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Regulation of Jak1 and Jak2 Synthesis through Non-Classical Progestin Receptors

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REGULATION OF JAK1 AND JAK2 SYNTHESIS THROUGH NON-CLASSICAL
PROGESTIN RECEPTORS

A Thesis Presented

By

HILLARY L. ADAMS

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
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ABSTRACT

REGULATION OF JAK1 AND JAK2 SYNTHESIS THROUGH NON-CLASSICAL PROGESTIN RECEPTORS

SEPTEMBER 2015

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The anteroventral periventricular (AVPV) nucleus of the hypothalamus integrates estradiol (E₂) and progesterone (P₄) feedback signals from the ovaries to stimulate gonadotropin releasing hormone (GnRH) neurons and trigger an ovulatory surge in luteinizing hormone (LH). E₂ maintains the daily cyclic LH surge and P₄ quickly amplifies the surge and limits it to one day. P₄ amplification of the surge and rapid signaling in the AVPV may occur through its non-classical progestin receptors. Previous *in vitro* studies using a microarray analysis with N42 mouse embryonic hypothalamic neurons suggest that progesterone membrane component 1 (Pgrmc1) regulates genes linked to the janus kinase (Jak)/signal transducer and activator of transcription (Stat) signaling pathway. I hypothesized that P₄ alters Jak/Stats through Pgrmc1 regulation of one or more Jak or Stat molecules and then performed a set of *in vitro* and *in vivo* studies to test this. I transfected N42 cells with either scramble or Pgrmc1 siRNA followed by treatment with either ethanol vehicle control or 10 nM P₄ and measured Jak1, Jak2, Stat3, Stat5a, Stat5b, and Stat6 mRNA levels via quantitative polymerase chain reaction

(QPCR). Jak1 and Jak2 mRNAs increased with P₄ treatments, and this upregulation required Pgrmc1. Silencing Pgrmc1 in the cells also produced an increase in Jak1 and Jak2 mRNA, suggesting that Pgrmc1 constitutively suppressed *jak1* and *jak2* in the absence of P₄. None of the Stats were significantly regulated by P₄ or Pgrmc1 silencing. To determine how Pgrmc1 regulates Jak/Stat *in vivo*, I took AVPV microdissections from Pgrmc1 and Pgrmc2 double conditional knockout (DCKO) mice and looked at gene expression of *jak/stat*. Transcript levels of Jak2, but not Jak1, were severely downregulated in the DCKO animals and Stat mRNAs were not significantly changed. Discrepancies from *in vitro* and *in vivo* data prompted me to analyze the role of the class II progestin and adipoQ (Paqr) receptors in Jak/Stat signaling. P₄ treatments and siRNA experiments in N42 cells showed that Paqr8, but not Paqr7, was required for P₄ upregulation of Jak1 and Jak2 mRNAs. Overall, these findings show that Pgrmc1 regulates Jak1 and Jak2 synthesis in a P₄-dependent and -independent manner that requires interaction with Paqr8.

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CHAPTER 1

INTRODUCTION

1.1 Progesterone and its neuroendocrine role in ovulation

Inducing ovulation involves intricate signaling mechanisms regulated by the brain and the cycling of progesterone (P_4) and estradiol (E_2) in the hypothalamic pituitary gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus triggers the pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Clark and Cummins, 1985). LH and FSH bind receptors in the ovaries, advancing their production of E_2 and P_4 . E_2 positive feedback to the hypothalamus is necessary to stimulate GnRH neurons and initiate an LH surge, but the surge is not maximal without P_4 (Christian et al., 2005; DePaolo and Barraclough, 1979).

1.2 The anteroventral periventricular (AVPV) nucleus

It has long been understood that the anteroventral periventricular nucleus (AVPV) of the preoptic area (POA) is the control center in the hypothalamus that integrates ovarian E_2 -positive feedback and neural P_4 signaling to directly stimulate GnRH neurons and generate an LH surge for ovulation (Petersen et al., 2003). Estrogen receptors (ERs) within the POA are most densely populated in the AVPV and estrogen deprivation to the AVPV prevents LH release (Ottem et al., 2004; Petersen et al., 1989). E_2 also induces expression of nuclear progesterone receptor (Pgr) in the AVPV, the activation of which is required for the LH surge (Simerly et al., 1996; Chappell and Levine, 2000). While P_4 signaling in the AVPV is necessary for the LH surge, directly stimulating GnRH neurons

with P₄ inhibits LH release (Richter et al., 2001), confirming the dominant role of the AVPV in GnRH surge release.

Although P₄ activation of Pgr in the AVPV may be required for gonadotropin release, other studies have shown that Pgr stimulation in this region may be ligand-independent (Mani et al., 1996) and P₄ can also signal in the absence of Pgr (Frye et al., 2006; Sleiter et al., 2009). Moreover, Pgr-mediated genomic effects do not fully explain the rapid signaling that P₄ wields on the system (Ke and Ramirez, 1987). Therefore it is quite possible that rapid P₄ signaling in the AVPV involves other P₄ mediators.

1.3 Non-classical P₄ receptors

Two different groups of non-classical P₄ receptors exist: the membrane P₄ receptors (mPR) that belong to the class II progestin and adipoQ (PAQR) family comprising of mPR α (Paqr7), mPR β (Paqr8), mPR γ (Paqr5), mPR δ (Paqr6), mPR ϵ (Paqr9), and the membrane-associated P₄ receptor (MAPR) family comprised of P₄ receptor membrane component 1 (Pgrmc1), Pgrmc2, and neudesin (Thomas and Pang, 2012). All of the mPRs and Pgrmc1 display high binding affinity for P₄ (Peluso et al., 2006). Several studies in non-neural tissue have verified the localization of mPRs to plasma membranes where they mediate P₄ signaling through G protein activation (Zhu et al., 2003; Pang and Thomas, 2011) and current findings suggest that Paqr7 and Pgrmc1 interact to regulate P₄ effects (Thomas et al., 2014; Sueldo et al., 2015)

Recent studies have mapped *pgrmc1*, *pgrmc2*, *paqr7* and *paqr8* family members to several regions in the brain, including but not limited to the hypothalamus, thalamus, hippocampus, and cortex (Zuloaga et al., 2012; Intlekofer and Petersen, 2011). However, *in situ* hybridization experiments in the adult rat forebrain revealed that *pgrmc1* and *pgrmc2*, but not *paqr7* and *paqr8*, were highly abundant in various neuroendocrine nuclei, and most notably in the AVPV (Intlekofer and Petersen, 2011a). Although *Pgrmc1* has been widely studied in granulosa and cancer cells, its function is quite elusive in the POA, thus making it a vital candidate to study rapid P₄ effects in the AVPV.

Pgrmc1's structure allows it to perform a variety of functions. It contains a cytochrome b5-like heme binding domain that activates P450 proteins involved in steroidogenesis (Hughes et al., 2007). *Pgrmc1* is also known to form complexes with various proteins, such as steroid regulatory element-binding protein cleavage activating protein (Scap), insulin-induced gene (Insig1), and epidermal growth factor receptor (Egfr) (Ahmed et al., 2010a, 2010b). While *Pgrmc2*, neudesin, and serpine1 mRNA binding protein 1 (Serbp1) do not display P₄-binding activity, they can bind *Pgrmc1* and may affect its affinity for P₄. SH2 and SH3 target motifs are also important components of *Pgrmc1* that regulate tyrosine kinases and activate signaling cascades, such as those involving protein kinase G and mitogen-activated protein kinase/extracellular signal-regulated kinase 1 and 2 (Mapk/Erk) (Peluso et al., 2006; Cahill, 2007). Such activation of signaling cascades may be the key to which P₄ exerts its fast effects. A recent study revealed that P₄ rapidly inhibits GnRH neurons via *Pgrmc1* activation of protein kinase G (Bashour and Wray,

2012). Pgrmc1's versatility is also underscored by the fact that it has been localized to the cell membrane, cytoplasm, endoplasmic reticulum, mitochondria, and nucleus (Cahill, 2007).

1.4 Janus kinase/Signal transducer and activator of transcription (Jak/Stat): targets of Pgrmc1

Interested in Pgrmc1's role in the AVPV and GnRH release, Intlekofer and Petersen performed a series of microarray analyses using N42 mouse embryonic hypothalamic neurons with and without Pgrmc1 silencing to identify its downstream targets. Four out of the five pathways regulated by Pgrmc1 knockdown in these experiments involved signal transducer and activator of transcription (Stat) (Intlekofer, unpublished). ChIP-Seq analyses in granulosa cells supported these findings and also identified Pgrmc1 as a regulator of the Janus kinase (Jak)/Stat family (Peluso, personal communication).

The Jak/Stat pathway is a conserved intracellular signaling mechanism found in most eukaryotes. Hormone or cytokine activation of receptors initiate Jak phosphorylation of Stats, which dimerize and translocate to the nucleus to regulate transcription (Linnekin et al., 1997). While Jak/Stat proteins are ordinarily expressed at lower levels in the brain than other tissues, they are known to have roles in neuronal development (Garza et al., 2008), hormone release (Wu et al., 2011), inflammation (Yu et al., 2009), and tumorigenesis (Hussain et al., 2007). Energy homeostasis regulated by the hypothalamus also relies heavily on leptin-mediated Jak/Stat signaling (Gorska et al., 2010). In non-

neural tissues, including breast cancer cells, P₄ activates Jak, recruiting Stat5a to induce Pgr expression (Subtil-Rodriguez et al., 2008).

1.5 Rationale and Objectives

The evidence described above supports the hypothesis of the current study: **P₄ regulates Jak/Stat signaling in the AVPV through Pgrmc1**. This novel idea linking Pgrmc1 and Jak/Stat signaling builds on previous data illustrating that these molecules are crucial for similar physiological and cellular processes. In non-neural tissues, both Pgrmc1 and Jak/Stat are involved in cholesterol metabolism and steroid hormone synthesis, inflammatory response, and tumorigenesis involving EGFR signaling. (Leung et al., 2003; Proietti et al., 2005; Ahmed et al., 2010a; Lange et al., 1999). Both molecules are also important players in reproduction and GnRH release. Low levels of *pgrmc1* have been associated with premature ovarian failure and polycystic ovary syndrome (Schuster et al., 2010), while neuronal Jak2 conditional knockout mice have reduced GnRH mRNA levels and experience decreased fertility (Wu et al., 2011). Investigating Pgrmc1 control of Jak/Stat molecules and intracellular signaling mechanisms in the AVPV is important for elucidating their roles in these processes.

In these studies, I used *in vitro* and *in vivo* models to investigate regulation of Jak/Stat signaling through non-classical P₄ receptors. The N42 mouse embryonic hypothalamic cell line was used to perform siRNA experiments with Pgrmc1 and Paqrs and P₄ treatments to assess their downstream effects on Jak/Stat mRNA. N42 cells model the AVPV because they contain abundant ER α , ER β , Pgrmc1, Pgrmc2, and Serbp1. In

addition they have no detectable Pgr, making them suitable for studying P₄ effects on Pgrmc1. Jak/Stat and Paqr mRNAs were also measured in AVPV microdissections from Pgrmc1 and Pgrmc2 double conditional knockout (DCKO) mice.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study 1: Pgrmc1 regulation of Jak/Stat mRNA in the absence and presence of P₄

To determine whether P₄ and Pgrmc1 regulate Jak/Stat synthesis, Pgrmc1 silencing and regulation by P₄ was first verified in N42 cells via qPCR and western blot. Jak1, Jak2, Stat3, Stat5a, Stat5b, and Stat6 mRNA levels were measured in these cells lacking Pgrmc1 and treated with and without P₄.

siRNA transfection and P₄ treatments

N42 cells were seeded at a density of 1.2×10^5 per reaction in Dulbecco's Modification of Eagle's Medium (DMEM; Thermo Fisher Scientific, Rockford, IL) supplemented with 20% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1 % penicillin streptomycin glutamine (PSG). They were transfected immediately with AllStars Negative Control siRNA or Mm_Pgrmc1_1 Flexitube siRNA; (Qiagen, Valencia, CA) to a final concentration of 20 nM with HiPerfect Transfection Reagent (Qiagen) according to the manufacturer's protocol. Cells were incubated at 37°C in 5% CO₂. Forty-eight hours later, media was removed and cells were washed with phosphate buffer saline (PBS) and replaced with DMEM containing either ethanol vehicle control or 10 nM P₄. Cells were harvested in Trizol 3 hours later and stored at -80°C.

RNA isolation and quantitative polymerase chain reaction (QPCR)

RNA was isolated in Trizol (Invitrogen) according to the manufacturer's protocol and reverse transcribed using the M-MLV Reverse Transcription kit (Promega, Madison, WI). For quantitative polymerase chain reaction (QPCR), cDNA was diluted 1:10 in nuclease-free water and combined with FastStart Universal SYBR Green (ROX) mix (Roche Applied Science, Indianapolis, IN) with the appropriate primer. Primers for *Pgrmc1*, *Jak1*, *Jak2*, *Stat5a*, *Stat5b*, and *Stat6* (Integrated DNA Technologies, Coralville, IA) are listed in Table 1. Primers for *Gapdh* and *Stat3* were pre-designed from Qiagen (Valencia, CA). Reactions were completed using the Stratagene MX3005P thermocycler (Agilent Technologies, Wilmington, DE) with the following settings: 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Reactions using Qiagen prevalidated primers were set to 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Threshold cycle (Ct) numbers were obtained with MxPro™ QPCR analysis software (Agilent Technologies) and the delta delta Ct method of analysis (Livak and Schmittgen, 2001) was used to compare treatment effects on target genes. Target genes were normalized to *Gapdh* housekeeping gene. All results were analyzed with GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA) using a one-way ANOVA with Newman-Keuls correction for *post hoc* analysis when an interaction of main effects was found.

Western Blotting

N42 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL) with 1% protease and phosphatase inhibitor cocktail (Thermo

Fisher Scientific) according to the manufacturer's protocol. Protein concentrations were obtained using the Pierce biocinchoninic acid (BCA) assay (Thermo Fisher Scientific). Ten micrograms of sample protein were loaded onto a 4-15% Tris-HCL SDS-PAGE precast gel (Bio-Rad Laboratories, Inc., Hercules, CA) and run at 150V for one hour. Protein was electrophoretically transferred to an Immobilon-P PVDF membrane (EMD Millipore, Billerica, MA) at 46V for 90 minutes. The membrane was blocked overnight in Tris buffered saline 0.05% Tween-20 (TBST) with 5% non-fat dry milk at 4°C and then probed with anti-Pgrmc1 antibody (1:1000; Abcam, Cambridge, MA) or anti- β -actin antibody (1:5000; Abcam) in TBST with 5% milk for 1 hour at room temperature. After washing the membrane three times for 10 minutes in TBST, the secondary antibody solution, containing anti-rabbit horseradish peroxidase conjugated antibody (1:15,000; Abcam) in TBST with 5% milk, was applied for 1 hour at room temperature. The membrane was washed three times for 10 minutes and chemiluminescence was performed using the Clarity ECL Western Blotting Substrate kit (Bio-Rad Laboratories, Inc.) and ImageQuant Las 4000 mini (GE Healthcare, Waukesha, WI).

2.2 Study 2: Pgrmc1 and Pgrmc2 regulation of *jak/stat* in the AVPV

To determine whether Pgrmc1 regulated Jak/Stat mRNA levels *in vivo*, expression of *jak1*, *jak2*, *stat3*, *stat5a*, *stat5b*, and *stat6* was measured in microdissections of the AVPV from control and Pgrmc1 and Pgrmc2 DCKO mice.

Tissue Preparation

Brains were removed from Pgrmc1 and Pgrmc2 DCKO and control female mouse heads obtained from the John J. Peluso laboratory (UConn Health Center, Farmington, CT).

Coronal cryosections were obtained at 12µm using the Microm HM 525 Cryostat (Thermo Fisher Scientific) at -12°C. Once the AVPV was reached, as was determined by the appearance of the optic recess and anterior commissures, a 300µm coronal section was collected and the AVPV was immediately excised using a 2mm circular Harris Uni-Core™ stainless steel tissue micropunch needle (Ted Pella Inc., Redding, CA).

Micropunches were transferred to a 1.5 mL microcentrifuge tube on dry ice. Two AVPV samples were pooled for total RNA isolation using Trizol (Invitrogen, Carlsbad, CA) and the Qiagen RNeasy Lipid Kit (Qiagen, Valencia, CA). RNA was reversed transcribed with the Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol and validated on the QPCR as described above. Statistical analysis was performed on each gene independently using student two-tailed t-tests.

2.3 Study 3: Effects of Paqr7 and Paqr8 on synthesis of Jak1, Jak2 and non-classical P₄ receptors

To determine whether other P₄ mediators regulate Jak synthesis, Paqr7 and Paqr8 siRNA experiments with and without P₄ were carried out in N42 cells as in Study 1. Knockdown experiments used Qiagen Flexitube siRNAs for Paqr7 (Mm_MGI:1919154_1) and Paqr8 (Mm_1700019B16Rik_1). Pre-validated primers for Paqr7 and Paqr8 mRNAs were obtained from Qiagen (Valencia, CA). Levels of Paqr7, Paqr8, Pgrmc1, Jak1, and Jak2 mRNAs were measured using QPCR as in Study 1.

2.4 Study 4: Pgrmc1 regulation of Paqr7 and Paqr8.

To determine whether Pgrmc1 regulates Paqr7 and Paqr8 mRNAs in a P₄-dependent or -independent manner, Paqr7 and Paqr8 mRNA levels were measured in N42 cells transfected with scramble or Pgrmc1 siRNA in the presence or absence of P₄ as in Study 1. Paqr7 and Paqr8 mRNAs were also assessed in the DCKO animals as in Study 2.

2.5 Study 5: Pgrmc1-dependent targets linked to Jak/Stat signaling

I used the Mouse JAK/STAT RT² Profiler PCR Array (Qiagen, Valencia, CA) and siRNA technology to identify targets of Pgrmc1 linked to Jak/Stat signaling in N42 cells. The PCR array reports on 84 genes that are closely related to Jak/Stat-mediated signaling. Cells were transfected with and without Pgrmc1 siRNA as described above. RNA isolation was performed in Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RT² First Strand Kit (Qiagen) was used to reverse transcribe 1µg RNA. Synthesized cDNA was prepared with RT² SYBR Green Mastermix (Qiagen) according to the manufacturer's protocol and loaded onto the Jak/Stat PCR array plate for QPCR analysis. Cycling conditions were as follows: 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. C_t values were exported and loaded into SABiosciences Web-based PCR Array Data Analysis Software (Qiagen) for analysis. I then used QPCR with prevalidated primers from Qiagen to verify these findings in N42 cells treated as described in Study 1.

Finally, I tested whether the identified genes were affected by knockdown of *Pgrmc1* and *Pgrmc2* in the AVPV of DCKO animals as in Study 2.

Table 1. Primers for QPCR validation

Gene	5' – 3' Sequence		Accession Number
Mouse Pgrmc1	Forward	CCTCTGCATCTTCCTGCTCTA	NM_016783
	Reverse	CGAGCTGTCTCGTCTTTTGG	
Mouse Jak 1	Forward	CAGAAAAGCAGCCAACAACA	NM_146145
	Reverse	CTTGACAGCTACCTGCTCCC	
Mouse Jak 2	Forward	TGTGAACTGTTTTCCCTCCC	NM_008413
	Reverse	TTCAGCTTGCCCAAGAGAAT	
Mouse Stat5A	Forward	CCGTGGGATGCTATTGACTT	NM_011448
	Reverse	TCTGGAGCTGTGTGGCATAG	
Mouse Stat5B	Forward	CTTGGCTTTCGATCCACTGT	NM_001113563
	Reverse	GGCAGAACGAGGTTGTGAAC	
Mouse Stat6	Forward	GGCTTTCCGGAGTCACTATAA	NM_009284
	Reverse	GTCCCTTTCCACAGTCATCTT	
Mouse Nr4a1	Forward	TTCCCACCACCAGCCACCCA	NM_010444
	Reverse	CTCGCTGCCACCTGAAGCCC	

CHAPTER 3

RESULTS

3.1 Study 1

P₄ does not regulate Pgrmc1 mRNA levels

In order to test P₄ and Pgrmc1 downstream effects, Pgrmc1 silencing was first verified in N42 cells along with Pgrmc1 response to P₄ treatments. Transfection with Pgrmc1 siRNA and ethanol vehicle control (veh) reduced Pgrmc1 mRNA levels to 36.7% of controls and also significantly reduced protein levels (Fig.1 A and B). Subsequent P₄ treatments did not affect Pgrmc1 mRNA levels in scramble or Pgrmc1 siRNA transfected cells (Fig.1 B).

Pgrmc1 control of Jak1 and Jak2 synthesis is both P₄ dependent and independent

To determine whether Jak/Stat synthesis is affected by Pgrmc1 and P₄, Jak1, Jak2, Stat3, Stat5a, Stat5b, and Stat6 mRNA levels were validated in N42 cells transfected with either scramble control or Pgrmc1 siRNA and treated with either veh or P₄. Jak1 and Jak2 mRNA levels significantly increased with Pgrmc1 silencing alone (Fig.2 A and B). P₄ treatments in scramble-transfected cells increased Jak1 and Jak2 synthesis. When treated with Pgrmc1 siRNA and P₄ together, Jak1 mRNA levels significantly decreased compared to that of both scramble-vehicle and scramble-P₄ controls (Fig.2 A). Jak2 mRNA levels also decreased significantly compared to that of scramble-P₄ (Fig.2 B). Stat3, Stat5a, and Stat6 synthesis did not appear to be significantly regulated by the absence of Pgrmc1 or presence of P₄ (Fig.2 C-F).

3.2 Study 2

Jak2 mRNA levels drastically decrease in the absence of Pgrmc1 and Pgrmc2 in the AVPV

To assess Pgrmc1 regulation of Jak/Stat molecules *in vivo*, mRNA levels of Jak/Stats were analyzed in mouse AVPV microdissections from control and Pgrmc1 and Pgrmc2 DCKO mice. Pgrmc1 mRNA levels in DCKO animals were decreased to 24.5% that of the controls (Fig.3 A). Jak1 did not appear to be regulated by Pgrmc1 and Pgrmc2 silencing, but Jak2 mRNA levels were almost undetectable in DCKO animals at 1.9% that of controls (Fig. 3B). Stat3, Stat5a, Stat5b, and Stat6 mRNA levels did not appear to be significantly regulated by Pgrmc1 and Pgrmc2 knockdown (Fig 3C).

3.3 Study 3

Absence of Paqr8, but not Paqr7, affects Jak1, and Jak2 synthesis

Results suggesting that another component may be involved in P₄ and Pgrmc1 regulation of Jak1 and Jak2 (Fig.2 A and B; Fig.3 B) prompted me to look at Paqr7 and Paqr8 involvement. N42 cells were transfected with either scramble control, Paqr7 or Paqr8 siRNA and treated with either veh or P₄ to assess Paqr7 and Paqr8 regulation of Pgrmc1, Jak1, and Jak2 (Figs. 4 and 5). Paqr7 mRNA levels were knocked down to 29.7% that of scramble/veh control. Treatment with P₄ in scramble and Paqr7-siRNA transfected cells also appeared to downregulate Paqr7 synthesis (Fig.4 A). Pgrmc1, Jak1, and Jak2 basal transcription levels were not affected by the absence of Paqr7 (Fig.4 B-D).

Paqr8 mRNA levels were knocked down to 34.7% that of scramble/veh control. P₄ treatment significantly upregulated Paqr8 synthesis in scramble-transfected cells, but had no effect in cells transfected with Paqr8 siRNA (Fig.5 A). Pgrmc1 mRNA levels were not affected by the absence of Paqr8 (Fig.5 B). Paqr8 silencing alone did not cause any significant changes in Jak1 and Jak2 basal level transcription, but in the presence of P₄, it abrogated Jak1 and Jak2 upregulation by P₄ (Fig.5 C and D).

3.4 Study 4

Pgrmc1 regulates Paqr7 and Paqr8 synthesis

P₄ and Pgrmc1 regulation of Paqr7 and Paqr8 was first assessed in N42 cells transfected with either scramble or Pgrmc1 siRNA and treated with either veh or P₄. Pgrmc1 silencing alone did not affect Paqr7 mRNA levels, but P₄-treated cells showed slight downregulation in basal levels of Paqr7 (Fig.6 A). Paqr8 mRNA levels increased significantly with Pgrmc1 silencing, suggesting a suppressive role of Pgrmc1 on Paqr8 synthesis (Fig.6 B). Paqr8 transcript levels were further upregulated with P₄ treatments alone, and significantly decreased when P₄ was administered in the absence of Pgrmc1; hence, Pgrmc1 was necessary for P₄ upregulation of Paqr8. Examination of Paqr7 and Paqr8 *in vivo* showed that Paqr7 mRNA levels in the AVPV significantly increased in the absence of Pgrmc1 and Pgrmc2 (Fig.6 C). Paqr8 mRNA levels appeared to increase in the DCKO animals, but not significantly; therefore, I performed a power analysis and determined that an n of 8 would be necessary to detect differences based on the variability within treatments.

3.5 Study 5

Identification of Pgrmc1 and Jak/Stat downstream gene targets

The Mouse JAK/STAT Signaling Pathway RT² Profiler PCR Array (Qiagen) was used to find putative gene targets regulated by Pgrmc1 and Jak/Stat. In the absence of Pgrmc1, four genes were identified as significantly upregulated and eight genes downregulated by at least 1.5 fold (Table 2). Genes picked for further analysis were those known from the literature to be involved in P₄ signaling or present in neurons. They included nuclear factor of kappa light polypeptide gene enhancer in B-cells I (Nfkb1), protein tyrosine phosphatase, non-receptor type I (Ptpn1), SMAD family member 5 (Smad5), and Stat2.

Nfkb1 and Smad5 are potential targets of Pgrmc1 and Jak/Stat signaling

To determine whether Pgrmc1 silencing and P₄ affect synthesis of the PCR array-identified downstream targets, mRNA levels of Nfkb1, Ptpn1, Smad5, and Stat2 were validated on the QPCR from N42 cells transfected with scramble or Pgrmc1 siRNA and treated with either veh or P₄ (Fig.7). Nuclear receptor subfamily 4, group A, member 1 (Nr4a1), a gene detected on the same microarray as the Stats for regulation by Pgrmc1 (Intlekofer, unpublished), was also evaluated (Fig.7 B). Of the genes investigated, only Nfkb1 mRNA decreased as a result of Pgrmc1 silencing, but no effect was seen in the presence of P₄ (Fig.7 A). Smad5 mRNA levels significantly decreased as a result of Pgrmc1 siRNA transfection and P₄ treatment, but no effect was seen with Pgrmc1 silencing or P₄ treatment alone (Fig.7 D). This may suggest a Pgrmc1-independent effect of P₄, but further verification must be performed. Nr4a1, Ptpn1, and Stat2 were not significantly regulated by either Pgrmc1 silencing or P₄ treatment (Fig.7 B, C and E).

Transcript levels of the target genes were also analyzed in the AVPV of control and Pgrmc1 and Pgrmc2 DCKO mice. None of the genes were significantly regulated in DCKO animals, but this may be due to insufficient power of the analysis (Fig.7 F).

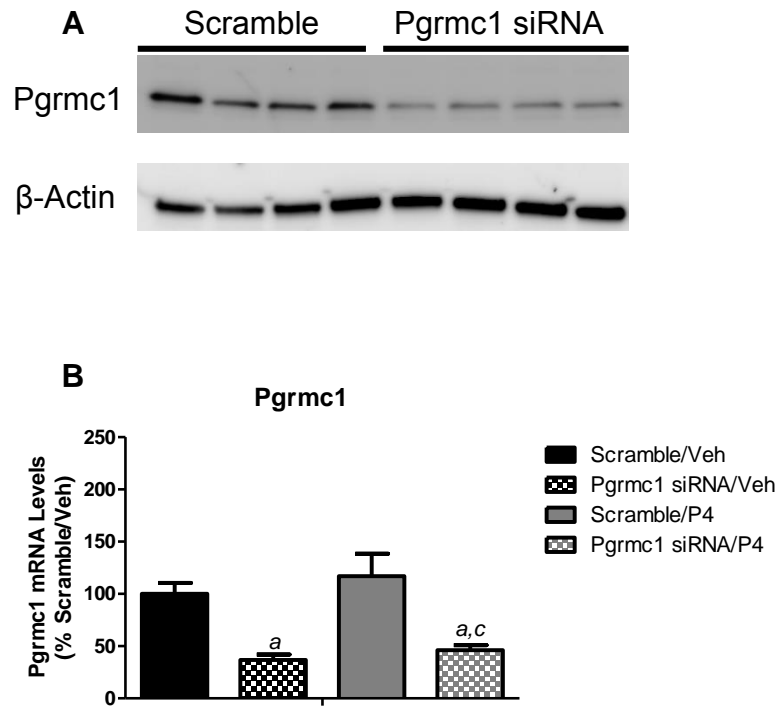


Figure 1. Pgrmc1 siRNA experiments and P4 treatments

Pgrmc1 silencing was detected by western blot in N42 cells transfected with either scramble control or Pgrmc1 siRNA and compared with β-actin (A). Pgrmc1 mRNA levels were validated on the QPCR from N42 cells transfected with either scramble control or Pgrmc1 siRNA for 48 hours followed by a 3-hour treatment with either ethanol vehicle control (Veh) or 10 nM P4 (B). Treatment with Pgrmc1 siRNA/Veh produced a decrease in Pgrmc1 mRNA to 36.7% that of Scramble/Veh (B). Results were analyzed using a one-way ANOVA with Newman-Keuls correction; bars=means±SEM.

^aSignificantly different from Scramble/Veh; ^bsignificantly different from Pgrmc1 siRNA/Veh; ^csignificantly different from Scramble/P4.

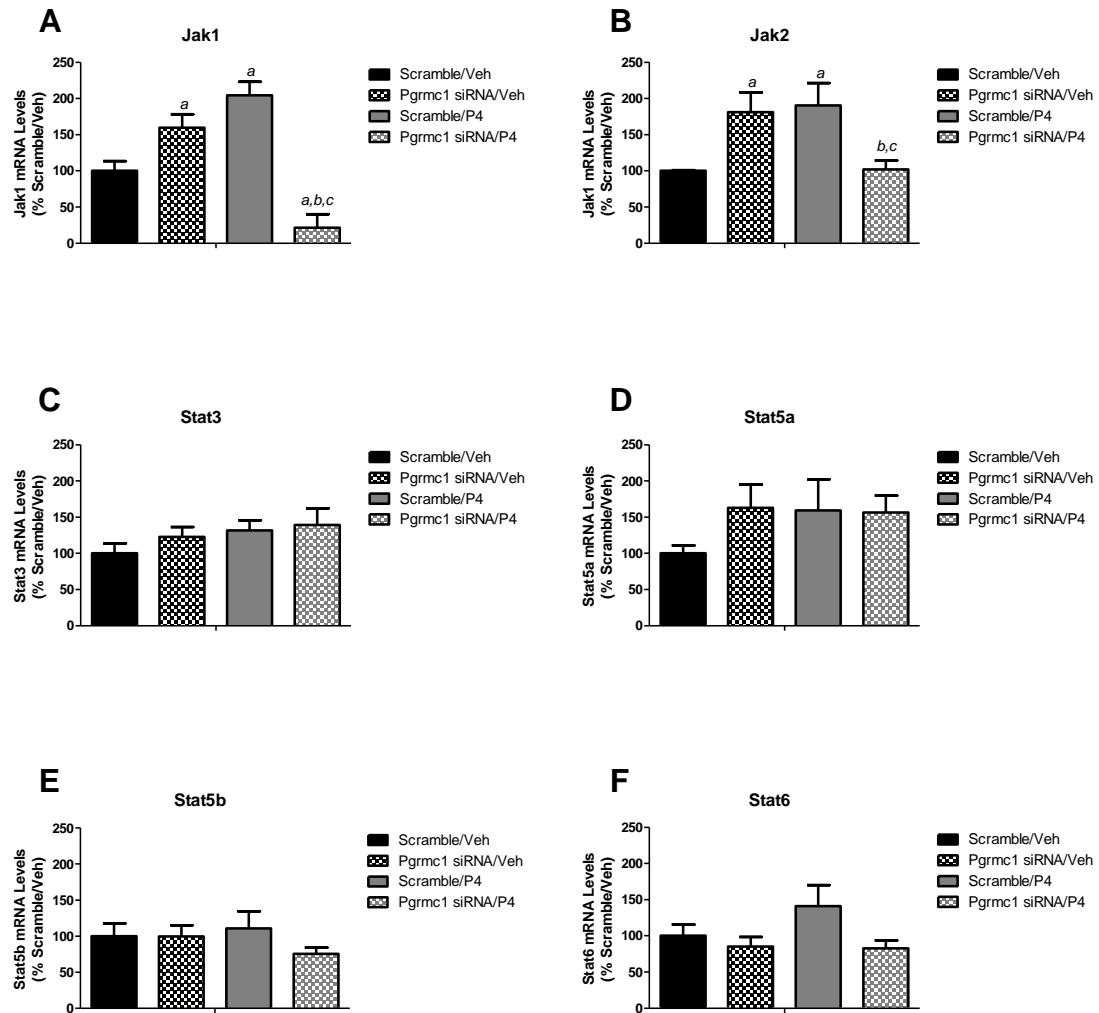


Figure 2. Effects of Pgrmc1 siRNA and P4 on Jak/Stat gene expression

Jak/Stat mRNA levels were validated on the QPCR in N42 cells transfected with either scramble control or Pgrmc1 siRNA for 48 hours followed by a 3-hour treatment with either ethanol vehicle control (Veh) or 10 nM P4 (A-F). Results were analyzed using a one-way ANOVA with Newman-Keuls correction; bars=means±SEM. ^aSignificantly different from Scramble/Veh; ^bsignificantly different from Pgrmc1 siRNA/Veh; ^csignificantly different from Scramble/P4.

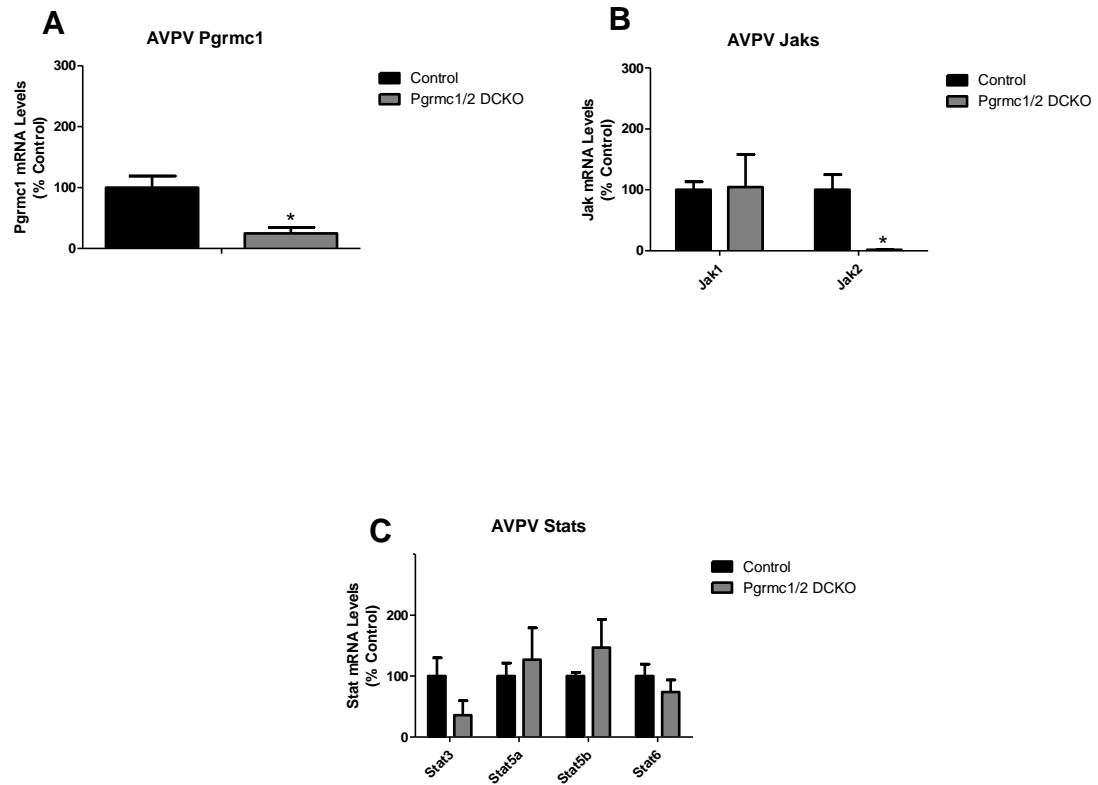


Figure 3. Gene regulation in the AVPV of Pgrmc1 and Pgrmc2 DCKO mice
Pgrmc1 and Jak/Stat mRNA levels were validated on the QPCR from AVPV microdissections of control and Pgrmc1 and 2 DCKO animals (n=5, A-D). Jak2 mRNA was knocked down to 1.9% in DCKO animals as compared to controls (B). Each gene was analyzed independently with a student t-test; bars=means±SEM. *Significantly different from control; $p < 0.05$.

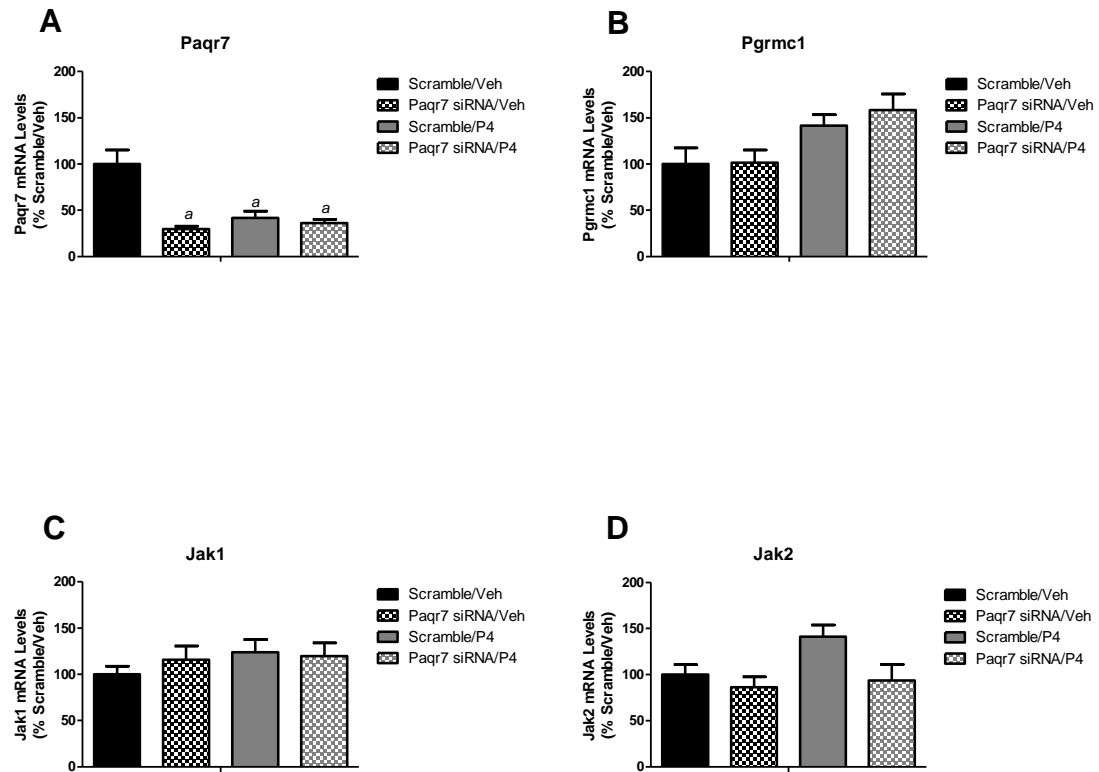


Figure 4. Paqr7 siRNA experiments with P4 treatments

Paqr7, Pgrmc1, Jak1, and Jak2 mRNA levels were analyzed via QPCR in N42 cells transfected with either scramble control or Paqr7 siRNA for 48 hours followed by a 3-hour treatment with either ethanol vehicle control (Veh) or 10 nM P4 (A-E). Paqr7 mRNA levels were knocked down to 29.7% that of the Scramble/Veh control (A). Results were analyzed using a one-way ANOVA with Newman-Keuls correction; bars=means±SEM. ^aSignificantly different from Scramble/Veh; ^bsignificantly different from Pgrmc1 siRNA/Veh; ^csignificantly different from Scramble/P4.

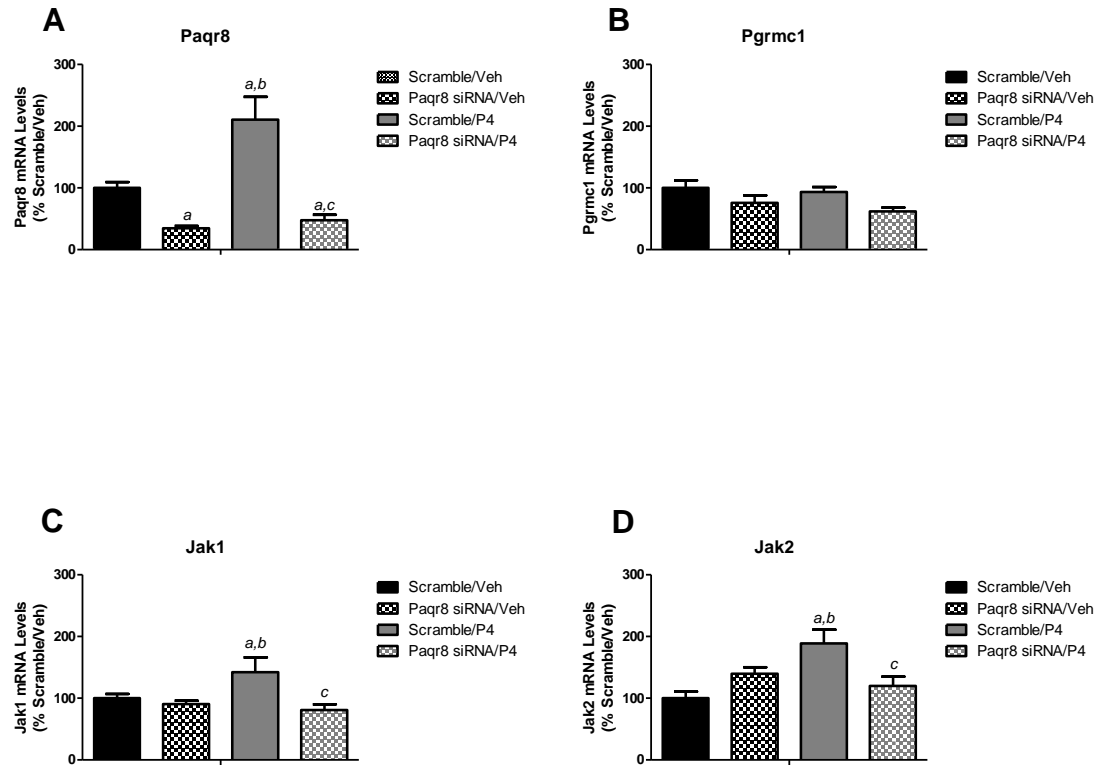


Figure 5. Paqr8 siRNA and P4 regulation of Jak1 and Jak2

mRNA levels for Paqr8, Pgrmc1, Jak1, and Jak2 were validated on the QPCR from N42 cells transfected with either scramble control or Paqr8 siRNA for 48 hours followed by a 3-hour treatment with either ethanol vehicle control (Veh) or 10 nM P4 (A-E). Paqr8 mRNA levels were knocked down to 34.7% that of scramble vehicle control (A). Results were analyzed using a one-way ANOVA with Newman-Keuls correction; bars=means±SEM. ^aSignificantly different from Scramble/Veh; ^bsignificantly different from Pgrmc1 siRNA/Veh; ^csignificantly different from Scramble/P4.

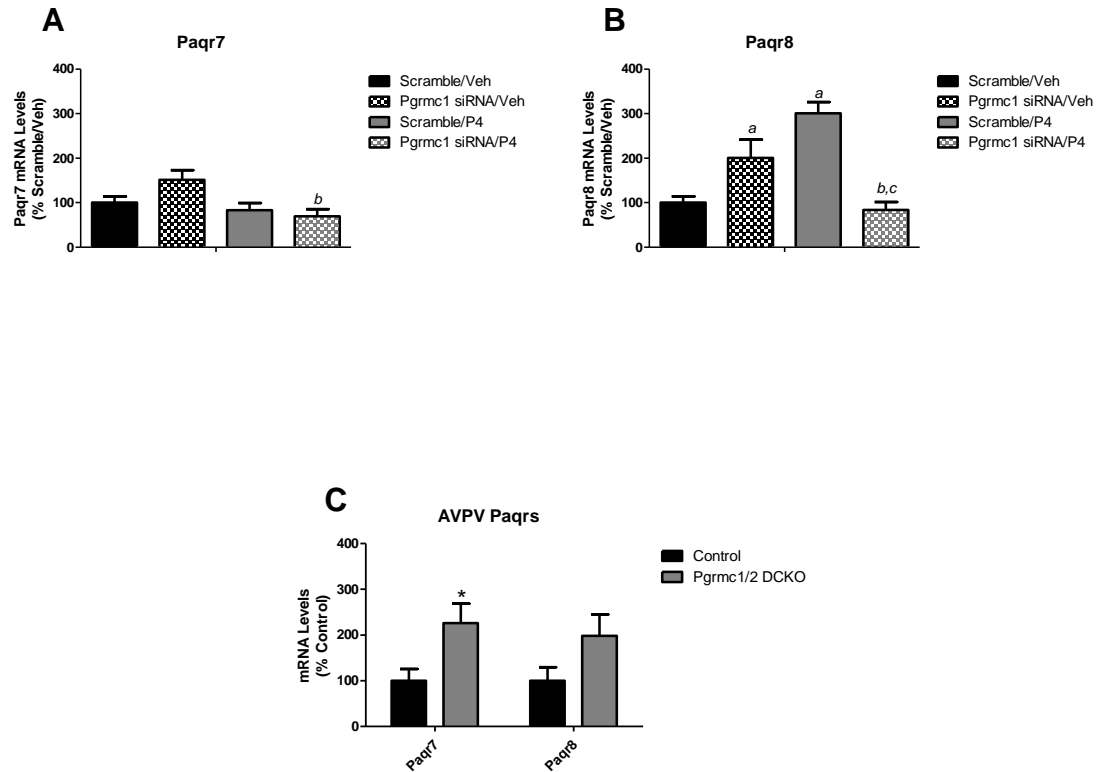


Figure 6. Pgrmc1 regulation of Paqr7 and Paqr8

Paqr7 and Paqr8 mRNA levels were analyzed in N42 cells transfected with either scramble control or Pgrmc1 siRNA for 48 hours followed by a 3-hour treatment with either ethanol vehicle control (Veh) or 10 nM P4 (A and B). Paqr7 and Paqr8 mRNA levels were analyzed in the AVPV of control and Pgrmc1/2 DCKO mice (C). Results in A and B were analyzed using a one-way ANOVA with Newman-Keuls correction and a student t-test was used to analyze each gene separately in C; bars=means±SEM.

^aSignificantly different from Scramble/Veh; ^bsignificantly different from Pgrmc1 siRNA/Veh; ^csignificantly different from Scramble/P4. *Significantly different from control; $p < 0.05$.

Table 2. Genes regulated by Pgrmc1 and Jak/Stat from PCR array

Symbol	Gene Name	Fold Change
Ptpn1	Protein tyrosine phosphatase, non-receptor type I	7.3279
Fcgr1	Fc receptor, IgG, high affinity I	2.0187
Socs2	Suppressor of cytokine signaling II	1.6975
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B cells I, p105	1.5088
Il4ra	Interleukin 4 receptor, alpha	-2.023
Csf2rb2	Colony stimulating factor 2 receptor, beta, low affinity	-1.995
Tyk2	Tyrosine kinase 2	-1.8875
Stam	Signal transducing adaptor molecule	-1.836
Smad5	SMAD family member 5	-1.737
Stat2	Signal transducer and activator of transcription 2	-1.713
Saa3	Serum amyloid A 3	-1.587
Gusb	Glucuronidase, beta	-1.501

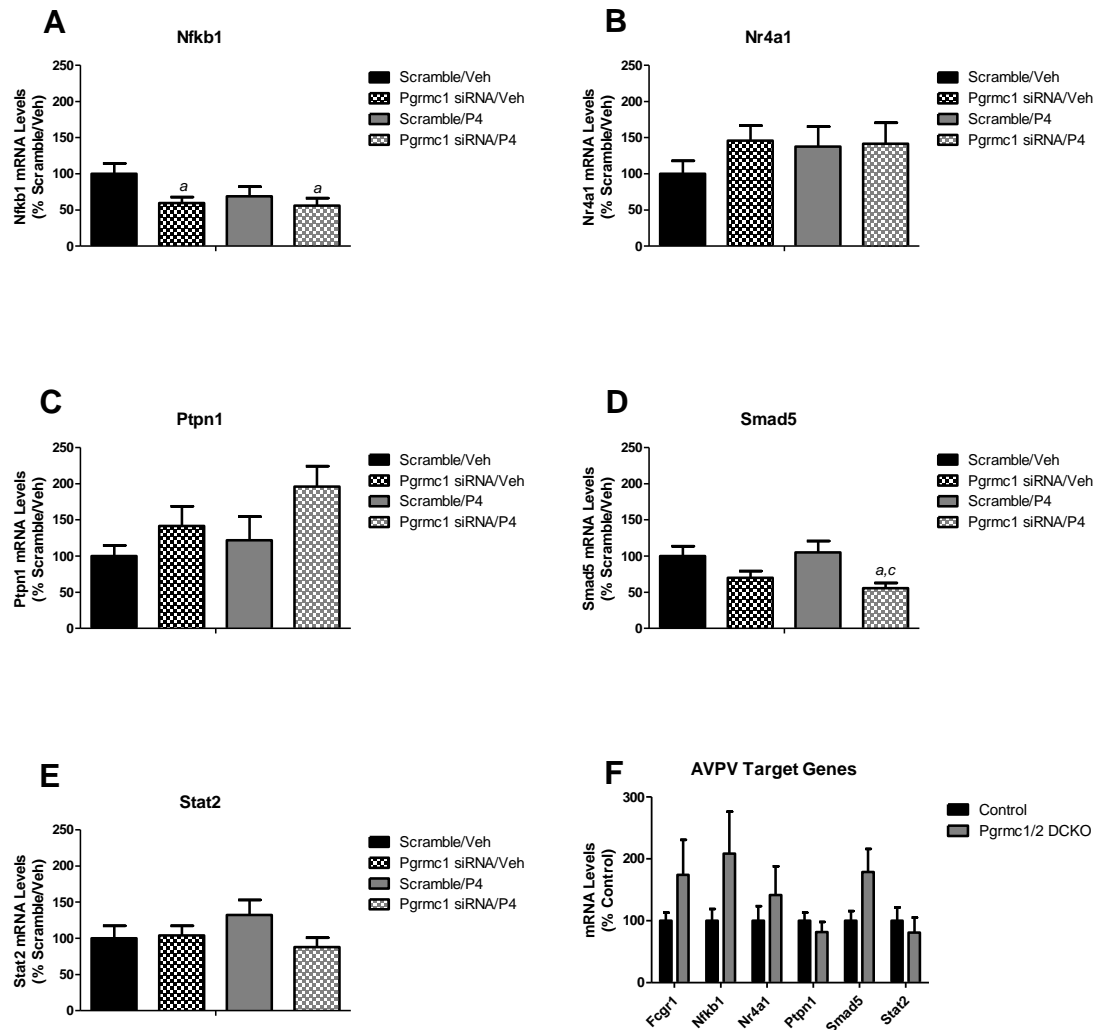


Figure 7. QPCR validation of Pgrmc1 and Jak/Stat downstream target genes
mRNA levels of selected genes from the PCR array data set and microarray data (Intlekofer, unpublished) were assessed in N42 cells treated with either scramble control or Pgrmc1 siRNA and ethanol vehicle control (Veh) or 10 nM P4 (A-E). mRNA levels of the target genes were assessed in AVPV microdissections of control and Pgrmc1 and Pgrmc2 DCKO mice (F). Results in A-E were analyzed using a one-way ANOVA with Newman-Keuls correction and a student t-test was used to analyze each gene separately in F; bars=means±SEM. ^aSignificantly different from Scramble/Veh; ^bsignificantly different from Pgrmc1 siRNA/Veh; ^csignificantly different from Scramble/P4.

CHAPTER 4

DISCUSSION

My *in vitro* findings are the first to show that P₄ increases synthesis of Jak1 and Jak2 through a mechanism that requires both Pgrmc1 and Paqr8. In the absence of P₄, Pgrmc1 alone suppresses Jak1 and Jak2 mRNA levels. My *in vivo* findings suggest that Paqr8 and/or Pgrmc2 act in concert with Pgrmc1 to regulate Jak1 and Jak2 synthesis. Together, these results indicate that P₄ in hypothalamic neural cells regulates Jak1 and Jak2 signaling by a complex interaction among Pgrmc1, Pgrmc2 and Paqr8. Further studies are necessary to determine the downstream targets of Pgrmc1 and Jak/Stat, but Nfkb1 is a promising candidate.

P₄ upregulation of Jak1 and Jak2 mRNA was an important finding because many studies have only shown P₄ to regulate Jak/Stat phosphorylation (Proietti et al., 2005; Sagare-Patil and Modi, 2013). However, steroid hormone regulation of Jak synthesis is not completely surprising based on a previous finding in breast cancer cells that demonstrated E₂ upregulation of Jak2 mRNA (Gupta et al., 2012). Thus, P₄ regulates both Jak/Stat signaling and its synthesis.

The coordinated regulation of *jak1* and *jak2* by P₄ through Pgrmc1 and Paqr8 supports the newly emerging idea that P₄ wields its rapid effects through Pgrmc1 and Paqr interactions. Work from the Thomas lab showed that Pgrmc1 acts as an adaptor for Paqr7 and forms complexes at the plasma membrane, resulting in increased P₄ binding (Thomas et al., 2014). Overlapping results from the Peluso lab showed that Pgrmc1,

Pgrmc2, and Paqr7 interact in the cytoplasm and all three components are necessary to block P₄ anti-mitotic effects (Sueldo et al., 2015). In the Thomas and Peluso studies, Paqr8 was not studied because it is in low abundance in ovarian and breast cancer cells. In contrast, my findings showed that Paqr8, rather than Paqr7, mediated the effect of P₄ and Pgrmc1 on Jak1 and Jak2 synthesis in neural cells. This finding supports previous data indicating that Paqr8 is more abundant and responsive to P₄ than Paqr7 in the hypothalamus (Zuloaga et al., 2012; Intlekofer and Petersen, 2011a).

My *in vitro* findings indicate that Pgrmc1 constitutively represses both *jak1* and *jak2* expression. Although the mechanism is not yet clear, previous evidence has shown that Pgrmc1 can activate several signaling pathways. In GnRH neurons, P₄ binds Pgrmc1 to activate protein kinase G and rapidly inhibit GnRH secretion (Bashour and Wray, 2012). In pancreatic β cells, Pgrmc1 increases adenylyl cyclase/EPAC and regulates the EGF receptor-PI3K signal transduction pathway (Zhang et al., 2014). Pgrmc1 may also operate like a transcription factor, as it has been shown in spontaneously immortalized rat granulosa cells (SIGCs; Peluso et al., 2012).

The apparent discrepancy between my *in vitro* and *in vivo* findings where Jak2 mRNAs increased in N42 cells lacking Pgrmc1, but decreased in animals lacking Pgrmc1 and Pgrmc2 suggest that Pgrmc2 and Paqr8 may also play roles in P₄ upregulation of *jak1* and *jak2* gene expression. This idea that Pgrmc1, Paqr8, and Pgrmc2 interact together to regulate gene expression correlates with the findings in ovarian cells where Pgrmc1, Paqr7, and Pgrmc2 form complexes with each other in the cytoplasm (Sueldo et al.,

2015). On the other hand, Pgrmc1, Pgrmc2 and Paqr8 could work independently of each other to regulate the same molecules. For example, Paqr8 alone is known to activate G proteins and Jak/Stats are known downstream targets of G-protein coupled receptors (Zhu et al., 2003; Pelletier et al., 2003).

Identification of Nfkb1 as a downstream target of Pgrmc1 and Jak/Stat is an interesting finding because Nfkb1 is anti-apoptotic in the developing female AVPV (Petersen et al., 2012). Nfkb1 is the p105 precursor to the p50 transcription factor in canonical Nfkb signaling (Sun, 2011). Although not much is known about Nfkb1 signaling in the hypothalamus, it does have roles in learning and memory (Lehmann et al., 2010) and synaptic plasticity (Li et al., 2015) in the central nervous system. These are intriguing findings because both P₄ and Pgrmc1 regulate the same functions (Petersen et al., 2013). Although Pgrmc1 and Nfkb1 have never been linked before, both inhibit inflammation (Rolova et al., 2014) and regulate MAPK (Yang et al., 2011). Thus, manipulation of Pgrmc1 signaling may broaden the clinical approaches to treating brain injuries and neuroinflammation.

In conclusion, our results demonstrate that Pgrmc1 and Paqr8 are significant mediators of P₄-dependent and -independent regulation of Jak1 and Jak2 synthesis in neuroendocrine cells and nuclei. Further studies are needed to determine the mechanisms through which Pgrmc1 and Paqr8 interact to regulate Jak1 and Jak2 mRNA levels. Similarly, the role of Pgrmc2 in controlling Pgrmc1 and Paqr8 interactions remains to be determined.

Unveiling the mechanisms through which P_4 regulates Jak/Stat signaling will increase our understanding of how P_4 rapidly amplifies the LH surge.

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