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Implications of Pgrmc1 Regulation of Kit Ligand Synthesis in the Hippocampus

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Implications of Pgrmc1 Regulation of Kit Ligand Synthesis in the Hippocampus

A Thesis Presented

by

HALEY A. WOODS

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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Implications of Pgrmc1 Regulation of Kit Ligand Synthesis in the Hippocampus

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ABSTRACT

IMPLICATIONS OF PGRMC1 REGULATION OF KIT LIGAND SYNTHESIS IN THE HIPPOCAMPUS

SEPTEMBER 2017

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The mammalian hippocampus is responsible for many crucial brain functions such as learning, memory, and neurogenesis in adults. Its degeneration is a pathology associated with the early stages of Alzheimer’s disease. A variety of genes have been associated with both neuroprotection and neurogenesis in the brain, some of which include progesterone membrane component 1 (Pgrmc1) and kit ligand (KitL). Pgrmc1 is recognized for mediating hormonal functions in both the ovary and neuroendocrine regions such as the anteroventral periventricular nucleus (AVPV), but its functions in the hippocampus are not well known. Both Pgrmc1 and KitL share downstream targets, the most strongly supported being genes in the Janus kinase (Jak)/signal transducer and activator of transcription (Stat) pathway. I hypothesized that Pgrmc1 regulates neural targets through KitL/c-Kit signaling. To investigate this hypothesis I used a variety of in vivo and in vitro techniques. These techniques included mapping both KitL and receptor c-Kit in the adult female rat brain using in situ hybridization. I used Pgrmc1 silencing
with siRNA in hippocampal-derived mHe-18 cells and Pgrmc1/2 double conditional knock out mouse brains to study Pgrmc1 regulation of KitL synthesis. To determine common downstream targets of KitL and Pgrmc1 I then treated mHe-18 cells with soluble KitL protein. Finally, to determine whether c-Kit mediated effects of Pgrmc1, I treated cells with both Pgrmc1 siRNA and AG-1296, a c-Kit inhibitor. The results show that Pgrmc1 regulates KitL expression, as well as downstream targets Pias1, 2, 3, and 4. However, AG-1296 did not abrogate Pgrmc1 regulation of the downstream targets, demonstrating regulation independent of KitL signaling. Taken together, these results suggest that while Pgrmc1 alters KitL expression and regulates the same genes as KitL/c-Kit, the mechanism of action likely differs. Considering that these two genes are involved in neurogenesis and neuroprotection, as well as memory and learning, a better understanding of the pathways may help lead the way in treating neurodegenerative diseases in the future.
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CHAPTER 1

INTRODUCTION

1.1 Pgrmc1

Progesterone receptor membrane component 1 and 2 (Pgrmc1 and Pgrmc2) are part of a larger group of molecules that function in nonclassical progesterone (P4) signaling (Petersen, 2013). Pgrmc1 has one transmembrane domain and an extracellular binding site that is believed to bind hemes, sterols, and progestogens (Cahill & Medlock, 2016). Pgrmc1 can interact with both Pgrmc2 and plasminogen activator inhibitor RNA-binding protein 1 (PAIRBP1), but also self-dimerizes (Peluso, 2008). Though the ability of Pgrmc1 to directly bind P4 has been widely debated, there are many examples of Pgrmc1 mediating P4 effects. In spontaneously immortalized granulosa cells (SIGCs), Pgrmc1 levels directly relate to sensitivity of P4 anti-apoptotic effects shown in cell viability assays (Peluso, et al., 2006). Studies done with partially purified liver membranes containing Pgrmc1 have shown P4 binding with Kd estimates of 11nM (Meyer, et al. 1996).

Though Pgrmc1 is shown to mediate P4-dependent effects, it has other functions that are P4-independent, such as heme-binding and membrane trafficking (Cahill, et al., 2016). Recent findings regarding the structure of Pgrmc1 suggest that the membrane trafficking function may be regulated by phosphorylation of Pgrmc1 at position Y113 (Cahill, et al., 2016). Unfortunately, sub-cellular localization is not yet fully understood and Pgrmc1 has been found to localize in many places. Pgrmc1 has been found in the nucleus of SIGCs (Peluso, et al., 2008), the cell membrane of spinal cord neurons (Labombarda, et al., 2003), and the organellar membranes of the endoplasmic reticulum.
and Golgi apparatus in Purkinje cells (Sakamotoa, et al., 2004). It has also been confirmed that Pgrmc1 can localize to the nucleus and act as a transcription factor (Peluso, 2012). The diverse localization of Pgrmc1 supports the notion that it can function in a variety of manners from being a $P_4$ receptor to entering the nucleus as a transcription factor.

### 1.2 The role of Pgrmc1 in neuroprotection and neurogenesis

Work by Intlekofer and Petersen (Petersen, 2013) detected Pgrmc1 and Pgrmc2 in the hypothalamus and hippocampus, as well as other areas linked to $P_4$-regulated functions. Pgrmc1 has been shown to mediate neuroprotective effects of $P_4$ following spinal cord injury or traumatic brain injury, linking it to functions of neuroprotection (Guennoun, et al. 2008). In the hippocampus, neurogenesis fluctuates over the course of the estrous cycle and this response can be recapitulated with different doses of estradiol ($E_2$) and $P_4$, this is thought to be regulated by Pgrmc1 (Bali, et al., 2012). Moreover, Pgrmc1 was recently shown to reduce cognitive deficits and neuroinflammation in animal models of Alzheimer’s (Yi, et al. 2016).

It is not yet clear how Pgrmc1 might regulate neurogenesis or hippocampal functions, but we previously identified *kit ligand* (*kitl*; also known as stem cell factor; Adams and Petersen, unpublished) as a target of Pgrmc1. *KitL* encodes a cytokine that seems to share a variety of functions with Pgrmc1.

### 1.3 The role of Kit Ligand in neuroprotection and neurogenesis

KitL is a growth factor and cytokine that is found in membrane-bound and soluble forms, both of which interact with the KitL tyrosine kinase receptor, c-Kit (Lennartsson
and Rönnstrand 2012). In the brain, KitL signaling has been tied to neural stem/progenitor cell (NSPC) migration from the subgranular zone (SGZ) to the dentate gyrus of the hippocampus, as well as migration to damaged neuronal areas where cell death has occurred (Sun, Lee and Fine 2004). Mice with a mutation in the tyrosine kinase domain of the c-Kit receptor show poor performance in the Morris water maze when compared to the wild type (Katafuchi, et al. 2000), suggesting a role in spatial learning and memory functions controlled by the hippocampus. Like Pgrmc1, KitL is also connected to neuroprotection as induction of kit ligand expression can be seen in brain cells within the vicinity of brain injury brought on by a lack of blood flow and oxygen (Sun, Lee and Fine 2004). It has also been suggested that KitL promotes neurogenesis and formation of new neural networks to replace ones damaged due to events such as stroke (Zhao, et al., 2013). A 2005 study showed that KitL protected rat cortical neurons from both apoptotic and excitotoxic cell death in a PI3K/Akt-dependent mechanism likely influencing NFκB activity (Dhandapani, et al., 2005). This is interesting, as unpublished data from our laboratory shows knock down of Pgrmc1 in hypothalamic cells increases NFκB synthesis (Adams and Petersen, unpublished).

1.4 Implications of sex hormone signaling in the hippocampus

Electrophysiological changes in the female hippocampus as a result of hormone cycles have been investigated as early as 1968 (Terasawa and Timiras 1968), when it was seen that synaptic changes occurred in association with difference phases of the estrous cycle. E_2 and P_4 can also drastically change the number of dendritic spines on CA1 pyramidal neurons in rats (Woolley 1998). In ovariectomized female rats, cell proliferation in the dentate gyrus is significantly decreased; however, E_2 injections rescue
this phenotype (Tanapat, et al., 1999). The physiological relevance of these studies can be seen in research showing that P₄ injections in old mice improve their memory and learning skills (Frye, 2008). P₄ upregulates genes that promote mitosis in neural progenitor cells (NPCs) of the dentate gyrus (Liu, et al. 2009). These cells do not express the classical progesterone receptor, but they do express Pgrmc1 and 2. This proliferation is abrogated by the use of an Erk₁/₂ inhibitor and this is interesting because the Erk₁/₂ pathway is found downstream of c-Kit signaling (Lennartsson and Rönnstrand 2012). The mechanisms behind these hormonal changes in synaptic plasticity and neurogenesis are still not very well understood.

1.5 Functions of the hippocampus

As in most mammals, the rodent hippocampus is made up of four different areas, the CA1, CA2, CA3, and dentate gyrus. The subregions CA1, CA2 and CA3 are primarily made up of pyramidal neuronal cells, while the dentate gyrus is made up of granule cells. These granule cells project solely to cells residing in the CA3 section of the hippocampus (Witter and Amaral 1995). Generally, both the CA1 and CA3 function in episodic memory encoding, as lesions in these areas cause mice to perform significantly worse in memory performance tests (Farovik, 2010). Recently, published work shows that the CA2 is functionally important to social memory in mice. Social memory is the ability to recognize familiar mice or littermates. When mice deficient in CA2 activity are exposed to the same mouse continually at one hour intervals, they explore the other mouse as if it was novel every time (Hitti, 2014). The dentate gyrus is involved in sensory experiences as well as memory sequencing and pattern separation. An area of the dentate gyrus known as the subgranular zone is one of only two areas in the brain where
adult neurogenesis occurs, the other being the subventricular zone (Drew, 2013). As both Pgrmc1 and KitL mRNA are detected in the hippocampal formation, they may be involved in mediating neurogenesis in this area.

1.6 Shared pathways of Pgrmc1 and Kit Ligand signaling

The same bioinformatics study of the Pgrmc1-dependent transcriptome in neural cells that identified KitL as a target of Pgrmc1 showed Jak/Stat signaling to be a key downstream pathway regulated by Pgrmc1 (Intlekofer et al, unpublished). KitL has also been connected to regulation of the Jak/Stat pathway, interacting with and phosphorylating both Jak2 and Stat1 (Deberry, 1997) and phosphorylating Stat3 (Gotoh, 1996). Jak/Stat signaling contributes to cell viability, resulting in either cell death or protection (Planas, et al., 2006) and it is predominantly activated through phosphorylation cascades, allowing for rapid effects. Modulation of the Jak/Stat pathway in the form of post-translational inhibition allows for Jak/Stat responses to be controlled and transient. Cytokine activation of the Jak/Stat pathway may play an important role in the brain’s immune response following an ischemic event. Stat1 and Stat3 are both activated in neurons after being subjected to ischemia and Stat1 knockout mice are more resistant to ischaemic brain injury (Planas, et al. 2006). The Jak/Stat pathway is a modulator of long-term potentiation (LTP) in the hippocampus (Nicolas, et al. 2012). LTP is an activity in the brain that causes for strengthening of synapses and is heavily tied to learning. Importantly, knock down or inhibition of Jak2 can impair induction of the LTP. This may have clinical relevance as studies of Alzheimer’s disease pathology also show that inactivation of Jak2/Stat3 phosphorylation can lead to memory loss and a deficit in
working spatial memory (Chiba, et al., 2009). It remains to be determined if Pgrmc1 and Kit Ligand are involved in the same mechanisms regulating Jak/Stat signaling.

Commonalities between Kit Ligand and Pgrmc1 have also been seen in regards to aromatase (Cyp19). KitL has been shown to upregulate aromatase mRNA and protein levels (Jin, et al. 2005), and Pgrmc1 is also involved in regulation and activation of aromatase (Petersen, 2013). Aromatase has very important neuroendocrine functions in the brain and its main responsibility is the metabolism of testosterone to estradiol. Aromatase has been found to be neuroprotective as well. In mice, its expression is induced following stroke and mice with a conditional aromatase knock out have more damage following stroke than wild type counterparts (Roselli 2007).

1.7 Rationale and Objectives

Altered neurogenesis is an early critical event in the Alzheimer’s Disease (AD) process and the hippocampus is specifically vulnerable to this phenomenon as the subgranular zone is one of the only places adult neurogenesis occurs. A suggested strategy in combating and possibly slowing AD is to preserve this neurogenesis (Mu & Gage, 2011). Thus, understanding mechanisms in the hippocampus that regulate neurogenesis and neuroprotection is important to better understanding neurodegenerative disease. In view of evidence that Pgrmc1 may play a role in these functions, identifying the underlying mechanisms may lead to new treatments for AD and other neurodegenerative diseases.

Based on the information presented above, the hypothesis tested in these studies is: **Pgrmc1 regulates neural targets through KitL/c-Kit signaling in a P₄-dependent**
and/or -independent manner. I tested this hypothesis by performing both in vitro and in vivo studies. I first used in situ hybridization to map KitL and cKit mRNA in the female rat brain to determine whether the pattern of distribution was similar to that of Pgrmc1 mRNA, a condition that must be met if my hypothesis is correct. I next performed in vitro studies in the immortalized mouse hippocampal cell line mHe-18. These cells contain Pgrmc1, KitL and c-Kit, but do not have detectable levels of either progesterone receptor (Pgr) mRNA or protein, making them suitable models to study non-classical P₄ signaling in the hippocampus. MHe-18 cells were treated with Pgrmc1 siRNA to test whether Pgrmc1 alters KitL or c-Kit expression. I next treated these cells with Kit Ligand recombinant protein to study downstream regulation of c-Kit signaling in the absence of Pgrmc1. Finally, the cells were treated in combination with Pgrmc1 siRNA and a c-Kit inhibitor AG-1296 to test if Pgrmc1 regulation of downstream targets depended on c-Kit signaling.
CHAPTER 2

MATERIALS AND METHODS

2.1 Study 1: Localization of c-Kit receptor and Kit Ligand \textit{in vivo}

Before investigating a possible relationship between Pgrmc2 and KitL/c-Kit signaling, I mapped the distribution of these genes throughout the brain and compared the distribution to that of Pgrmc1. Mapping studies of both Kit Ligand and c-Kit were performed using S$^{35}$-labeled cRNA probes and assessed using radiographic films and emulsion autoradiography. The localization pattern of both ligand and receptor were then compared to a previous Pgrmc1 mapping study (Petersen, et al. 2013) to find areas of shared localization.

2.1.1 Tissue Collection

Brains of female Sprague-Dawley adult (n=3, approximately 60 days of age) and neonatal pups (n=3, post-natal day 2) were previously collected and stored at -80°C. Using a microtome (Leica CM3000, Nussloch, Germany), all six brains were cryosectioned and 14-μm coronal forebrain sections were thaw-mounted onto gelatin coated slides and stored at -80°C until use.

2.1.2 Complimentary RNA (cRNA) Probe Preparation

Primer sets with an amplicon of approximately 600 base pairs were designed for both Kit Ligand and c-Kit, this was done using the National Center for Biotechnology Information (NCBI) online nucleotide database in conjunction with Primer-Blast (NCBI). Forward and reverse primers (Integrated DNA Technologies, Coralville, IA) can be
found in Table 2. Using cDNA from an AVPV microdissected punch sample, a PCR reaction was run using GoTaq® DNA Polymerase (Promega) according to manufacturer’s instructions on a Mastercycler® Gradient Thermal Cycler (Eppendorf) for 1 cycle of 95°C for 2 minutes, followed by 39 cycles of 95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute with a final 72°C extension cycle of 5 minutes. Confirmation of amplification of sequences was done by running PCR products on a 1% agarose gel with ethidium bromide in TAE buffer. Cloning of sequences and bacterial transformation were done using the TOPO® TA Cloning® Kit (Invitrogen) according to manufacturer’s instructions. Sequences were cloned into the pCR™II-TOPO® Vector (Invitrogen) and transformed using One Shot® TOP10 chemically competent E. coli cells (Invitrogen). Cells were plated on LB agar (Sigma-Aldrich) plates spread with X-Gal (VWR) to screen for incorporation of plasmids, plates were incubated overnight at 37°C. White colonies were then lysed and presence of plasmid was confirmed via PCR. One white colony per plasmid was used to inoculate LB broth with 100µg/mL Ampicillin (VWR) and incubated for 16 hours at 37°C on shaker. Plasmids were then isolated from bacterial cultures using QIAGEN Plasmid Midi Kit (Qiagen) according to manufacturer’s instructions. Concentration and purity of sample was then measured using the Nanodrop 8000 (Thermo Scientific) and plasmids were sequenced to confirm identity of sequence. Plasmids were linearized by restriction digest and the enzymes HindIII (New England Biolabs, Ipswich, MA) and EcoRV (Fisher) were used at a concentration of 5U of enzyme per microgram of DNA. Efficacy of digest was confirmed by running digest product and uncut product (control) on 1% Agarose gel. Digest was cleaned up by means of a phenol/chloroform extraction. In vitro transcription of cRNA probes (both sense and
anti-sense for KitL and c-Kit) was done in the presence of $\text{S}^{35}$-labelled UTP (Perkin Elmer, Shelton, CT) followed by a phenol chloroform extraction and ethanol precipitation.

2.1.3 Prehybridization of Tissue

Prehybridization was done using all RNAse-free solutions prepared with $\text{H}_2\text{O}$ treated with diethylpyrocarbate (DEPC $\text{H}_2\text{O}$) (Fisher). Tissue slides were removed from -80°C freezer and allowed to thaw for no more than 10 minutes. Slides were loaded into slide racks and washed in phosphate buffered saline/4% formalin (Fisher) (15 minutes), 2X SSC (1X SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 2 minutes), triethanolamine HCL (VWR) with 0.25% acetic anhydride (Sigma) (10 minutes) all on a platform shaker. This was followed by a brief 2X SSC rinse and then an ethanol dehydration using 70%, 80%, 95%, and 100% ethanol in DEPC $\text{H}_2\text{O}$. Then a 5-minute chloroform wash was done before the final 100% and 95% ethanol washes, slides were then allowed to air dry.

2.1.4 Hybridization of Tissue

Localization was carried out using $\text{S}^{35}$-labeled antisense cRNA probes and the sense cRNA probes were used as negative controls. Twenty $\mu$L of hybridization buffer composed of 50% formamide (Fisher), 10% dextran sulfate (Sigma), 1X Denhardt’s solution (Thermo Fisher Scientific), 2X SSC, 500$\mu$g/mL heparin sodium salt (Sigma), 035mg/mL yeast tRNA (Fisher), 0.1% sodium pyrophosphate (Sigma), and freshly added 0.4M dithiothreitol (Fisher) containing 1 X $10^6$ cpm $\text{S}^{35}$ was applied to each section.
Slides were then covered with glass cover slips and placed in humidified large square Nunc petri dishes and incubated 16-18 hours at 55°C.

2.1.5 Post-Hybridization of Tissue

Slides were removed from incubation and allowed to come to room temperature. All washes/rinses were performed on an orbital shaker unless stated otherwise. Cover slips were removed in a 1X SSC soak and slides were washed twice in 1X SSC at room temp (15 minutes each), then washed in 50% formamide in 2X SSC for 20 minutes in a 52°C water bath and then rinsed twice in 2X SSC at room temperature (10 minutes each). Slides were then incubated in RNase buffer (0.5 M NaCl, 10mM Tris pH 8.0, 1mM EDTA pH 8.0) with 25µg/mL of RNase A (Fisher) in a 37°C water bath (30 minutes) followed by a 10 minute 2X SSC rinse at room temperature. Slides are then incubated in a 52°C water bath in 50% formamide in 2X SSC (10 minutes), then washed at room temp in 2X SSC (10 minutes), followed by a brief deionized H₂O rinse. Slides are then dehydrated first in 70% ethanol, then 80% ethanol, followed by 95% ethanol and left to air dry.

2.1.6 Detection

Once dry, slides were placed in autoradiography cassettes (8x10in, FisherBiotech) against an 8x10inch sheet of KODAK® BioMax® Maximum Resolution (MR) Autoradiography Film (Carestream Health, VWR) and film was then developed within 10-14 days. Subsequently, slides were analyzed using emulsion autoradiography. NTB emulsion (VWR, Pennsylvania) was warmed in a 42°C water bath and diluted 1:1 with ddH₂O. Slides were then dipped in diluted emulsion in a dark room and left to dry on
aluminum foil for 1-2 hours. Slides were then placed in light-tight boxes (covers off) with
desiccant tablets up against the side of a metal container with tissue sections parallel to
the ground and emulsion side up. These were left to dry in the dark room overnight. The
following day, covers were added to light-tight boxes and they were taped with electrical
tape and placed inside the metal box with slides perpendicular to the ground. Metal boxes
were then placed in a 4°C refrigerator free of radiation sources. Slides were developed
10-14 days later in the dark room using Kodak Dektol developer at 15°C (Fisher) using a
1:1 dilution prepared according to manufacturer’s instructions then fixed in and Kodak
fixer (Fisher) for 5 min. Slides were loaded into slide racks and placed in developer for 2
minutes, water for 30 seconds, and finally fixer for 5 minutes. Slides were dried and then
stained in filtered Toluidine blue for 5 minutes, allowed to dry, and cover-slipped.

Localization was determined using reference atlases such as the Allen Mouse Brain
Reference Atlas (Allen Institute for Brain Science, 2017) and Brain Maps: Structure of
the Rat Brain (Swanson, 1998). Atlases were used in conjunction light microscopic
analysis of toluidine blue stained tissue sections corresponding to the film
autoradiographic images.

2.2 Study 2: Pgrmc1 regulation of Kit Ligand mRNA in vitro and in vivo

To determine whether Pgrmc1 levels affect KitL and c-Kit synthesis in vitro, Pgrmc1
knockdown in a mouse embryonic hippocampal (mHe-18) cell line was verified via
qPCR. Kit Ligand and c-Kit mRNA levels were then measured in these cells with
Pgrmc1 knock down. To determine whether Pgrmc1 levels affect KitL and c-Kit
synthesis in vivo, Pgrmc1 and 2 double-conditional knockdown adult mice brains were
analyzed via qPCR.
2.2.1 SiRNA Transfection

I seeded mHe-18 cells at a density of 1.2 x 10^5 cells/mL in a 24-well plate in DMEM supplemented with 5% FBS and 1% PSG. They were then immediately transfected with Mm_Pgrmc1_1 Flexitube siRNA or AllStars Negative Control siRNA (Qiagen, Valencia, CA) to a final concentration of 20nM with HiPerfect Transfection Reagent (Qiagen) according to the manufacturer’s protocol. Cells were incubated at 37°C in 5% CO₂ for 48 hours and then harvested in Trizol and stored at -80°C until RNA isolation.

2.2.2 Tissue Collection

Brains were collected from Pgrmc1 and Pgrmc2 dcKO and control female mouse heads received from the James Pru laboratory (Washington State University, Pullman WA). Brains were cryosectioned coronally using a microtome (Leica CM3000, Nussloch, Germany) until the hippocampus was reached. A 150µm section was then taken and the entire hippocampus (CA1, CA2, CA3, and the dentate gyrus) was removed using a scalpel. Frozen tissue was transferred to a 1.5mL microcentrifuge tube on dry ice.

2.2.3 RNA isolation, reverse transcription, and quantitative polymerase chain reaction (qPCR)

Cellular RNA was isolated using Trizol (Invitrogen, Carlsbad, California) according to manufacturer’s instructions and reverse transcription was completed using the M-MLV Reverse Transcription Kit (Promega, Madison, WI). Following reverse transcription, cDNA was diluted 1:10 in nuclease-free water.
RNA from tissue was isolated using Trizol and the Qiagen RNeasy Lipid Kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was then completed using the Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer’s protocol and cDNA was diluted 1:10 in nuclease free water.

For qPCR, the 1:10 dilution was combined with FastStart Universal SYBR Green (ROX) mix (Roche Applied Science, Indianapolis, IN) with the correct primer. See Table 1 for Kit Ligand, c-Kit receptor, Pgrmc1, and Gapdh forward and reverse primer (Integrated DNA Technologies, Coralville, IA) sequences. The qPCR reactions were accomplished using the Stratagene MX3005P Thermocycler (Agilent Technologies, Wilmington, DE) with the settings: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. MxPro™ qPCR analysis software (Agilent Technologies) was used to obtain threshold cycle (Ct) values which were then used to analyze various treatment effects on target genes using the delta delta Ct method of analysis (Livak & Schmittgen, 2001). Normalization of target genes was done using the housekeeping gene Gapdh. Results of each gene were then analyzed statistically using GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA) using student two-tailed t-tests.

**2.3 Study 3: Regulation of Kit Ligand as a sex hormone independent mechanism**

To determine whether Pgrmc1 regulation of KitL or c-Kit mRNA levels is affected by ovarian sex hormones, mHe-18 cells were treated with 10nM estradiol (E2) and 10nM P4. Pgrmc1, KitL, and c-Kit mRNA levels were then assessed using qPCR as in Study 1.
2.3.1 E₂ and P₄ Treatments

The mHe-18 cells were plated in Dulbecco’s Modification of Eagle’s Medium (DMEM; Thermo Fisher Scientific, Rockford, IL) supplemented with 5% fetal bovine serum (FBS; Thermo Fisher) and 1% penicillin streptomycin glutamine (PSG; Thermo Fisher). When cell density reached approximately 65% confluency, media was changed to DMEM with no phenol red (Thermo Fish Scientific) containing 5% charcoal-stripped FBS (Atlanta Biologicals) and 1% PSG. Twenty-four hours later cells were treated with 10nM P₄, 10nM E₂, both 10nM P₄ and 10nM E₂, or vehicle control. Cells were then harvested in Trizol 24 hours later and stored at -80°C until RNA isolation.

2.4 Study 4: Shared downstream targets of Kit Ligand/c-Kit and Pgrmc1 regulation

To determine if Kit Ligand/c-Kit signaling shared downstream targets, mHe-18 cells were treated with soluble Kit Ligand and then RNA was isolated and reverse transcribed. Validation of KitL treatment effect was done by analyzing Bcl2 expression levels using qPCR. Bcl2 is a known c-Kit signaling target and has been used previously for validating treatments (Carson, et al., 1994; Dhandapani, et al., 2005). The cDNA from this treatment was analyzed using a JAK/STAT RT² Profiler PCR Array. This array had been used previously in our laboratory to identify targets affected by Pgrmc1 knockdown, allowing for comparison of regulated targets between the two different treatments. Targets taken from the qPCR array were then validated individually. See Table 1 for Bcl2 and Pias1, 2,3 and 4 forward and reverse primer (Integrated DNA Technologies) sequences.
2.4.1 Soluble Kit Ligand Treatments

The mHe-18 cells were plated in DMEM supplemented with 5% FBS and 1% PSG. When cell density reached approximately 65% confluency, cells were treated with 1ng/mL, 10ng/mL, and 50ng/mL Recombinant Murine SCF (PeproTech, Rocky Hill, NJ) or vehicle control. Cells were then harvested in Trizol 24 hours later and stored at -80°C until RNA isolation.

2.4.2 PCR Array

The mouse JAK/STAT RT² Profiler PCR Array (Qiagen) was used in conjunction with cDNA from soluble Kit Ligand treated mHe-18 cells to identify Kit Ligand/c-Kit signaling targets that are linked to the Jak/Stat pathway. cDNA was mixed with RT² SYBR Green Mastermix (Qiagen) according to the JAK/STAT RT² Profiler PCR Array protocol and loaded onto the array plate for qPCR analysis. The cycling conditions used were : 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. C_t values were then analyzed using SABiosciences Web-based PCR Array Data Analysis accessed on the Qiagen website.

2.5 Study 5: Effects of c-Kit signaling and Pgrmc1 on the synthesis of Pias1, 2, 3, and 4

To determine whether Pgrmc1 mediates changes in the expression level of Pias genes through KitL/c-Kit signaling, the known c-Kit inhibitor AG-1296 (Ueda, et al., 2002; Letard, et al., 2008) was used in conjunction with siRNA experiments. Pias1, 2, 3, and 4 expression levels were then assessed using qPCR as done in previous studies. Data for each gene were analyzed using two-way ANOVA with multiple comparisons.
2.5.1 SiRNA transfection and AG-1296 treatment

I seeded mHe-18 cells at a density of $1.5 \times 10^5$ cells/mL in a 24-well plate in DMEM supplemented with 5% FBS and 1% PSG. They were then immediately transfected with Mm_Pgrmc1_1 Flexitube siRNA or AllStars Negative Control siRNA to a final concentration of 10nM with Lipofectamine® RNAiMax Transfection Reagent (Invitrogen) according to the manufacturer’s protocol. Cells were incubated at 37°C in 5% CO$_2$ for 24 hours and then media containing siRNA was replaced with DMEM supplemented with 5% FBS, 1% PSG, and 10nM AG-1296 (Cayman Chemicals, Ann Arbor, Michigan). Incubation continued for 24 hours and cells were then harvested in Trizol and stored at -80°C until RNA isolation.
Table 1. Primer pairs used in qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>5’ – 3’ Sequence</th>
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<tr>
<td>Mouse Gapdh</td>
<td>Forward: GTGGAGTCATACTGGAACATGTAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AATGGTGAAAGGTCGTGTG</td>
</tr>
<tr>
<td>Mouse Kit Ligand</td>
<td>Forward: GCTGCAACAGGGGTAACAT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAATCTCCGAAGAGGCCAGAA</td>
</tr>
<tr>
<td>Mouse c-Kit</td>
<td>Forward: GTGACGGTACATGCTGCAT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGTGCAACCACCACGTAAATG</td>
</tr>
<tr>
<td>Mouse Pgrmc1</td>
<td>Forward: CCTGCTCTACAAGATCGTTGC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAACACCTTGCCGTGATGG</td>
</tr>
<tr>
<td>Mouse Pias1</td>
<td>Forward: AGAACTTCTACAGGAGCGGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTGTGGAAATGGTGGATGGA</td>
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<tr>
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<td>Forward: AATGCGGGATTTCGAGGAGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: TATTCCGTCCAGCAACGCC</td>
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<tr>
<td>Mouse Pias3</td>
<td>Forward: CTAGGAAGATGCGCTGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCGACTCATAGGAGCCCTT</td>
</tr>
<tr>
<td>Mouse Pias4</td>
<td>Forward: GCAGGAGGACCAATACCCAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGCCTCTTAGGTTCCACACC</td>
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Table 2. Primer pairs used to make cRNA probes

<table>
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<tr>
<th>Gene</th>
<th>5’ – 3’ Sequence</th>
</tr>
</thead>
<tbody>
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<td>Rat Kit Ligand</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GCTCCAGAACAGCTAAACGG</td>
</tr>
<tr>
<td>Reverse</td>
<td>TGTTGAAGAGAGCACACAAATCAC</td>
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<tr>
<td>Rat c-Kit</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GTCTCCACCATCCATCCAGC</td>
</tr>
<tr>
<td>Reverse</td>
<td>ACCGTAATGTGTCCCCCTTCC</td>
</tr>
</tbody>
</table>
CHAPTER 3

RESULTS

3.1 Study 1

3.1.1 KitL and c-Kit mRNA is localized throughout the female rat brain

Both KitL and c-Kit mRNAs are present throughout the brain (Fig. 1 A1-13, B1-13) in adults. KitL has robust expression in the outer layers of the cortex (Co; A1-A12) as well as in the anterodorsal preoptic nucleus (ADP; A3), the piriform cortex (PIR; A2), the bed nucleus of the anterior commissure (BAC; A4), and the endopiriform nucleus (EP; A5). It is expressed highly in many thalamic nuclei such as the triangular septal nucleus (TS; A4), the paraventricular nucleus (PVA; A6), the anteroventral nucleus (AV; A6), the centromedian nucleus (CM; A6), the reuniens thalamic nucleus (Re; A9), the medial habenula (MH) and the lateral habenula (LH; A9), and the parafascicular nucleus (PF; A11). It can also be seen in all areas of the hippocampal formation, most notably in the dentate gyrus (DG; A10), as well as in the amygdalar region in the lateral amygdaloid nucleus (LA; A11). Though c-Kit is found in some of the same areas as KitL, the expression levels and patterns differ strikingly. C-Kit can be found more evenly distributed throughout the cortical layers (B1-12), except the outer layer where it is highest. Robust expression of c-Kit can also be seen in the MH and LH, as well as the Subthalamic Nucleus (STN; B11). In the hippocampal formation, c-Kit is robustly expressed throughout all tissues (B10).

Both KitL and c-Kit mRNAs are present throughout the brain (Fig. 2 A1-11, B1-11) in PND2 animals. KitL has robust expression in both the outer layer of the cortex, as
well as the inner area (A2). It can also be seen in the PIR (A2), EP (A4), and the BAC (A5). It is seen in a variety of thalamic nuclei such as the TS (A7), the PVA (A8), and the AV (A8). It can also be seen in all areas of the hippocampal formation, most notably in the DG (A9), as well as in the amygdalar region in the LA (A9).

The distribution patterns for c-Kit and KitL differed, with c-Kit showing a wider distribution such that there was some overlap. In addition, expression levels differ. C-Kit can be found more evenly distributed throughout the cortical layers (B1-12). Robust expression of c-Kit can also be seen in the septohippocampal nucleus (SHi; B3), as well as the subthalamic nucleus (STN; B9). In the hippocampal formation c-Kit is expressed throughout all tissues (B10), though in the DG it is expressed in the outer layer of cells. Further analysis using emulsion radiography on the adult brain sections shows both KitL and c-Kit to be located in the pyramidal cell layer of the dentate gyrus (Fig. 3).

3.2 Study 2

3.2.1 Pgrmc1 silencing regulates KitL mRNA levels, but not c-Kit mRNA levels in vitro and in vivo

Pgrmc1 siRNA treatments were first verified in mHe-18 cells and expression levels of Pgrmc1, KitL, and c-Kit were measured using qPCR. Transfection with Pgrmc1 siRNA reduced Pgrmc1 levels when compared to scramble controls (Fig. 4A). Pgrmc1 knockdown significantly upregulated KitL mRNA when compared to scramble controls, though it had no effect on c-kit gene expression (Fig. 4B). In the hippocampal tissue of Pgrmc1/2 dcKO animals, KitL mRNA was significantly increased when compared to controls and there was no effect on c-Kit mRNA levels (Fig. 4C).
3.3 Study 3

3.3.1 Ovarian hormones regulate c-Kit and Pgrmc1 mRNA levels, but not KitL mRNA levels

Treatment of mHe-18 cells with 10nM E₂ significantly upregulated c-Kit mRNA levels when compared to vehicle controls, but had no effect on KitL or Pgrmc1 mRNA levels (Fig. 5A). Treatment with 10nM P₄ significantly downregulated Pgrmc1 mRNA levels when compared to vehicle controls, but had no effect on KitL or c-Kit mRNA levels (Fig. 5B). E₂ had no effect on P₄ downregulation of Pgrmc1, but P₄ completely blocked upregulation of c-Kit by E₂ (Fig. 4C).

3.4 Study 4

3.4.1 KitL/c-Kit signaling affects the expression level of genes associated with the Jak/Stat pathway

The expression of bcl2, a known target of c-Kit signaling, was significantly upregulated in the 10ng/mL and the 50ng/mL treatments (Fig. 6A). Levels of c-Kit mRNA were also measured using qPCR (Fig. 6B).

I then used cDNA from control mHe-18 cells and cells treated with 50ng/mL KitL to perform the Mouse JAK/STAT Signaling Pathway RT² Profiler PCR Array (Qiagen). Table 3 shows preliminary data of the top five upregulated genes and the top five downregulated genes. Unpublished data from our laboratory show Pgrmc1 knock down also upregulates protein inhibitor of activated stat (Pias) family members. Therefore the pias genes were picked for further analyses.
3.4.2 KitL/c-Kit signaling mediates expression of Pias1, 2, and 3

Results of KitL treatment of mHe-18 cells are shown in Fig. 7A-C. Levels of Pias 2 mRNA were significantly upregulated when compared to vehicle controls in the 10ng/mL KitL treatment (Fig. 7B). Levels of Pias 3 mRNA were significantly upregulated when compared to vehicle controls in the 50ng/mL KitL treatment (Fig. 7A-C). Pias4 mRNA was not significantly upregulated in any of the three treatments (Fig. 7D). A biphasic response is seen in response to KitL treatment across Pias1, 2, and 3 (7A-C).

3.4.3 Pgrmc1 silencing upregulates Pias1-4 expression in vitro, but only affects Pias1 expression in vivo

Pgrmc1 silencing significantly upregulated Pias1, 2, 3, and 4 mRNA levels when compared to the scramble controls (Fig. 8A). In the Pgrmc1/2 dcKO animals, Pias1 mRNA levels was upregulated, while Pias2-4 levels were not significantly affected (Fig. 8B).

3.5 Study 5

3.5.1 Pgrmc1 regulates the Pias family expression independently of KitL/c-Kit signaling

To determine whether Pgrmc1 regulation of Pias1-4 mRNA expression is mediated through c-Kit signaling, Pgrmc1 siRNA experiments were performed on mHe-18 cells in the presence and absence of 10nM AG-1296 (Cayman Chemicals), a known c-Kit inhibitor. Pgrmc1, Pias1, Pias2, Pias3, and Pias4 mRNA levels were then measured using qPCR. The Pgrmc1 knock down in the presence of siRNA were first validated.
Pgrmc1 silencing significantly decreased Pgrmc1 expression levels in the presence of vehicle, as well as in the presence of AG-1296 (Fig. 9A).

Neither Pgrmc1 nor AG-1296 had a significant effect on Pias1 mRNA levels after any treatments (Fig. 9B). Pias2 and 3 mRNAs were increased by Pgrmc1 silencing in the presence of vehicle (Fig. 9C,D), while Pias2 and 4 mRNA was increased by Pgrmc1 silencing in the presence of AG-1296 (Fig. 9C,E). Pias2 mRNA was decreased in the presence of AG-1296 when compared to scramble/vehicle controls (Fig. 9C), but it did not significantly affect levels of Pias1, 2, and 4. Silencing in the presence of vehicle did not significantly upregulate Pias4 mRNA levels, nor did silencing in the presence of AG-1296 increase Pias3 levels.
Figure 1. Hippocampal localization of KitL and c-Kit in adult rat brain using $^{35}$S-labeled cRNA probes

(A) KitL localization in the female adult rat brain using (A2-11) anti-sense and (A1, A12) sense probes. (A13) Enlarged image of the hippocampal formation from A10. (B) c-Kit
Figure 2. Hippocampal localization of KitL and c-Kit in PND2 rat brain using S^{35}-labeled cRNA probes

(A) KitL localization in the female developing rat brain using (A2-9) anti-sense and (A1, A10) sense probes. (A11) Enlarged image of the hippocampal formation from A9. (B) c-Kit localization in the female adult rat brain using (B2-9) anti-sense and (B1, B10) sense probes. (B11) Enlarged image of the hippocampal formation from B9. Darkened areas
denote radio-labeled probe binding and presence of specified mRNA by anti-sense. Sense probes signify background signal. (ADP: anterodorsal preoptic nucleus; AV: anteroventral thalamic nucleus; BAC: bed nucleus of the anterior commissure; CA1: Cornu Ammonis, Pyramidal Cell Layer 1; CA2: Cornu Ammonis, Pyramidal Cell Layer 2; CA3: Cornu Ammonis, Pyramidal Cell Layer 3; Co: cortical layers; CP: caudate putamen; DG: dentate gyrus; EP: endopiriform nucleus; LA: lateral amygdaloid nucleus; PVA: paraventricular nucleus; SHi: septohippocampal nucleus; TS: triangular septal nucleus)
Figure 3 KitL and c-Kit mRNA signals are found in the adult cell layer of the dentate gyrus

Shown are KitL and c-Kit localization in the dentate gyrus of the hippocampal formation. Signal is indicated by silver grains, these depict $^{35}$S-labeled anti-sense cRNA probe binding. Black arrows indicate areas of silver grain signal. Toluidine blue staining allows for visualization of cell nuclei.
Figure 4. Pgrmc1 regulation of KitL synthesis in vitro and in vivo

(A) Validation of in vitro Pgrmc1 knockdown using siRNA in mHe-18 cells. (B) KitL and c-Kit mRNA levels following 48 hour Pgrmc1 siRNA treatment on mHe-18 cell lines. (C) KitL and c-Kit mRNA levels in micro dissected hippocampal tissue of control and Pgrmc1/2 dcKO mice. (A-C) Genes were analyzed independently using a student t-test; Bars = means±SEM. *Significantly different from scramble or control; * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
Figure 5. Effects of ovarian hormones on KitL, c-Kit, and Pgrmc1 expression levels

(A) Effects of estradiol (10nM) on KitL, c-Kit, and Pgrmc1 mRNA expression levels in mHe-18 cells. (B) Effects of progesterone (10nM) on KitL, c-Kit, and Pgrmc1 mRNA expression levels in mHe-18 cells. (C) Effects of combined estradiol (10nM) and progesterone (10nM) on KitL, c-Kit, and Pgrmc1 mRNA expression levels in mHe-18 cells. (A-C) Genes were analyzed independently using a student t-test; Bars = means±SEM.. *Significantly different from vehicle control; * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
Figure 6. Bcl2 and c-Kit mRNA levels in response to soluble KitL treatment

(A) Bcl2 and (B) c-Kit mRNA levels following soluble KitL dose concentrations of 1ng/mL, 10ng/mL, and 50ng/mL on mHe-18 cells. Gene was analyzed using a student t-test; Bars = means±SEM. *Significantly different from scramble or control; * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
Table 3. Genes found to be regulated by Pgrmc1 using JAK/STAT PCR Array

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<th>Gene</th>
<th>Abbreviation</th>
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<th>Fold Change</th>
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<tr>
<td>Protein inhibitor of activated STAT 1</td>
<td>Pias1</td>
<td>NM_019663</td>
<td>3.0525</td>
</tr>
<tr>
<td>MAD homolog 1 (Drosophila)</td>
<td>Smad1</td>
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<td>1.366</td>
</tr>
<tr>
<td>SFFV proviral integration 1</td>
<td>Sp1</td>
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<td>1.1892</td>
</tr>
<tr>
<td>Interferon regulatory factor 9</td>
<td>Irf9</td>
<td>NM_008394</td>
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<td>MAD homolog 3 (Drosophila)</td>
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<td>Tyrosine kinase 2</td>
<td>Tyk2</td>
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<td>Socs3</td>
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<td>MAD homolog 2 (Drosophila)</td>
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<td>YY1 transcription factor</td>
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Figure 7. Pias family mRNA levels in response to soluble KitL treatment

Effects of varying concentrations of soluble KitL on (A) Pias1 mRNA, (B) Pias2 mRNA, (C) Pias3 mRNA, and (D) Pias4 mRNA levels in mHe-18 cells. (A-D) Genes were analyzed independently using a student t-test; Bars = means±SEM. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
**Figure 8. Pgrmc1 regulation of Pias1, 2, 3, and 4 synthesis in vitro and in vivo**

(A) Pias1-4 mRNA levels following 48 hour Pgrmc1 siRNA treatment on mHe-18 cell lines. (B) Pias1-4 mRNA levels in micro dissected hippocampal tissue of control and Pgrmc1/2 dcKO mice. (A-B) Genes were analyzed independently using a student t-test; Bars = means±SEM. *Significantly different from scramble or control; * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
Figure 9. Pgrmc1 regulation of Pias family synthesis independent of KitL/c-Kit signaling

(A) Validation of in vitro Pgrmc1 knockdown using siRNA in mHe-18 cells. (B) Pias1, (C) Pias2, (D) Pias3, and (E) Pias4 mRNA levels following 48 hour Pgrmc1 siRNA treatment in the presence and absence of AG-1296 on mHe-18 cell lines. (A-D) Gene values were analyzed using two-way ANOVA followed by multiple comparisons. Bars = means±SEM. 

*a* Significantly different from Scramble/Vehicle. 

*b* Significantly different from Pgrmc1 siRNA/Vehicle. 

*c* Significantly different from Scramble/AG-1296.
CHAPTER 4

DISCUSSION

My *in vitro* and *in vivo* findings demonstrate a connection between Pgrmc1 and KitL/c-Kit signaling in hippocampal neurons. First, KitL and c-Kit are localized to many regions related to specific neural functions in which Pgrmc1 also localizes. Of particular interest is the hippocampal formation where each of these molecules is expressed highly. Second, Pgrmc1 knockdown upregulates KitL *in vivo* and in immortalized hippocampal neurons. The protein inhibitor of activated stats (Pias) family members were identified as downstream targets of KitL and Pgrmc1, implicating both Pgrmc1 and KitL in Jak/Stat signaling. Consistent with the idea that Pgrmc1 represses KitL expression, Pgrmc1 suppresses, but KitL upregulates Pias 1, 2, and 3 mRNA levels. However, c-Kit antagonists do not alter the effects of Pgrmc1 knockdown on pias gene expression. Finally my results suggest the intriguing possibility that observed estradiol stimulation of neurogenesis and progesterone reversal of this effect may be through c-Kit.

My findings show that although KitL and c-Kit are in many of the same regions, the difference in expression levels is immediately obvious in both adult and PND2 brains. A similarly divergent pattern has been described previously in embryos (Keshet, et al., 1991). Differences between KitL and the c-Kit receptor localization may be explained by the fact that the ligand can be secreted. Several lines of evidence suggest that secreted KitL plays a role in neurogenesis. In the developing brain, KitL mRNA is detected in the main areas of neurogenesis, such as the subventricular zone and the inner layers of the cortex (McConnell, 1995). These mRNA distribution patterns were not seen in the adult brains. This is expected as the rat brain continues developing until day 60 (Semple, et al.,
2013) at which point it has a much lower level of neurogenesis. It is interesting to note that the c-Kit pattern remains the same in developing and adult brains except in the dentate gyrus. In the PND2 brain, c-Kit is localized to the outermost regions of the dentate gyrus, while in the adult it is detected within the structure. Though it is impossible without cellular markers to confirm what cell types c-Kit is in, a similar pattern of ligand and receptor distribution are also seen in the developing cortex. In the this region, KitL mRNA is detected in Purkinje cell bodies and c-Kit mRNA is detected in the interneuron basket cells surrounding the Purkinje cells (Manova, et al., 1992). This evidence builds a case supporting KitL and c-Kit involvement in synaptic organization. Similarly, KitL and c-Kit signaling may affect the development of the dentate gyrus.

Bone morphogenic protein (Bmp) induces kitl expression (West, et al., 2010) (Otsuka & Shimasaki, 2002) and silencing of bmp leads to a smaller dentate gyrus (Caronia, et al., 2010). My in situ hybridization data adds to the notion that KitL/c-Kit signaling is involved in specific neural functions such as neurogenesis and synaptogenesis.

My data supports the idea that pgrmc1, kitl and c-kit may interact, as their expression patterns overlap throughout the forebrain in areas with specific functions such as neurogenesis. Though it is widely established that the major area of the adult brain that undergoes neurogenesis is the dentate gyrus, many of the nuclei in which KitL was detected may also be sites of adult neurogenesis. Among these areas are the amygdala (Fowler, et al., 2009), the arcuate (McNay, et al., 2012), the piriform cortex (Shapiro, et al., 2007), and areas of the hypothalamus (Pierce & Xu, 2011). Pgrmc1 has also been detected in all of these areas, as well as the dentate gyrus (Intlekofer and Petersen 2011). The dentate gyrus does not contain the classical progesterone receptor, and it has been
suggested that P₄ effects of neurogenesis in this area are mediated by Pgrmc1 (Bali, et al. 2012). Pgrmc1 has been shown to be regulated by E₂ and P₄ in other brain nuclei as well (Intlekofer & Petersen, 2011), supporting the idea that sex hormones may mediate some of these mechanisms.

My in vitro findings are the first to show Pgrmc1 regulation of KitL synthesis. Though there is a Pgrmc1 binding site on the KitL promoter (Peluso, personal communication), more studies must be done to show if Pgrmc1 has a direct effect on KitL synthesis. It is possible that Pgrmc1 affects the mRNA levels of other genes, which in turn regulate KitL. The varying sub cellular localization of the Pgrmc1 protein allow for abundant possibilities for its regulatory mechanisms. Recent x-ray crystallography work has made the structure of the cytosolic domain of Pgrmc1 known (Kabe, et al., 2016), providing further insight into its functions. For example, it is now known that Pgrmc1 has a predicted SH2-domain target sequence (Cahill, et al., 2016). This sequence would allow Pgrmc1 to interact and be phosphorylated by proteins with SH2 domains, such as the c-Kit receptor. This may suggest a feedback loop involving phosphorylation of Pgrmc1 by the c-Kit receptor.

My data are the first to link KitL/c-Kit signaling and Pgrmc1 with the regulation of the pias family expression levels. The Pias family is composed of four members (Pias1, Pias2, Pias3, and Pias4) and they repress various STATs by binding to their DNA binding domain (J. Palvimo 2007). The biphasic trend seen in the KitL dose response may be explained by the down regulation of the receptor in response to ligand binding. The down regulation can occur through decreased mRNA levels, intracellular degradation, and inactivation of the kinase domain (J. &. Lennartsson 2012). A
connection between Pgrmc1 and the Pias family is intriguing, as our laboratory has shown that Pgrmc1 may be involved in regulating \textit{jak1} and \textit{jak2} (Adams and Petersen, Unpublished). Though Pgrmc1 knock down upregulated all four members of the Pias family, only Pias1 was upregulated \textit{in vivo}. Pgrmc2 is silenced in the dcKO brain tissue as well and it is unknown if this contributes to the conflicting data. The combination treatment of Pgrmc1 siRNA and AG-1296 did not abrogate Pgrmc1 effects on the Pias1, 2, 3, or 4. This suggests that Pgrmc1 and KitL are mediating Pias expression levels through independent mechanisms. Pias1 expression levels did not change in response to siRNA treatment as they did in the previous study. An explanation for this may be that mHe-18 cells used were at a later passage than previous cells and this may have affected their expression profiles. Both Pias2 and Pias4 were significantly downregulated in the presence of AG-1296. Unfortunately AG-1296 also inhibits platelet derived growth factor (PDGF), so further studies need to be done to confirm that this down regulation is a direct effect of c-Kit signaling and not a caveat of the inhibitor. Though the role of Pias family members in hippocampal functions has not been thoroughly investigated, one group has found Pias1 to be upregulated in “fast learners” compared to “slow learners” when looking at hippocampal mRNA. Follow-up studies revealed that overexpression of Pias1 in rats enhanced their spatial learning abilities (Tai, et al., 2011). My data therefore suggest a mechanism by which Pgrmc1 is involved in learning and memory through both KitL and Pias1, 2, 3, and 4. Specifically, regulation of \textit{pias} family expression levels by Pgrmc1 and KitL may modify and control the transient responses seen from Jak/Stat signaling.
The dramatic upregulation of c-Kit by E$_2$ is consistent with previous evidence that E$_2$ may mediate neurogenesis. Although my findings are the first to show E$_2$ effects on c-Kit in the brain, similar effects have been observed in the testes and prostate (Correia, et al., 2014) (Figueira, et al., 2016). No previous work studied the P$_4$ regulation of E$_2$-mediated c-Kit signaling, but other work shows that either E$_2$ or P$_4$ alone doubled the number of proliferating neural progenitor cells (NPCs) in the dentate gyrus while administration of simultaneous E$_2$ and P$_4$ has no effect (Bali, et al., 2012). Similarly, E$_2$ increases dendritic spine number and density in hippocampal CA1 pyramidal cells, while addition of P$_4$ reverses the effect (Woolley & McEwen, 1993). The ability of P$_4$ to increase the proliferation of NPCs has also been attributed to Pgrmc1 (Liu, et al., 2009), this finding contradicts my data as no change was seen in c-Kit following P$_4$ treatment. This does not rule out a connection between P$_4$ and c-Kit signaling, as there may be non-genomic effects such as phosphorylation triggered by P$_4$ through Pgrmc1. As c-Kit signaling and estradiol have also already been implicated in neurogenesis, these data give insight into possible cellular mechanisms behind it.

Knowledge of the KitL/cKit pathway can be applied to many clinical situations as well. In 2010, Northwestern University filed a patent on an invention using KitL polypeptide “alone and in combination with granulocyte colony stimulating factor (G-CSF) polypeptide, in the prevention or treatment of injury to the brain after cerebral ischemia or neurological disorder.” (Zhao, et al., 2013). In neurodegenerative diseases such as Alzheimer’s disease (AD) and Parkinson’s, the hippocampus is very sensitive to damage. Induced neurogenesis has been suggested as a viable treatment to prolong the damages of AD (Mu and Gage 2011). Future directions for this work should include the
investigation of KitL regulation of cell proliferation in hippocampal neurons to more clearly establish this pathway in neurogenesis. Overall, my data builds a case for regulation of hippocampal functions through KitL and Pgrmc1 and their downstream neural targets such as Pias1, 2, 3, 4 and indicates that this mechanism may be influenced by ovarian hormones. Based on the mapping data provided by my work, there are many other potential areas to investigate regarding this pathway. The understanding of Pgrmc1 and KitL effects in the brain are still very new, leaving many exciting directions that can be taken with these data.

Figure 10. Suggested pathway based on experimental findings.
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