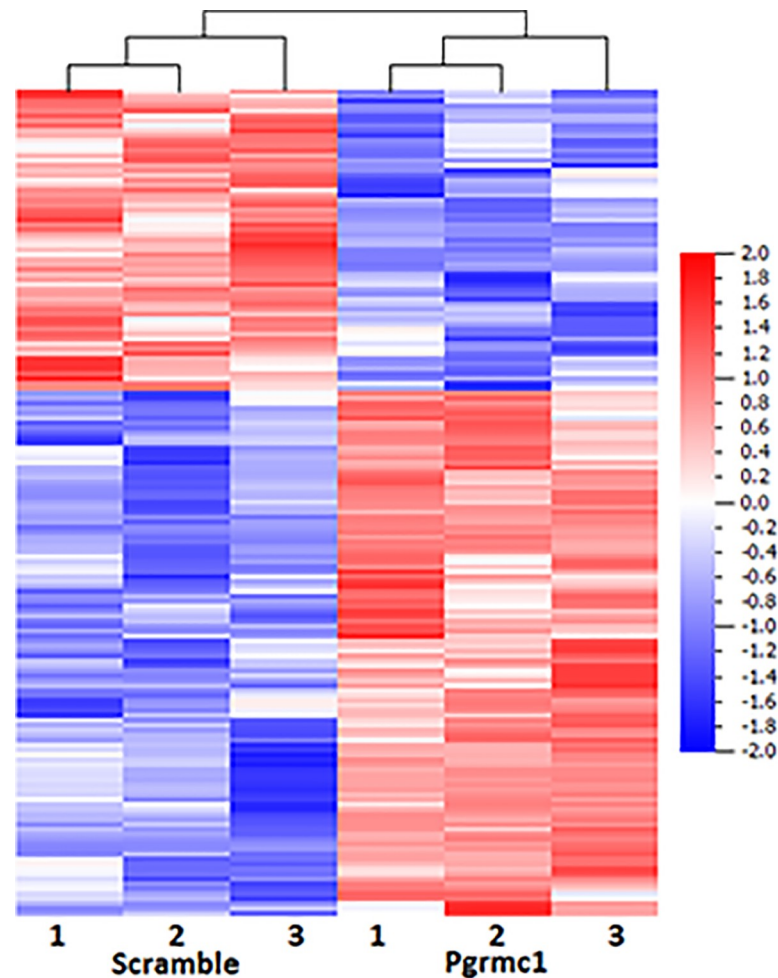
Fig 1 shows the heat map of genes significantly regulated by Pgrmc1 siRNA.

Fig 2 shows results of QPCR and western blots showing that Pgrmc1 was knocked down in Pgrmc1 siRNA-treated cells. S1 Dataset provides results of microarray analysis comparing annotated genes in transcriptomes of N42 cells treated with scramble or Pgrmc1 siRNA.

Compared with controls, Pgrmc1 knockdown in the absence of P<sub>4</sub> changed expression of 1905 annotated genes at the  $p < 0.05$  level and 590 at  $p < 0.01$ . Expression of eighteen of the identified genes (including *pgrmc1*) changed by at least 1.5-fold with  $p < 0.05$  (Table 2). Eleven of these genes have been linked to immune/inflammatory response functions, five to Jak/Stat signaling and three to steroid hormone synthesis or signaling (NIH Gene; [www.ncbi.nlm.nih.gov/gene](http://www.ncbi.nlm.nih.gov/gene) and GeneCards; [www.genecards.org](http://www.genecards.org)).

An unbiased GSEA Hallmark study of the 19301 individual genes (S1 Dataset) identified 14 pathways and processes with NOM  $p$  and FDR  $q$  values of  $p < 0.05$  (Table 3). Nearly half of the pathways and processes were related to Jak/STAT, TNF $\alpha$ , cytokines (interleukins and interferons) and inflammatory regulators (Table 3; shown in bold text). Similarly, results of our Ariadne pathway analysis identified five pathways significantly ( $p < 0.05$ ) regulated by Pgrmc1 knockdown and four involved STAT signaling (Table 4).

Table 5 shows results of our IPA study to identify upstream regulators of the same genes that are altered by Pgrmc1 knockdown. Targets were considered significant if the  $p$  value of



**Fig 1.** Heat map of microarray data showing relative expression of genes found to be differentially expressed ( $p < 0.05$ ) in all three samples for each treatment (scramble siRNA or Pgrmc1 siRNA).

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the overlap between dataset genes and genes regulated by a putative upstream regulator was  $< 0.05$ . A majority of the targets were steroids or molecules related to steroid action or synthesis. In addition, more than half have been linked previously to Pgrmc1 or P<sub>4</sub>, increasing confidence in our data and analyses.

DAVID 6.8 Functional Clustering analysis identified 2112 DAVID IDs and three significantly ( $p < 0.05$ ) enriched Annotation Clusters: Guanylate Binding (11.8-fold enrichment), MAPK Activity (1.8-fold enrichment) and SMAD Protein Signal Transduction (1.4-fold enrichment).

### QPCR studies verified microarray findings

We verified a significant ( $p < 0.0001$ ) decrease in Pgrmc1 mRNA levels after treatment of N42 cells with Pgrmc1 siRNA (scramble control =  $100.0 \pm 4.32$ ; Pgrmc1 siRNA treated =  $49.5 \pm 4.25$ ). Fig 3 shows results of QPCR studies using RNA from N42 cells treated with Pgrmc1 siRNA. Fig 4 shows similar studies verifying that Pgrmc1 knockdown also upregulates three proinflammatory cytokine gene targets, *gbp4*, *ifit3*, and *rtp4*, that were also among the genes identified as top targets on the microarray. Ten of 11 genes significantly regulated by Pgrmc1 in microarray analysis were verified by independent QPCR validation studies.



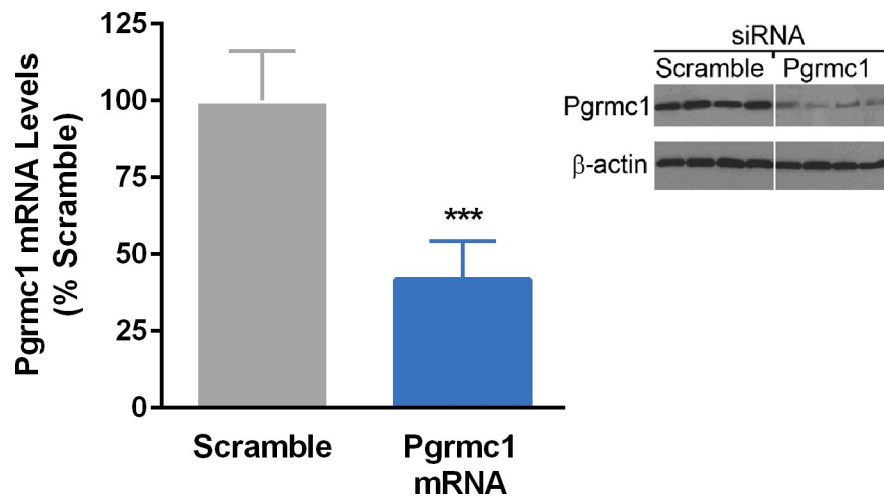


Fig 2. Results of QPCR (left) and western blots (right) verifying knockdown of Pgrmc1 mRNA and protein using siRNA in N42 hypothalamic cells.

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### Pgrmc1 receptor antagonist upregulates expression of cytokine target genes

Fig 5 shows that treatment of cells with the Pgrmc1 antagonist, AG-205, upregulates the expression of *gbp4*, *ifit3*, and *rtp4*, verifying that knockdown results were not an artifact of siRNA transfection.

### Pgrmc1 inhibits TNF $\alpha$ -induced upregulation of genes in N42 hypothalamic cells independent of P<sub>4</sub>

Treatment of N42 cells with TNF $\alpha$  did not alter Pgrmc1 mRNA levels; however, Pgrmc1 knockdown significantly increased both basal and TNF $\alpha$ -induced expression of *gbp4*, *ifit3* and

Table 2. Genes regulated by at least 1.5-fold and  $p < 0.05$  in N42 cells transfected with Pgrmc1 siRNA compared with cells transfected with scramble siRNA. Genes in bold have been linked to immune/interferon functions (NIH Gene, [www.ncbi.nlm.nih.gov/gene](http://www.ncbi.nlm.nih.gov/gene) and GeneCards, [www.genecards.org](http://www.genecards.org)).

Gene Name	Gene Symbol	Entrez Gene ID	Fold Change	P Value
<b>Progesterone receptor membrane component 1</b>	<i>Pgrmc1</i>	53328	-2.2	0.00046
<b>Spectrin, alpha erythrocytic 1</b>	<i>Spna1</i>	20739	-1.5	0.011
<b>Cell migration inducing protein, hyaluronan binding</b>	<i>Cemip</i>	80982	-1.5	0.040
<b>Tripartite motif-containing 5</b>	<i>Trim5</i>	319236	1.5	0.024
<b>Glycoprotein (transmembrane) nmb</b>	<i>GpnmB</i>	93695	1.5	0.024
<b>Ubiquitin specific peptidase 18</b>	<i>Usp18</i>	217109	1.5	0.002
<b>Olfactory receptor, family 51, subfamily B, member 2</b>	<i>Or51b2</i>	18366	1.5	0.044
<b>Nuclear receptor subfamily 4, group A, member 1</b>	<i>Nr4a1</i>	15370	1.5	0.004
<b>2'-5' Oligoadenylate synthetase-like 2</b>	<i>Oasl2</i>	23962	1.5	0.051
<b>Interferon-induced protein 44</b>	<i>Ifi44</i>	99899	1.5	0.0001
<b>Phospholipid scramblase 2</b>	<i>Plscr2</i>	18828	1.5	0.031
<b>Ubiquitously-expressed transcript</b>	<i>Utx</i>	22294	1.5	0.001
<b>Guanylate binding protein 4</b>	<i>Gbp4</i>	55932	1.6	0.029
<b>Guanylate binding protein 7</b>	<i>Gbp7</i>	229900	1.6	0.010
<b>Interferon-induced protein with tetratricopeptide repeats 3</b>	<i>Ifit3</i>	15959	1.6	0.005
<b>T-cell specific GTPase 1</b>	<i>Tgtp</i>	21822	1.7	0.007
<b>Ets variant 1</b>	<i>Etv1</i>	14009	1.8	0.010
<b>Receptor transporter protein 4</b>	<i>Rtp4</i>	67775	1.8	0.002

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**Table 3. Results of GSEA analysis (Hallmark Collection database) of pathways and biological processes that differed significantly between N42 cells transfected with scramble or Pgrmc1 siRNA.** Pathways were considered significant if the normalized enrichment score was at least 1.5-fold and nominal P values and false discovery rate Q values were significant ( $p < 0.05$ ). Pathways involved in inflammatory processes are shown in bold text.

Pathway or Process Name	Normalized Enrichment Score	Nominal P Value	False Discovery Rate Q Value
Interferon Alpha Response	2.64	0	0
TNF $\alpha$ Signaling via Nf $\kappa$ B	2.40	0	0
Interferon Gamma Response	2.31	0	0
E2F Targets	2.15	0	0
Kras Signaling (GTPase)	1.90	0	5.34E-04
Oxidative Phosphorylation	1.89	0	4.45E-04
Myc Targets V1 (transcription/ cell cycle)	1.87	0	3.82E-04
G2M Checkpoint	1.85	0	6.56E-04
DNA Repair	1.84	0	5.83E-04
P53 Pathway	1.75	0	0.0022
Myc Targets V2	1.69	0.004	0.0038
IL6 Jak STAT3 Signaling	1.61	0.010	0.0065
<b>Inflammatory Response</b>	1.57	0	0.010
IL2 STAT5 Signaling	1.51	0	0.017

<https://doi.org/10.1371/journal.pone.0215389.t003>

*rtp4* (Fig 6). Treatment of cells with the Pgrmc1 antagonist, AG-205, increased basal expression of *ifit3* and *rtp4* and significantly enhanced the ability of TNF $\alpha$  to induce expression of *gbp4*, *ifit3* and *rtp4* (Fig 7).

Conversely, Pgrmc1 overexpression in stably transfected N42 cells (Fig 8) completely blocked the ability of TNF $\alpha$  to induce expression of *ifit3*, *gbp4* and *rtp4* genes (Fig 9). P<sub>4</sub> had no effect on the ability of TNF $\alpha$  to increase the expression of these genes (Fig 10).

### Pgrmc1 inhibits cytokine upregulation of genes in mHippoE-18 hippocampal cells

As shown in Fig 11, Pgrmc1 knockdown also increased both basal and TNF $\alpha$ -induced expression of *gbp4*, *ifit3* and *rtp4* in hippocampal cells without altering *pgrmc1* expression. However, in contrast to our findings in N42 cells, TNF $\alpha$  did not significantly increase expression of any of these genes in control cells that contained Pgrmc1.

### Discussion

These microarray and bioinformatics findings provide evidence that Pgrmc111 acts independently of P<sub>4</sub> to regulate signaling important for neuroimmune functions. Among the genes most highly upregulated by Pgrmc1 depletion were *gbp4*, *ifit3*, and *rtp4*, genes induced by

**Table 4. Pathways found to contain significantly ( $p < 0.05$ ) more Pgrmc1-regulated genes than would be expected by chance.** Data were analyzed using Ariadne Pathway Studio Enrichment Analysis.

Rank	Pathway Name	P-Value
1	Interleukin 6 Receptor and STAT3 Signaling	0.019
2	Gonadotrope Cell Activation	0.026
3	Urokinase Receptor and STAT Signaling	0.040
4	Interferon $\alpha/\beta$ Receptor and STAT Signaling	0.041
5	Ciliary Neurotropic Factor and STAT3 Signaling	0.045

<https://doi.org/10.1371/journal.pone.0215389.t004>

**Table 5. Top 10 upstream regulators of genes also regulated by Pgrmc1 in N42 hypothalamic cells.** Regulators shown in boldface type are steroids, steroid receptors or molecules known to regulate steroid synthesis or activities. Those shown in italic boldface are known to be regulated by Pgrmc1 or P<sub>4</sub>.

Upstream Regulator	Direction of Change	Symbol	Molecule Type	P-Value of Overlap
$\beta$ -Estradiol	Activated	E <sub>2</sub>	<b>Steroid transcription regulator</b>	2.94E-14
<i>E2F transcription factor 8</i>		<i>E2f8</i>	<i>Transcription regulator</i>	6.94E-10
<b>Sterol regulatory element binding factor (Srebf) cleavage-activating protein</b>	Activated	Scap	<b>Chaperone protein</b>	8.38E-09
<i>Transforming growth factor <math>\beta</math>1</i>	<i>Activated</i>	<i>Tgfb1</i>	<i>Cytokine; growth factor</i>	1.42E-08
Huntington		Htt	Transcription regulator	1.92E-07
<b>Sterol regulatory element binding factor</b>	Activated	<b>Srebf2</b>	<b>Transcription regulator</b>	3.04E-07
<b>E2F7 transcription factor 7</b>		E2f7	Transcription regulator	5.99E-07
<i>Brain-derived neurotrophic factor</i>	<i>Activated</i>	<i>Bdnf</i>	<i>Nerve growth factor</i>	1.48E-06
<b>Estrogen receptor 1 (encodes estrogen receptor <math>\alpha</math>)</b>	Activated	Esr1	<b>Steroid transcription regulator</b>	3.39E-06
<b>Harvey rat sarcoma viral oncogene homolog</b>		Hras	Ras oncogene	3.73E-06

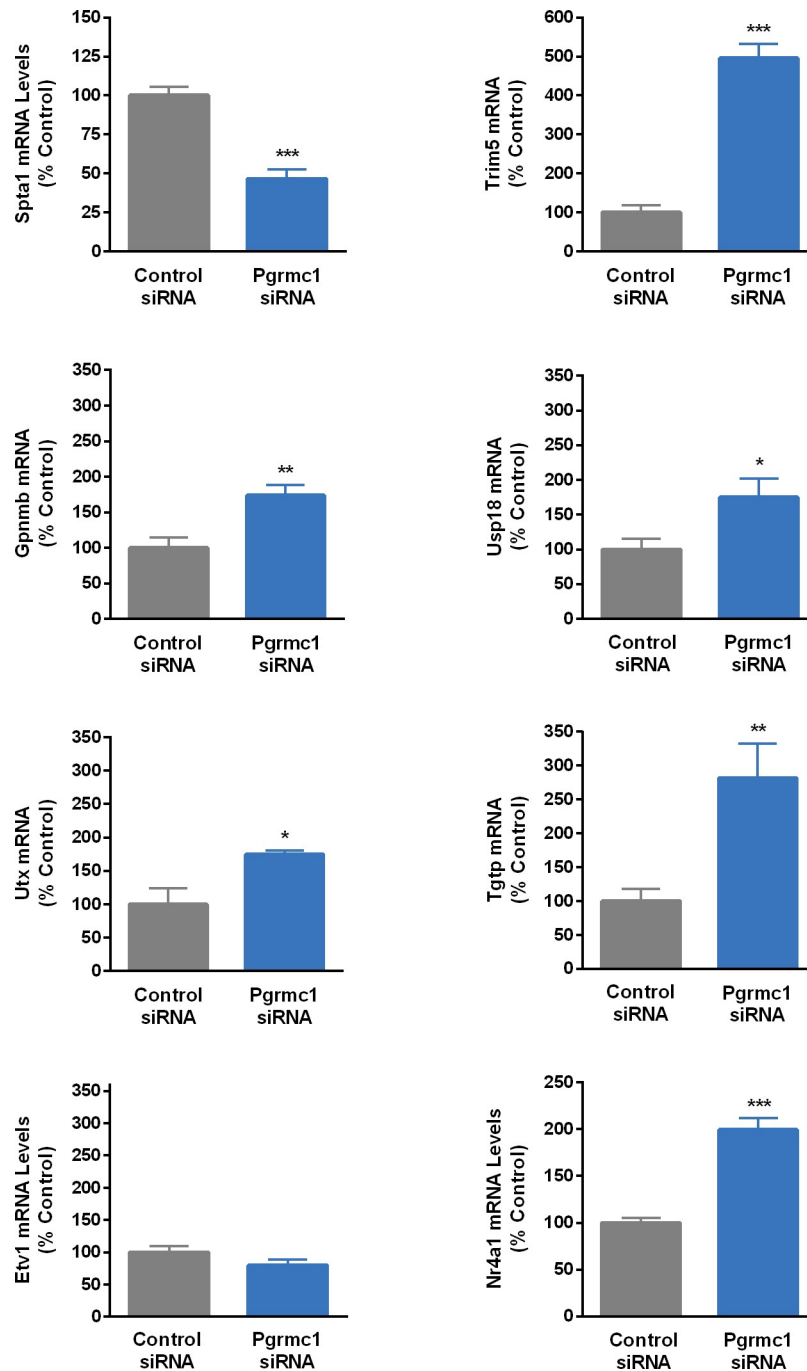
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proinflammatory cytokines such as interferons, interleukins and Tnf $\alpha$  [30–33]. Our follow-up studies verified that at least one of these cytokines, TNF $\alpha$ , upregulated expression of *gbp4*, *ifit3*, and *rtp4* and expression was inhibited by Pgrmc1 in both hypothalamic and hippocampal cell lines. The effect of Pgrmc1 on the expression of these genes was independent of P<sub>4</sub>, but our analysis of upstream regulators suggests that Pgrmc1 may alter synthesis or signaling of steroids that alter neuroinflammatory signals [34]. Overall, our findings provide new insights into how Pgrmc1 may exert neuroprotective effects.

Pathway analyses of the entire transcriptomes of Pgrmc1 siRNA- and scramble siRNA-treated N42 cells identified pro-inflammatory signaling pathways as regulatory targets of Pgrmc1. In addition, 60% of the most highly regulated genes in our data set were downstream of proinflammatory cytokines. It is unlikely that our findings are due primarily to activation of an innate immune response to siRNA for several reasons. First, *gbp4*, *ifit3*, and *rtp4* were upregulated by a Pgrmc1 antagonist in cells not treated with siRNA. Moreover, basal levels of expression were significantly higher in cells treated with Pgrmc1 siRNA than in those treated with scramble control siRNA. Finally, cells engineered to constitutively overexpress Pgrmc1 had lower levels of Gbp4 and Ifit3 mRNA. Instead, our findings indicate that Pgrmc1 suppresses proinflammatory cytokine signaling in neural cells.

One of the identified cytokines, TNF $\alpha$ , is particularly abundant in hypothalamic and hippocampal regions [35–37] wherein *pgrmc1* expression is also highest [21]. TNF $\alpha$  regulates a wide range of physiological functions controlled by the hypothalamus and hippocampus including sleep [38], food intake [39, 40], learning, memory and anxiety-like behaviors [41]. Moreover, dysregulation of these functions can occur when production of TNF $\alpha$  increases in response to infection or injury [40]. Little is known about Pgrmc1 regulation of these functions under normal conditions, but our findings suggest that Pgrmc1 may mitigate pathological effects of elevated TNF $\alpha$  levels by repressing expression of cytokine effector genes.

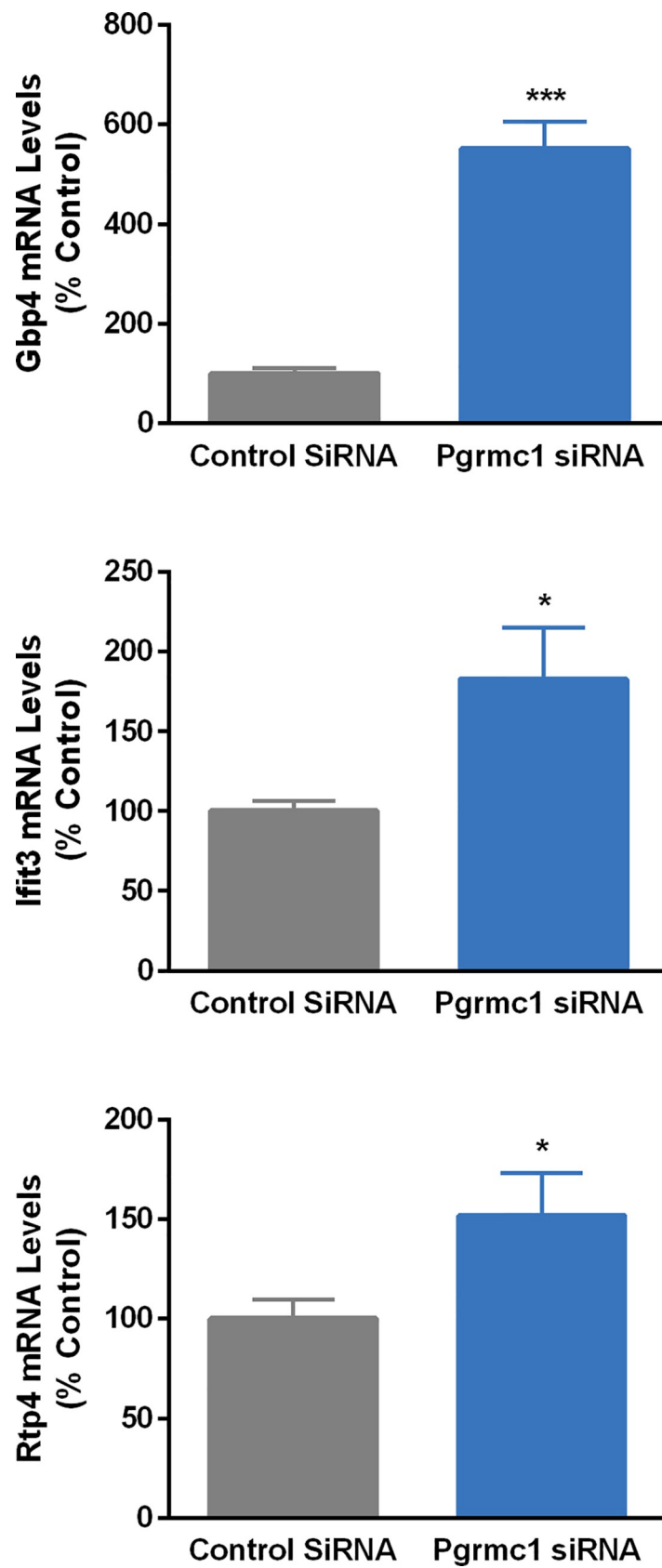
A neuropathology that involves TNF $\alpha$ -mediated neuroinflammation and Pgrmc1 signaling is Alzheimer's disease (AD), a progressive neurological disease that results in cognitive impairment and memory loss. There is some debate about the primary pathophysiology underlying AD. According to the amyloid cascade hypothesis [42], the pathology begins with dysregulation of amyloid beta (A $\beta$ ) production and clearance. A $\beta$  deposition, in turn, is thought to disrupt synaptic functions and induce a cascade that eventually produces neuronal loss and deficits in neural transmission underlying impaired memory and cognition. Others



**Fig 3. Results of QPCR verification of genes that were found to be regulated by Pgrmc1 knockdown in microarray analysis.** N42 cells were transfected with scramble control or Pgrmc1 siRNA. Bars represent mean  $\pm$  SEM. (\*Significantly different from scramble control,  $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ ).

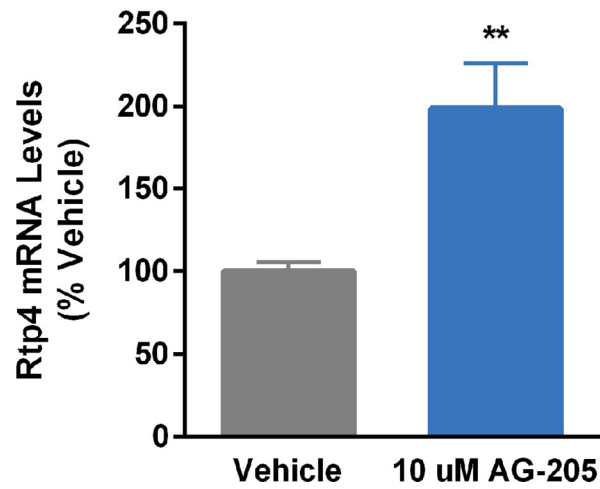
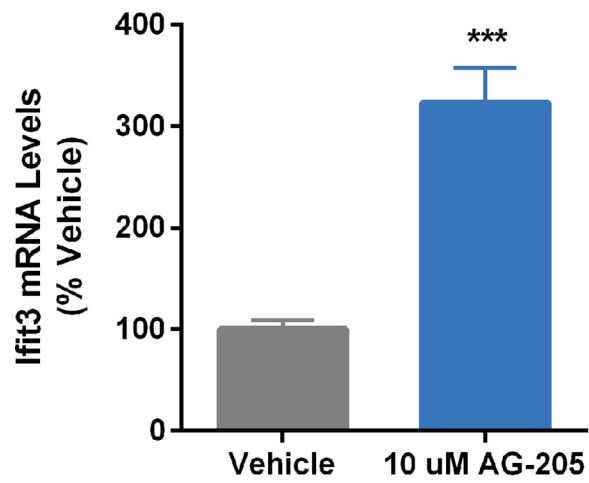
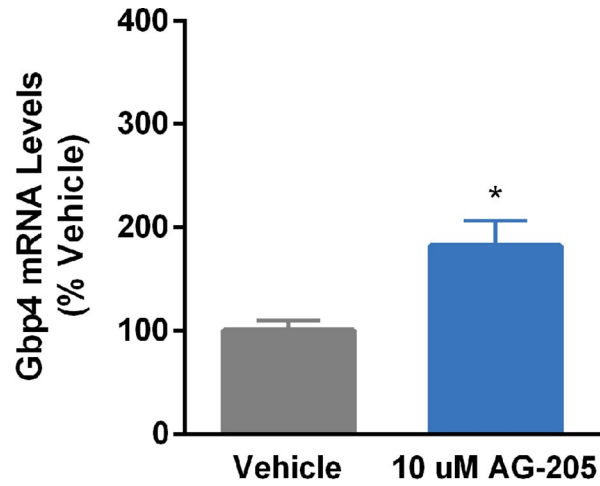
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argue that, rather than amyloid deposition triggering the disease, it may be a result of neuroinflammation mediated by TNF $\alpha$  [43–45]. Previous work combined with findings reported herein show that Pgrmc1 can impact both A $\beta$  deposition and TNF $\alpha$  signaling. The literature in this area is complicated because Pgrmc1 was thought for several years to be the same molecule as the sigma 2 receptor [46]. It is now clear that they are separate molecules encoded by



**Fig 4. Results of QPCR verification of proinflammatory cytokine gene targets found to be regulated by Pgrmc1 knockdown in microarray analysis.** N42 cells were transfected with scramble control or Pgrmc1 siRNA. Bars represent mean  $\pm$  SEM. (\*Significantly different from scramble control,  $p < 0.05$ ; \*\*\* $p < 0.0001$ ).

<https://doi.org/10.1371/journal.pone.0215389.g004>



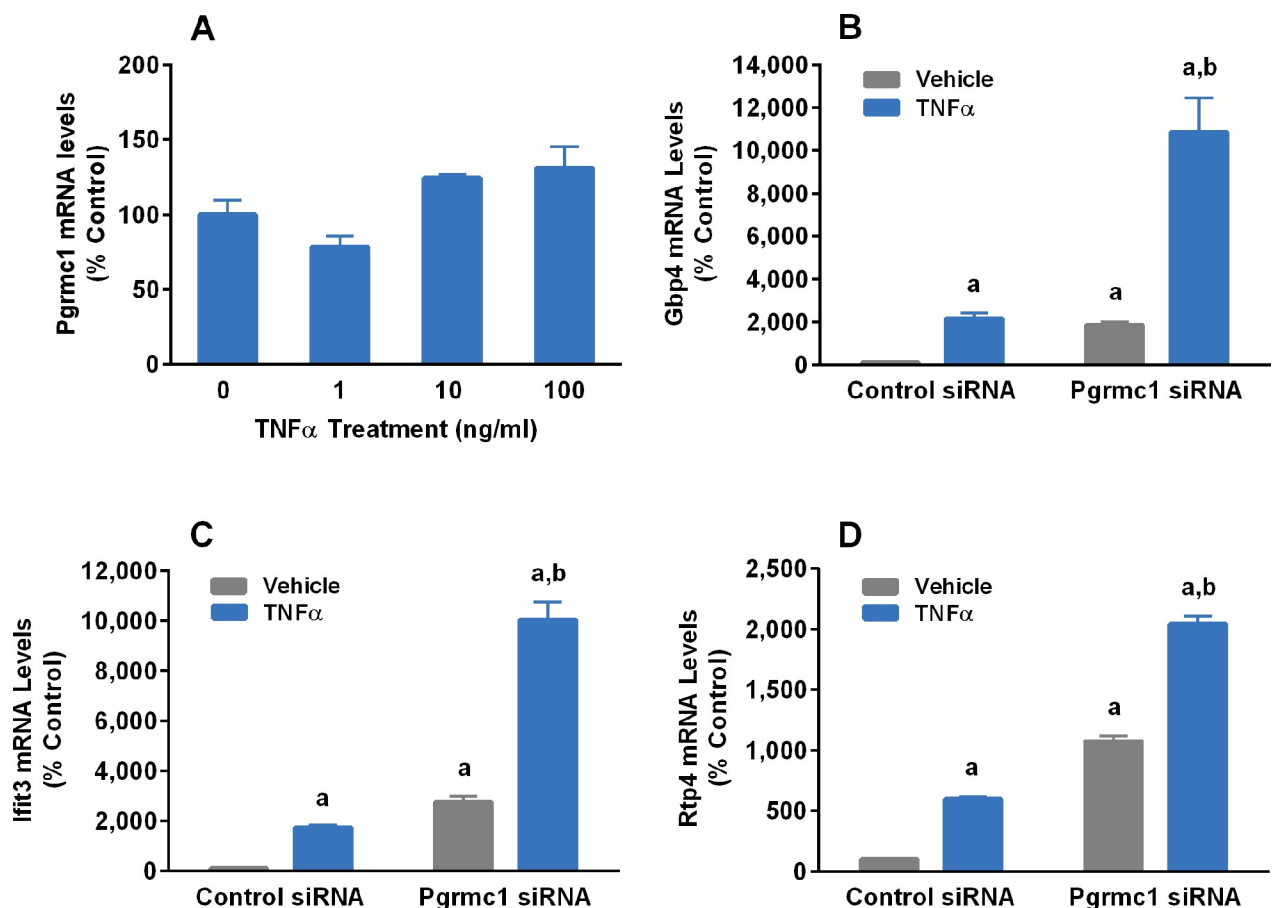


**Fig 5. Effects of Pgrmc1 antagonist, AG-205, on genes found to be upregulated by Pgrmc1 knockdown on microarray.** N42 cells were treated with 10  $\mu$ M AG-205 for 24 h before harvesting. Bars represent mean  $\pm$  SEM. (\*Significantly different from vehicle control,  $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ ).

<https://doi.org/10.1371/journal.pone.0215389.g005>

two different genes [47–49], but both alter A $\beta$  deposition and TNF $\alpha$  signaling. Importantly, Pgrmc1 antibodies or Pgrmc1 siRNA knockdown, as well as antagonists to sigma 2 receptor, prevent A $\beta$  deposition and improve synaptic functioning [50, 51]. Thus, they are logical therapeutic targets for AD treatment. However, such treatments may be complicated because in the present studies we found that Pgrmc1 likely represses TNF $\alpha$  signaling and others showed that sigma 2 agonists inhibit *tnf $\alpha$*  gene expression [52]. Consequently, while Pgrmc1 and sigma 2 antagonists decrease A $\beta$  binding to neurons, they may also block the protective effects of Pgrmc1 and sigma 2 agonists exerted through TNF $\alpha$  inhibition. Further studies will be required to determine how Pgrmc1 and sigma 2 receptor interactions might be manipulated to prevent both A $\beta$  deposition and TNF $\alpha$ -dependent inflammation associated with AD.

The downstream targets of TNF $\alpha$  are not entirely clear in neurons, but our work shows that TNF $\alpha$  upregulates *ifit3*, *gbp4* and *rtp4*, genes among the most highly upregulated when Pgrmc1 was depleted. In addition, both Pgrmc1 depletion and treatment with the Pgrmc1 antagonist, AG-205, enhanced the ability of TNF $\alpha$  to activate expression of *ifit3*, *gbp4* and *rtp4*.



**Fig 6. Effects of TNF $\alpha$  on Pgrmc1 mRNA levels (A) and effects of Pgrmc1 knockdown on TNF $\alpha$  (10 ng/ml) regulation of Gbp4 (B), Ifit3 (C) and Rtp4 (D) mRNA levels in N42 cells.** Bars represent mean  $\pm$  SEM. (<sup>a</sup>Significantly different from control cells treated with vehicle,  $p < 0.001$ ; <sup>b</sup>significantly different from cells treated with Pgrmc1 siRNA + vehicle,  $p < 0.0001$ ).

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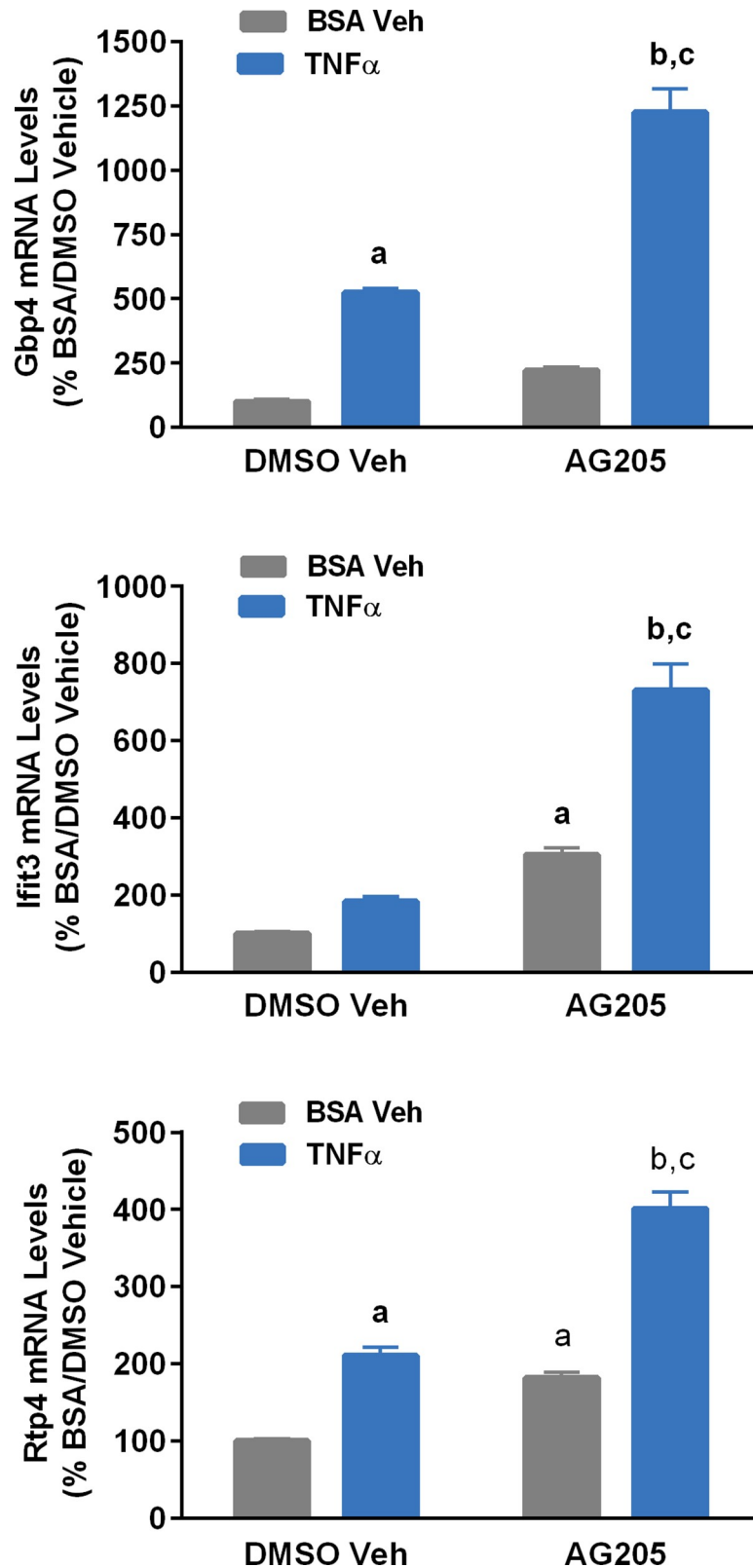
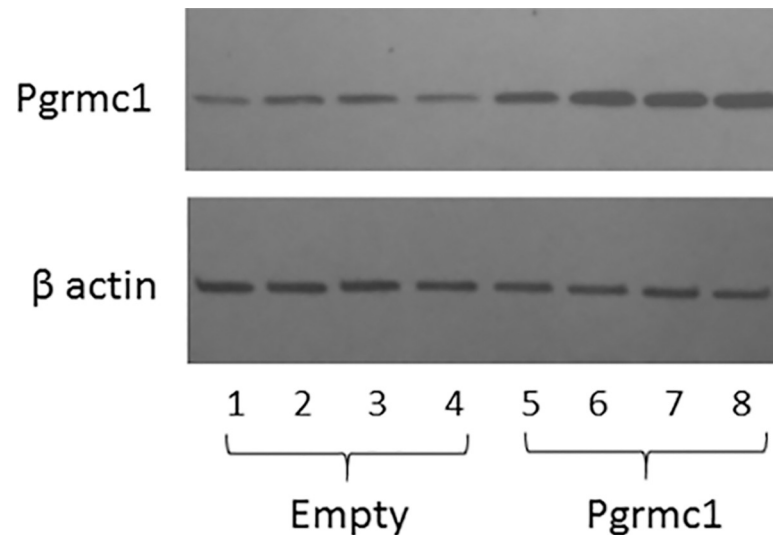


Fig 7. Effects of Pgrmc1 antagonist, AG-205 on TNF $\alpha$  (10 ng/ml) regulation of Gbp4 (top panel), Ifit3 (middle panel) and Rtp4 (bottom panel) mRNA levels in N42 cells. Bars represent mean  $\pm$  SEM. (<sup>a</sup>Significantly different from control cells treated with DMSO vehicle,  $p < 0.0001$ ; <sup>b</sup>significantly different from cells treated with TNF $\alpha$  + vehicle; <sup>c</sup>significantly different from cells treated with AG205+vehicle,  $p < 0.0001$ ).

<https://doi.org/10.1371/journal.pone.0215389.g007>



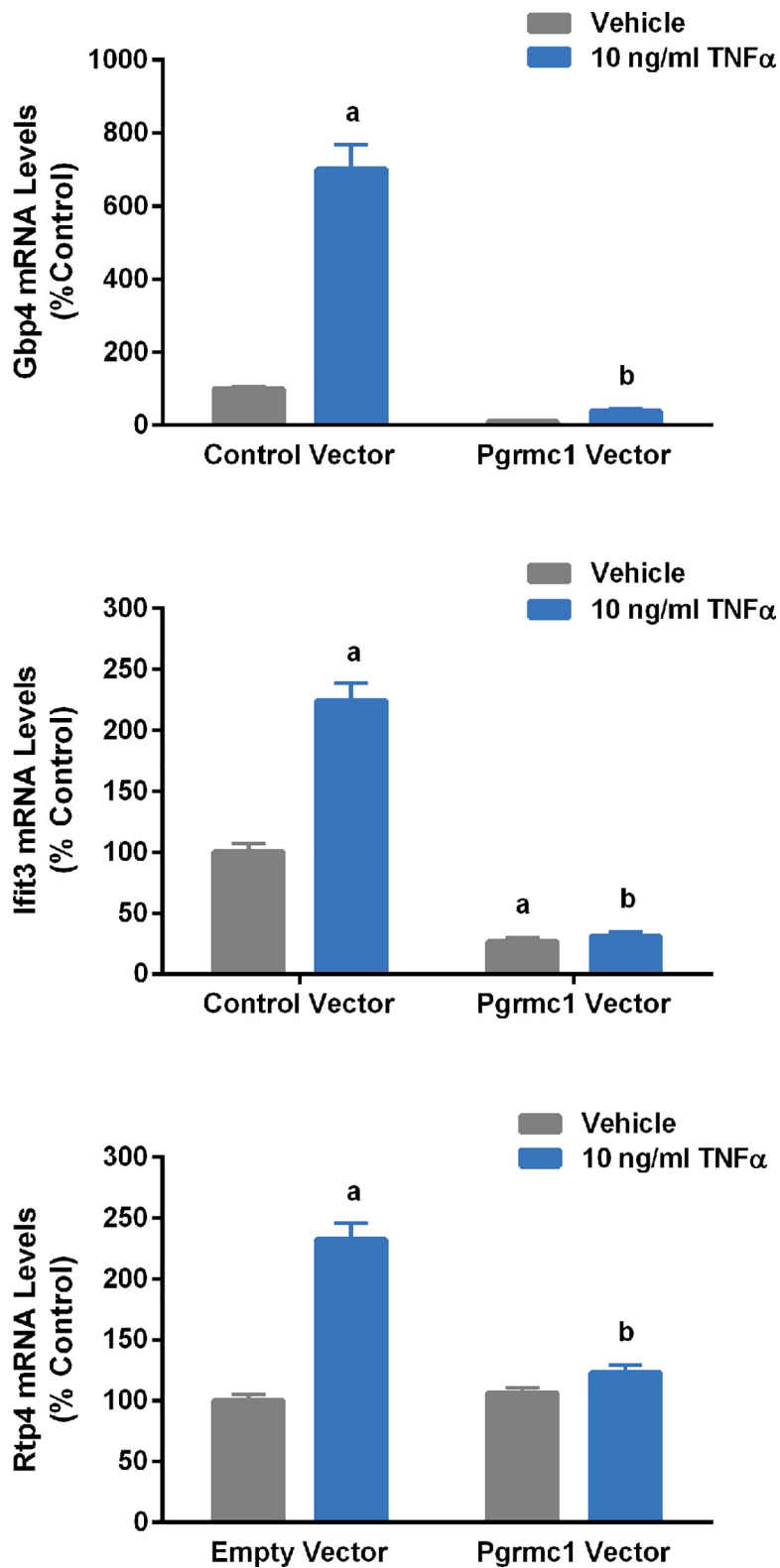
**Fig 8. Pgrmc1 protein levels in cells transfected with an empty vector or with a Pgrmc1 expression vector.**

<https://doi.org/10.1371/journal.pone.0215389.g008>

Unfortunately, these genes have not been studied extensively in neural cells, so it is difficult to predict how they may affect cellular functions during the inflammatory process. *Ifit3* (also known as retinoic acid induced gene G protein) is a Jak-Stat regulated target of interferon and retinoic acid that blocks cell proliferation by upregulating p21 [53]. The other two genes upregulated by TNF $\alpha$  and suppressed by Pgrmc1, *gbp4* and *rtp4* each play a role in G protein signaling. Rtp4 chaperones G-protein coupled receptors to the cell surface [54, 55] and Gbp4 hydrolyzes GTP to both GDP and GMP [56]. Rtp4 also increases trafficking of mu-delta heterodimeric opioid receptors to the cell surface, thereby changing the cellular responses to endogenous ligands [55]. Both mu and delta opioid agonists repress TNF $\alpha$  production [57, 58], suggesting a possible feedback mechanism in the system. Further studies are necessary to determine what role these genes might play in neuroinflammation.

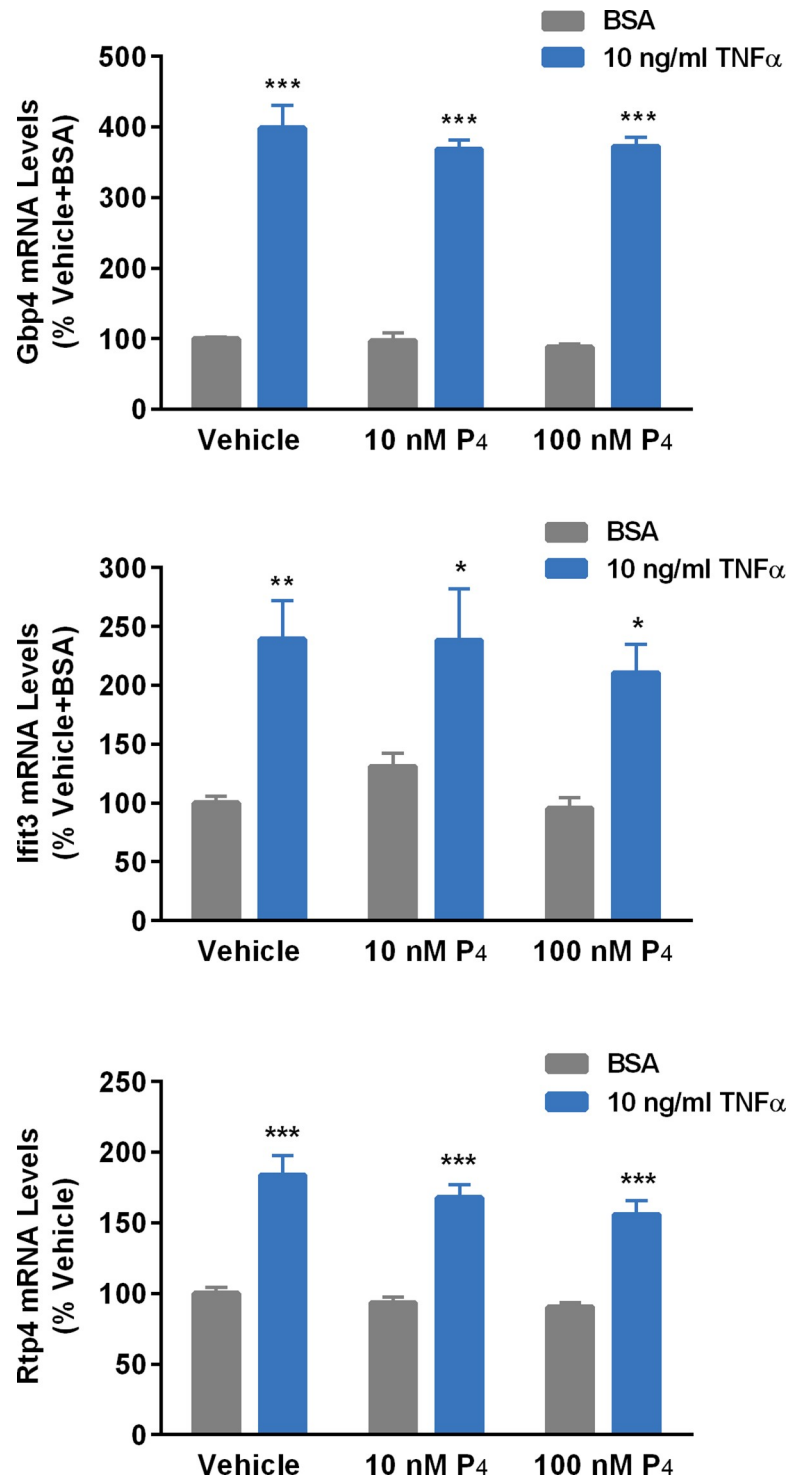
Although P<sub>4</sub> did not affect basal or TNF $\alpha$  induction of Pgrmc1 target genes, one interpretation of our bioinformatics data is that Pgrmc1 indirectly exerts neuroprotective effects by modulating neurosteroid synthesis or signaling. Interrogation of the entire Pgrmc1-dependent transcriptome without considering fold-change determined that over half of the top 20 upstream regulatory pathways identified involved steroid signaling or synthesis. It was beyond the scope of this paper to examine whether steroidogenesis occurs in N42 cells or if Pgrmc1 might alter the process, but previous work shows that Pgrmc1 directly binds to and activates cytochrome P450 enzymes critical for steroid synthesis [1, 2, 4, 59]. In addition, we found that Pgrmc1 regulates expression of *nr4a1* (also known as *nur77*, *tr3*, *nak-1* and *ngf1b*), an immediate early gene required for the induction of a number of genes encoding steroidogenic enzymes [60–63]. Thus, an important question to explore in future studies is whether Pgrmc1 alters synthesis of neurosteroids including progesterone, estrogen or steroid metabolites that are neuroprotective [63].

In summary, our findings suggest that Pgrmc1 may exert neuroprotective effects by blocking TNF $\alpha$  induction of downstream gene targets including *gbp4*, *ifit3*, and *rtp4*. In addition, Pgrmc1 may alter neuroinflammation by regulating neurosteroid synthesis, both directly through steroidogenic enzyme activation and indirectly through *nr4a1* regulation of steroidogenic enzyme expression. Our findings provide important new information about actions of



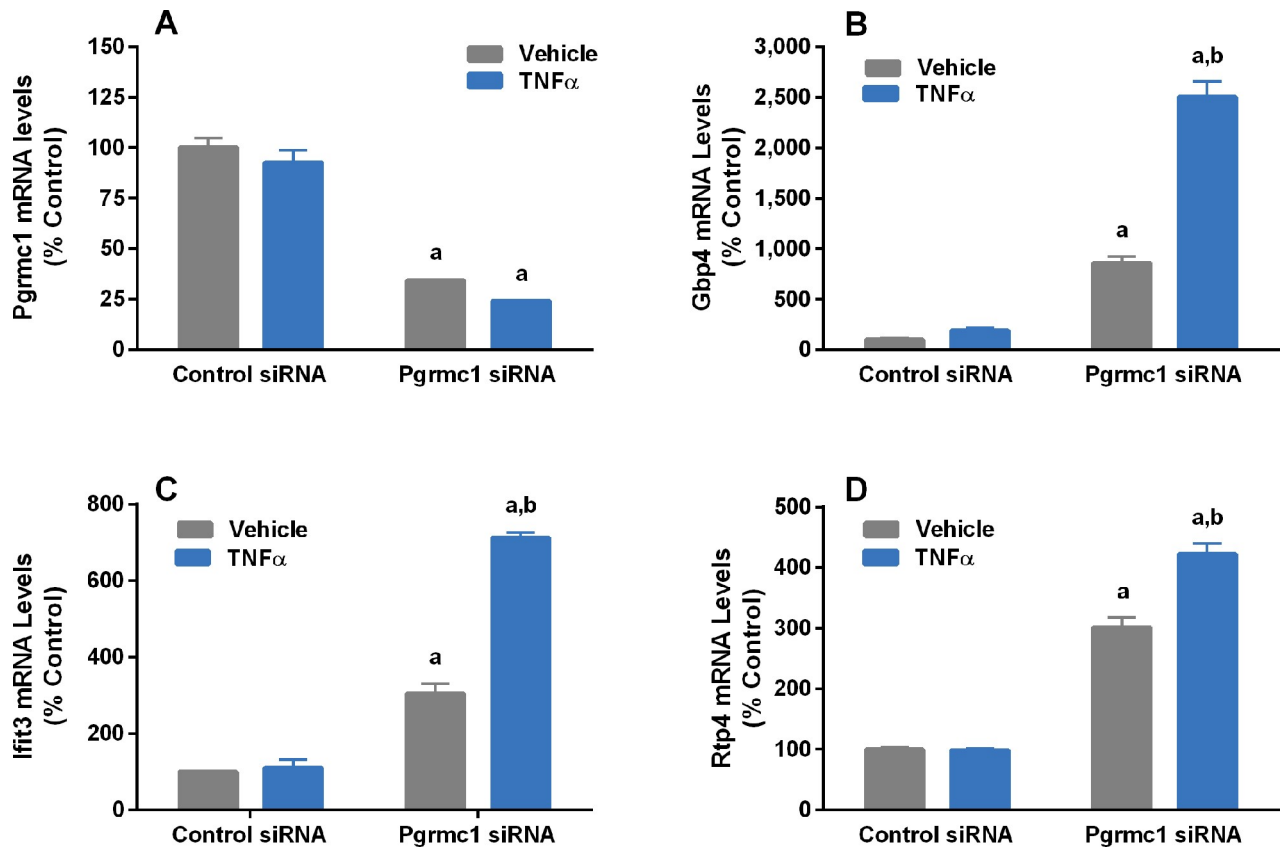
**Fig 9. Effects of Pgrmc1 overexpression on TNF $\alpha$  regulation of genes in N42 cells.** Bars represent mean  $\pm$  SEM. (\*Significantly different from cells with control vector treated with vehicle,  $p < 0.0001$ ; <sup>b</sup>significantly different from cells with control vector treated with TNF $\alpha$ ,  $p < 0.0001$ ).

<https://doi.org/10.1371/journal.pone.0215389.g009>



**Fig 10. Effects of progesterone (P<sub>4</sub>; 10 or 100 nM) on TNF $\alpha$  (10 ng/ml)-induced upregulation of genes in N42 cells.** Bars represent mean  $\pm$  SEM. (\*Significantly different from cells treated with vehicle,  $p < 0.05$ ; \*\*significantly different from vehicle-treated,  $p < 0.01$ ; \*\*\*significantly different from vehicle-treated,  $p < 0.0001$ ).

<https://doi.org/10.1371/journal.pone.0215389.g010>



**Fig 11.** Effects of Pgrmc1 knockdown on TNF $\alpha$  (10 ng/ml) regulation of Pgrmc1 (A), Gbp4 (B), Ifit3 (C) and Rtp4 (D) mRNA levels in mHippoE-18 cells. Bars represent mean  $\pm$  SEM. (<sup>a</sup>Significantly different from corresponding control cells,  $p < 0.001$ ; <sup>b</sup>significantly different from cells treated with Pgrmc1 siRNA + vehicle,  $p < 0.001$ ).

<https://doi.org/10.1371/journal.pone.0215389.g011>

Pgrmc1 in neural cells, and suggest new areas for exploration in the search for therapeutic targets to better treat neurodegenerative diseases.

## Supporting information

**S1 Dataset.** Full set of annotated genes derived from microarray analysis comparing transcriptomes of N42 cells treated with scramble or Pgrmc1 siRNA. This dataset was used for GSEA analysis.

(XLSX)

**S2 Dataset.** Genes found to change by 1.2-fold with  $p < 0.05$  in microarray analysis comparing transcriptomes of N42 cells treated with scramble siRNA or Pgrmc1 siRNA. This dataset was used for Ingenuity Pathway analysis of upstream regulators and for DAVID analysis.

(XLSX)

**S1 Fig.** Photomicrograph of PCR analysis using primers to Pgr (Forward, CTCCGGGACC-GAACAGAGT; Reverse, ACAACAACCCTTTGGTAGCAG) and cDNA reverse transcribed from mRNA obtained from anteroventral periventricular nucleus of female mice (lanes 1–3), lysates of N42 cells (lanes 4–6) or run with no template (Lane 7). Both animals and cells were treated with estradiol to maximize Pgr expression.

(TIF)



## Author Contributions

**Conceptualization:** Karlie A. Intlekofer, Kelsey Clements, Sandra L. Petersen.

**Data curation:** Sandra L. Petersen.

**Formal analysis:** Karlie A. Intlekofer, Alexander Suvorov, Sandra L. Petersen.

**Funding acquisition:** Sandra L. Petersen.

**Investigation:** Karlie A. Intlekofer, Kelsey Clements, Haley Woods, Hillary Adams.

**Methodology:** Kelsey Clements, Haley Woods, Hillary Adams, Alexander Suvorov.

**Project administration:** Sandra L. Petersen.

**Resources:** Sandra L. Petersen.

**Validation:** Kelsey Clements.

**Writing – original draft:** Sandra L. Petersen.

**Writing – review & editing:** Karlie A. Intlekofer, Kelsey Clements, Haley Woods, Hillary Adams.

## References

1. Hughes AL, Powell DW, Bard M, Eckstein J, Barbuch R, Link AJ, et al. Dap1/PGRMC1 binds and regulates cytochrome P450 enzymes. *Cell Metab.* 2007; 5(2):143–9. Epub 2007/02/06. S1550-4131(06)00424-4 [pii] <https://doi.org/10.1016/j.cmet.2006.12.009> PMID: 17276356.
2. Min L, Strushkevich NV, Harnastai IN, Iwamoto H, Gilep AA, Takemori H, et al. Molecular identification of adrenal inner zone antigen as a heme-binding protein. *FEBS J.* 2005; 272(22):5832–43. <https://doi.org/10.1111/j.1742-4658.2005.04977.x> PMID: 16279947.
3. Min L, Takemori H, Nonaka Y, Katoh Y, Doi J, Horike N, et al. Characterization of the adrenal-specific antigen IZA (inner zone antigen) and its role in the steroidogenesis. *Mol Cell Endocrinol.* 2004; 215(1–2):143–8. <https://doi.org/10.1016/j.mce.2003.11.025> PMID: 15026187.
4. Ryu CS, Klein K, Zanger UM. Membrane Associated Progesterone Receptors: Promiscuous Proteins with Pleiotropic Functions—Focus on Interactions with Cytochromes P450. *Front Pharmacol.* 2017; 8:159. <https://doi.org/10.3389/fphar.2017.00159> PMID: 28396637; PubMed Central PMCID: PMC5366339.
5. Falkenstein E, Meyer C, Eisen C, Scriba PC, Wehling M. Full-length cDNA sequence of a progesterone membrane-binding protein from porcine vascular smooth muscle cells. *Biochem Biophys Res Commun.* 1996; 229(1):86–9. <https://doi.org/10.1006/bbrc.1996.1761> PMID: 8954087.
6. Gerdes D, Wehling M, Leube B, Falkenstein E. Cloning and tissue expression of two putative steroid membrane receptors. *Biol Chem.* 1998; 379(7):907–11. PMID: 9705155.
7. Kaluka D, Batabyal D, Chiang BY, Poulos TL, Yeh SR. Spectroscopic and mutagenesis studies of human PGRMC1. *Biochemistry.* 2015; 54(8):1638–47. <https://doi.org/10.1021/bi501177e> PMID: 25675345; PubMed Central PMCID: PMC4533898.
8. Meyer C, Schmid R, Scriba PC, Wehling M. Purification and partial sequencing of high-affinity progesterone-binding site(s) from porcine liver membranes. *Eur J Biochem.* 1996; 239(3):726–31. PMID: 8774719.
9. Peluso JJ, Liu X, Gawkowska A, Johnston-MacAnanny E. Progesterone activates a progesterone receptor membrane component 1-dependent mechanism that promotes human granulosa/luteal cell survival but not progesterone secretion. *J Clin Endocrinol Metab.* 2009; 94(7):2644–9. Epub 2009/05/07. jc.2009-0147 [pii] <https://doi.org/10.1210/jc.2009-0147> PMID: 19417032; PubMed Central PMCID: PMC2708946.
10. Peluso JJ, Romak J, Liu X. Progesterone receptor membrane component-1 (PGRMC1) is the mediator of progesterone's antiapoptotic action in spontaneously immortalized granulosa cells as revealed by PGRMC1 small interfering ribonucleic acid treatment and functional analysis of PGRMC1 mutations. *Endocrinology.* 2008; 149(2):534–43. <https://doi.org/10.1210/en.2007-1050> PMID: 17991724; PubMed Central PMCID: PMC2219306.

11. Cahill MA, Jazayeri JA, Kovacevic Z, Richardson DR. PGRMC1 regulation by phosphorylation: potential new insights in controlling biological activity! *Oncotarget*. 2016; 7(32):50822–7. <https://doi.org/10.18632/oncotarget.10691> PMC5239438. PMID: 27448967
12. Piel RB, Shiferaw MT, Vashisht AA, Marcero JR, Praissman JL, Phillips JD, et al. A Novel Role for Progesterone Receptor Membrane Component 1 (PGRMC1): A Partner and Regulator of Ferrochelatase. *Biochemistry*. 2016; 55(37):5204–17. <https://doi.org/10.1021/acs.biochem.6b00756> PMID: 27599036
13. Hardt R, Winter D, Gieselmann V, Eckhardt M. Identification of progesterone receptor membrane component-1 as an interaction partner and possible regulator of fatty acid 2-hydroxylase. *Biochemical Journal*. 2018; 475(5):853–71. <https://doi.org/10.1042/BCJ20170963> PMID: 29438993
14. Ahmed IS, Rohe HJ, Twist KE, Craven RJ. Pgrmc1 (Progesterone Receptor Membrane Component 1) Associates with Epidermal Growth Factor Receptor and Regulates Erlotinib Sensitivity. *Journal of Biological Chemistry*. 2010; 285(32):24775–82. <https://doi.org/10.1074/jbc.M110.134585> PMID: 20538600
15. Hampton KK, Frazier H, Anderson K, Thibault O, Craven RJ. Insulin receptor plasma membrane levels increased by the progesterone receptor membrane component 1. *Molecular Pharmacology*. 2018. <https://doi.org/10.1124/mol.117.110510> PMID: 29674524
16. Kim JY, Kim SY, Choi HS, Kim MK, Lee HM, Jang Y-J, et al. Progesterone Receptor Membrane Component 1 suppresses the p53 and Wnt/ $\beta$ -catenin pathways to promote human pluripotent stem cell self-renewal. *Scientific Reports*. 2018; 8:3048. <https://doi.org/10.1038/s41598-018-21322-z> PMC5813096. PMID: 29445107
17. Peluso JJ. Progesterone receptor membrane component 1 and its role in ovarian follicle growth. *Frontiers in Neuroscience*. 2013; 7:99. <https://doi.org/10.3389/fnins.2013.00099> PMC3680780. PMID: 23781168
18. Clark NC, Friel AM, Pru CA, Zhang L, Shioda T, Rueda BR, et al. Progesterone receptor membrane component 1 promotes survival of human breast cancer cells and the growth of xenograft tumors. *Cancer Biology & Therapy*. 2016; 17(3):262–71. <https://doi.org/10.1080/15384047.2016.1139240> PMID: 26785864
19. Sakamoto H, Ukena K, Takemori H, Okamoto M, Kawata M, Tsutsui K. Expression and localization of 25-Dx, a membrane-associated putative progesterone-binding protein, in the developing Purkinje cell. *Neuroscience*. 2004; 126(2):325–34. <https://doi.org/10.1016/j.neuroscience.2004.04.003> PMID: 15207350.
20. Bali N, Morgan TE, Finch CE. Pgrmc1: new roles in the microglial mediation of progesterone-antagonism of estradiol-dependent neurite sprouting and in microglial activation. *Front Neurosci*. 2013; 7:157. <https://doi.org/10.3389/fnins.2013.00157> PMID: 24027494; PubMed Central PMCID: PMC3759828.
21. Intlekofer KA, Petersen SL. Distribution of mRNAs encoding classical progesterone receptor, progesterone membrane components 1 and 2, serpine mRNA binding protein 1, and progesterone and ADIPOQ receptor family members 7 and 8 in rat forebrain. *Neuroscience*. 2011; 172:55–65. Epub 2010/10/28. S0306-4522(10)01406-5 [pii] <https://doi.org/10.1016/j.neuroscience.2010.10.051> PMID: 20977928; PubMed Central PMCID: PMC3024713.
22. Krebs CJ, Jarvis ED, Chan J, Lydon JP, Ogawa S, Pfaff DW. A membrane-associated progesterone-binding protein, 25-Dx, is regulated by progesterone in brain regions involved in female reproductive behaviors. *Proc Natl Acad Sci U S A*. 2000; 97(23):12816–21. <https://doi.org/10.1073/pnas.97.23.12816> PMID: 11070092
23. Gomes S, Martins I, Fonseca ACRG, Oliveira CR, Resende R, Pereira CMF. Protective Effect of Leptin and Ghrelin against Toxicity Induced by Amyloid- $\beta$  Oligomers in a Hypothalamic cell Line. *Journal of Neuroendocrinology*. 2014; 26(3):176–85. <https://doi.org/10.1111/jne.12138> PMID: 24528254
24. Gingerich S, Kim GL, Chalmers JA, Koletar MM, Wang X, Wang Y, et al. Estrogen receptor alpha and G-protein coupled receptor 30 mediate the neuroprotective effects of 17 $\beta$ -estradiol in novel murine hippocampal cell models. *Neuroscience*. 2010; 170(1):54–66. <https://doi.org/10.1016/j.neuroscience.2010.06.076> PMID: 20619320
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25(4):402–8. Epub 2002/02/16. <https://doi.org/10.1006/meth.2001.1262> [pii]. PMID: 11846609.
26. Liu S, Lin L, Jiang P, Wang D, Xing Y. A comparison of RNA-Seq and high-density exon array for detecting differential gene expression between closely related species. *Nucleic Acids Res*. 2011; 39(2):578–88. <https://doi.org/10.1093/nar/gkq817> PMID: 20864445; PubMed Central PMCID: PMC3025565.
27. Thomas S, Bonchev D. A survey of current software for network analysis in molecular biology. *Human Genomics*. 2010; 4(5):353. <https://doi.org/10.1186/1479-7364-4-5-353> PMID: 20650822
28. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protocols*. 2008; 4(1):44–57. [http://www.nature.com/nprot/journal/v4/n1/supinfo/nprot.2008.211\\_S1.html](http://www.nature.com/nprot/journal/v4/n1/supinfo/nprot.2008.211_S1.html).

29. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research*. 2009; 37(1):1–13. <https://doi.org/10.1093/nar/gkn923> PMID: 19033363
30. Hoyo-Becerra C, Liu Z, Yao J, Kaltwasser B, Gerken G, Hermann DM, et al. Rapid Regulation of Depression-Associated Genes in a New Mouse Model Mimicking Interferon- $\alpha$ -Related Depression in Hepatitis C Virus Infection. *Molecular Neurobiology*. 2015; 52(1):318–29. <https://doi.org/10.1007/s12035-014-8861-z> PMID: 25159480
31. Martens S, Howard J. The Interferon-Inducible GTPases. *Annual Review of Cell and Developmental Biology*. 2006; 22(1):559–89. <https://doi.org/10.1146/annurev.cellbio.22.010305.104619> PMID: 16824009.
32. Thomson CA, McColl A, Cavanagh J, Graham GJ. Peripheral inflammation is associated with remote global gene expression changes in the brain. *Journal of neuroinflammation*. 2014; 11:73–. <https://doi.org/10.1186/1742-2094-11-73> PMID: 24708794.
33. Wachter C, Müller M, Hofer MJ, Getts DR, Zabarás R, Ousman SS, et al. Coordinated regulation and widespread cellular expression of interferon-stimulated genes (ISG) ISG-49, ISG-54, and ISG-56 in the central nervous system after infection with distinct viruses. *Journal of Virology*. 2007; 81(2):860–71. <https://doi.org/10.1128/JVI.01167-06> PMID: 17079283
34. Giatti S, Boraso M, Melcangi RC, Viviani B. Neuroactive steroids, their metabolites, and neuroinflammation. *J Mol Endocrinol*. 2012; 49(3):R125–34. <https://doi.org/10.1530/JME-12-0127> PMID: 22966132.
35. Bredow S, Guha-Thakurta N, Taishi P, Obal F Jr., Krueger JM. Diurnal variations of tumor necrosis factor alpha mRNA and alpha-tubulin mRNA in rat brain. *Neuroimmunomodulation*. 1997; 4(2):84–90. <https://doi.org/10.1159/000097325> PMID: 9483199.
36. Breder CD, Hazuka C, Ghayur T, Klug C, Huginin M, Yasuda K, et al. Regional induction of tumor necrosis factor alpha expression in the mouse brain after systemic lipopolysaccharide administration. *Proceedings of the National Academy of Sciences*. 1994; 91(24):11393–7. <https://doi.org/10.1073/pnas.91.24.11393>
37. Breder C, Tsujimoto M, Terano Y, Scott D, Saper C. Distribution and characterization of tumor necrosis factor- $\alpha$ -like immunoreactivity in the murine central nervous system. *Journal of Comparative Neurology*. 1993; 337(4):543–67. <https://doi.org/10.1002/cne.903370403> PMID: 8288770
38. Krueger JM, Majde JA, Rector DM. Cytokines in immune function and sleep regulation. *Handbook of clinical neurology*. 2011; 98:229–40. <https://doi.org/10.1016/B978-0-444-52006-7.00015-0> PMC5440845. PMID: 21056190
39. de Kloet AD, Pacheco-López G, Langhans W, Brown LM. The effect of TNF $\alpha$  on food intake and central insulin sensitivity in rats. *Physiology & behavior*. 2011; 103(1):17–20. <https://doi.org/10.1016/j.physbeh.2010.11.037> PMC3067978. PMID: 21163282
40. Santello M, Volterra A. TNF $\alpha$  in synaptic function: switching gears. *Trends in Neurosciences*. 2012; 35(10):638–47. <https://doi.org/10.1016/j.tins.2012.06.001> PMID: 22749718
41. Camara ML, Corrigan F, Jaehne EJ, Jawahar MC, Anscornb H, Koerner H, et al. TNF- $\alpha$  and its receptors modulate complex behaviours and neurotrophins in transgenic mice. *Psychoneuroendocrinology*. 2013; 38(12):3102–14. <https://doi.org/10.1016/j.psyneuen.2013.09.010> PMID: 24094876
42. Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Molecular Medicine*. 2016; 8(6):595–608. <https://doi.org/10.15252/emmm.201606210> PMID: 27025652
43. Cavanagh C, Wong TP. Preventing synaptic deficits in Alzheimer's disease by inhibiting tumor necrosis factor alpha signaling. *IBRO Reports*. 2018; 4:18–21. <https://doi.org/10.1016/j.ibror.2018.01.003> PMID: 30135948
44. Chang R, Yee KL, Sumbria RK. Tumor necrosis factor alpha Inhibition for Alzheimer's Disease. *J Cent Nerv Syst Dis*. 2017; 9:1179573517709278. <https://doi.org/10.1177/1179573517709278> PMID: 28579870; PubMed Central PMCID: PMC5436834.
45. McCaulley ME, Grush KA. Alzheimer's Disease: Exploring the Role of Inflammation and Implications for Treatment. *International Journal of Alzheimer's Disease*. 2015; 2015:515248. <https://doi.org/10.1155/2015/515248> PMC4664815. PMID: 26664821
46. Xu J, Zeng C, Chu W, Pan F, Rothfuss JM, Zhang F, et al. Identification of the PGRMC1 protein complex as the putative sigma-2 receptor binding site. *Nature communications*. 2011; 2:380–. <https://doi.org/10.1038/ncomms1386> PMID: 21730960.
47. Alon A, Schmidt HR, Wood MD, Sahn JJ, Martin SF, Kruse AC. Identification of the gene that codes for the  $\sigma(2)$  receptor. *Proceedings of the National Academy of Sciences of the United States of America*. 2017; 114(27):7160–5. Epub 05/30. <https://doi.org/10.1073/pnas.1705154114> PMID: 28559337.
48. Chu UB, Mavlyutov TA, Chu M-L, Yang H, Schulman A, Mesangeau C, et al. The Sigma-2 Receptor and Progesterone Receptor Membrane Component 1 are Different Binding Sites Derived From

- Independent Genes. *EBioMedicine*. 2015; 2(11):1806–13. <https://doi.org/10.1016/j.ebiom.2015.10.017> PMID: 26870805
49. Pati ML, Groza D, Riganti C, Kopecka J, Niso M, Berardi F, et al. Sigma-2 receptor and progesterone receptor membrane component 1 (PGRMC1) are two different proteins: Proofs by fluorescent labeling and binding of sigma-2 receptor ligands to PGRMC1. *Pharmacological Research*. 2017; 117:67–74. <https://doi.org/10.1016/j.phrs.2016.12.023> PMID: 28007569
  50. Izzo NJ, Staniszewski A, To L, Fa M, Teich AF, Saeed F, et al. Alzheimer's therapeutics targeting amyloid beta 1–42 oligomers I: Abeta 42 oligomer binding to specific neuronal receptors is displaced by drug candidates that improve cognitive deficits. *PLoS One*. 2014; 9(11):e111898. <https://doi.org/10.1371/journal.pone.0111898> PMID: 25390368; PubMed Central PMCID: PMC4229098.
  51. Izzo NJ, Xu J, Zeng C, Kirk MJ, Mozzoni K, Silky C, et al. Alzheimer's therapeutics targeting amyloid beta 1–42 oligomers II: Sigma-2/PGRMC1 receptors mediate Abeta 42 oligomer binding and synaptotoxicity. *PLoS One*. 2014; 9(11):e111899. <https://doi.org/10.1371/journal.pone.0111899> PMID: 25390692; PubMed Central PMCID: PMC4229119.
  52. Iñiguez MA, Punzón C, Nieto R, Burgueño J, Vela JM, Fresno M. Inhibitory effects of sigma-2 receptor agonists on T lymphocyte activation. *Frontiers in pharmacology*. 2013;4:23-. <https://doi.org/10.3389/fphar.2013.00004> PMID: 23494519.
  53. Xiao S, Li D, Zhu H-Q, Song M-G, Pan X-R, Jia P-M, et al. RIG-G as a key mediator of the antiproliferative activity of interferon-related pathways through enhancing p21 and p27 proteins. *Proceedings of the National Academy of Sciences*. 2006; 103(44):16448–53. <https://doi.org/10.1073/pnas.0607830103> PMID: 17050680
  54. Behrens M, Bartelt J, Reichling C, Winnig M, Kuhn C, Meyerhof W. Members of RTP and REEP gene families influence functional bitter taste receptor expression. *J Biol Chem*. 2006; 281(29):20650–9. <https://doi.org/10.1074/jbc.M513637200> PMID: 16720576.
  55. Décaillot FM, Rozenfeld R, Gupta A, Devi LA. Cell surface targeting of  $\mu$ - $\delta$  opioid receptor heterodimers by RTP4. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(41):16045–50. <https://doi.org/10.1073/pnas.0804106105> PMC2572960. PMID: 18836069
  56. Vestal DJ. Review: The Guanylate-Binding Proteins (GBPs): Proinflammatory Cytokine-Induced Members of the Dynamin Superfamily with Unique GTPase Activity. *Journal of Interferon & Cytokine Research*. 2005; 25(8):435–43. <https://doi.org/10.1089/jir.2005.25.435> PMID: 16108726.
  57. Begum MET, Sen D. DOR agonist (SNC-80) exhibits anti-parkinsonian effect via downregulating UPR/oxidative stress signals and inflammatory response in vivo. *Neuroscience Letters*. 2018; 678:29–36. <https://doi.org/10.1016/j.neulet.2018.04.055> PMID: 29727730
  58. Bonnet MP, Beloeil H, Benhamou D, Mazoit JX, Asehnoune K. The mu opioid receptor mediates morphine-induced tumor necrosis factor and interleukin-6 inhibition in toll-like receptor 2-stimulated monocytes. *Anesth Analg*. 2008; 106(4):1142–9, table of contents. <https://doi.org/10.1213/ane.0b013e318165de89> PMID: 18349186.
  59. Ahmed IS, Chamberlain C, Craven RJ. S2R(Pgrmc1): the cytochrome-related sigma-2 receptor that regulates lipid and drug metabolism and hormone signaling. *Expert Opin Drug Metab Toxicol*. 2012; 8(3):361–70. <https://doi.org/10.1517/17425255.2012.658367> PMID: 22292588.
  60. Cahill MA, Medlock AE. Thoughts on interactions between PGRMC1 and diverse attested and potential hydrophobic ligands. *The Journal of Steroid Biochemistry and Molecular Biology*. 2017; 171:11–33. <https://doi.org/10.1016/j.jsbmb.2016.12.020> PMID: 28104494
  61. Martin LJ, Boucher N, Brousseau C, Tremblay JJ. The orphan nuclear receptor NUR77 regulates hormone-induced StAR transcription in Leydig cells through cooperation with Ca(2+)/Calmodulin-dependent Protein Kinase I. *Molecular Endocrinology*. 2008; 22(9):2021–37. <https://doi.org/10.1210/me.2007-0370> PMC5419457. PMID: 18599618
  62. Martin LJ, Tremblay JJ. The Human 3 $\beta$ -Hydroxysteroid Dehydrogenase/ $\Delta$ 5- $\Delta$ 4 Isomerase Type 2 Promoter Is a Novel Target for the Immediate Early Orphan Nuclear Receptor Nur77 in Steroidogenic Cells. *Endocrinology*. 2005; 146(2):861–9. <https://doi.org/10.1210/en.2004-0859> PMID: 15498889
  63. Mendell AL, MacLusky NJ. Neurosteroid Metabolites of Gonadal Steroid Hormones in Neuroprotection: Implications for Sex Differences in Neurodegenerative Disease. *Frontiers in Molecular Neuroscience*. 2018; 11(359). <https://doi.org/10.3389/fnmol.2018.00359> PMID: 30344476